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Is the typical stage of *Penicillus capitatus* Lamarck (Bryopsidales, Halimedaceae) a possible indicator of climate warming?

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Abstract

In this study, through morphological and molecular analysis approaches, we documented the presence of the typical stage (adult stage) of *Penicillus capitatus* during the coldest seasons of the year, typically periods when this form had never been reported in the Mediterranean. Indeed, the typical stage is common throughout the year in the western Atlantic (native range of the species), whereas it is rare in the Mediterranean, where the *Espera* stage (juvenile stage) is the main stage. To date, in the Mediterranean, the adult stage has been found only in the warmer months of the year. Therefore, the observation of the typical stage in the Mediterranean Sea during the cold season might suggest a general increase in the seawater temperature in this basin, rendering this stage of *P. capitatus* a potential climate bioindicator. Furthermore, owing to the molecular analysis methods adopted, it was possible to assess the relationships between the Mediterranean and Atlantic populations of this species.

Keywords: climate change; warm-affinity species; molecular identification; Chlorophyta; Mediterranean Sea.

Introduction

During the 20th century, the Mediterranean Sea exhibited a process of increasing seawater temperatures, which caused the expansion of tropical and subtropical species, in turn notably affecting native assemblages and eroding local biodiversity (Piazzi & Balata, 2009). This process has been defined by Bianchi *et al.* (2017) as the tropicalisation of the Mediterranean. Indeed, since 1980, when the warming of Mediterranean waters became evident, the distribution ranges of many warm-affinity species have increased (Bianchi, 2007; Boero *et al.*, 2008); moreover, these species can live and, in some cases, even reproduce under these new conditions.

Penicillus capitatus Lamarck is one of the warm-affinity *taxa* occurring in the Mediterranean Sea today. This species, which is widely distributed throughout the Caribbean region, is the only example of the genus *Penicillus* found in different areas of the Mediterranean Sea (Huvé & Huvé, 1964; Meinesz, 1972; Mayhoub, 1976; Ben Maiz *et al.*, 1987; Bergin, 1987; Furnari *et al.*, 2003; Turna *et al.*, 2010; Bilecenoglu *et al.*, 2013; Guiry & Guiry, 2023; Boudouresque *et al.*, 2024) (Fig. 1). In nature, *P. capitatus* exhibits two very different morphological stages: a juvenile stage referred to as *Espera*, consisting of free filaments forming a shrub without a stipe, and an adult stage, defined as typical, consisting of a brush-like upper part, namely, the *capitulum*, an intermediate part, namely, the stalk, and a rhizoidal basal part (Cormaci *et al.*, 2014). The *Espera* stage was originally considered a distinct species, namely, *Espera mediterranea* Decaisne. Later, Huvé and Huvé (1964) reclassified this species as *Penicillus capitatus* f. *mediterraneus*, and finally, Friedmann *et al.* (1977) eliminated this distinction.

In the western Atlantic, the *Espera* stage is very rare, whereas in the Mediterranean Sea, it is more common throughout the year, occurring in waters off the coasts of Spain (Ballesteros, 1992), Algeria (Perret-Boudouresque & Seridi, 1989), Tunisia (Ben Maiz *et al.*, 1987), Egypt (Bergin, 1987), Syria (Mayhoub, 1976), Greece (Athanasiadis, 1987), and Corsica (Boudouresque & Perret-Boudouresque, 1987). Instead, the typical stage is relatively common mainly in both the eastern and southern Mediterranean (Turna *et al.*, 2010; Bilecenoglu *et al.*, 2013; Ben Maiz *et al.*, 1987; Mayhoub, 1976; Tsirika & Haritonidis, 2005), whereas in the western and northern Mediterranean, this stage has been observed only in the warmer months of the year at very few sites, including France

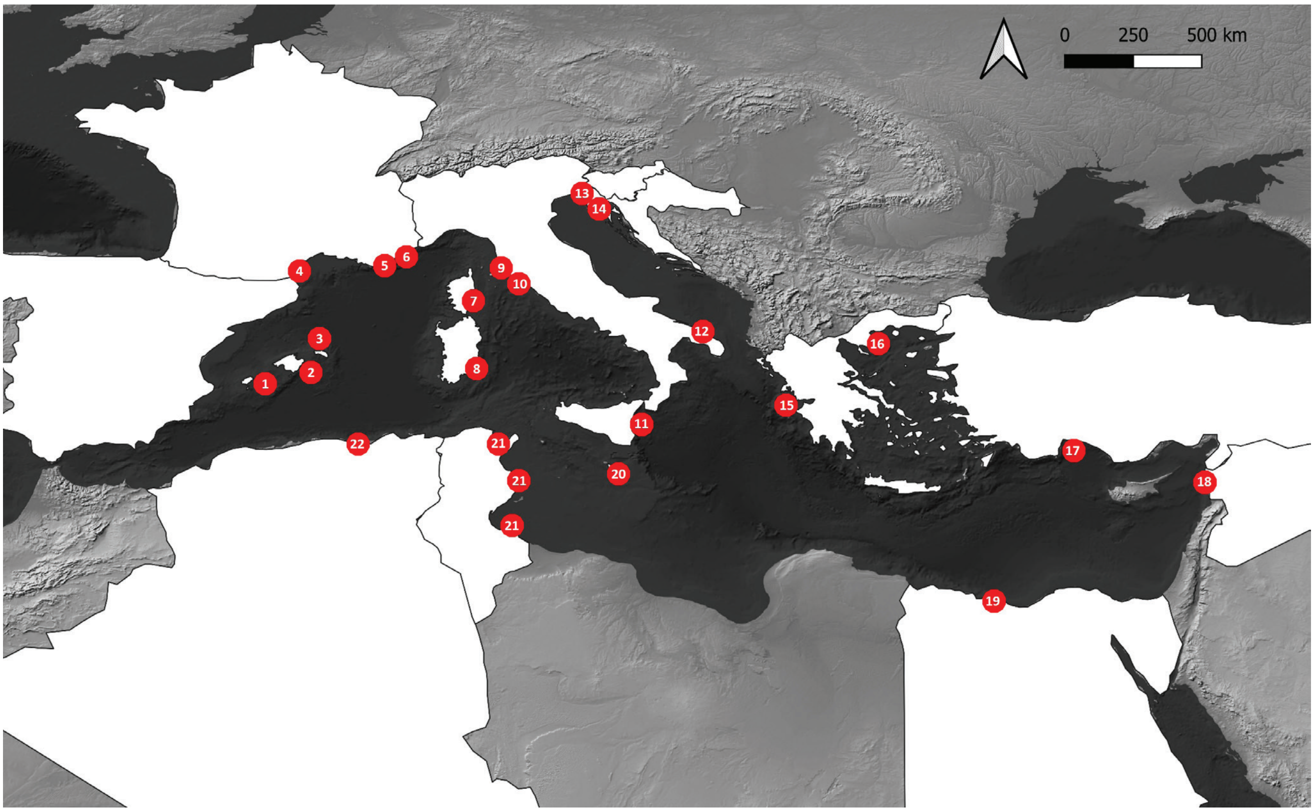

Fig. 1: Distribution of *P. capitatus* in the Mediterranean Sea on the basis of the following literature data: **Algeria** [(21) Perret-Boudouresque & Seridi, 1989]; **Croatia** [(14) Buosi, 2015]; **Egypt** [(19) Bergin, 1987]; **France** [(5) Huvé & Huvé, 1964; (6) Meinesz, 1972; (7) Boudouresque & Perret-Boudouresque, 1987; (4) Boudouresque *et al.*, 2024]; **Greece** [(16) Athanasiadis, 1987; (15) Tsirika & Haritonidis, 2005]; **Italy** [(8) Cossu & Gazale, 1996; (12) Furnari *et al.*, 1999; (9) Rindi *et al.*, 2002; (10) Sfriso 2010; This study (11)]; **Malta** [(20) Bilecenoglu *et al.*, 2013]; **Spain** [(1) Ballesteros 1992; (2) Ribera *et al.*, 1997; (3) Cerrato *et al.*, 2023]; **Slovenia** [(13) Digenis *et al.*, 2024]; **Syria** [(18) Mayhoub, 1976]; **Tunisia** [(21) Ben Maiz *et al.*, 1987]; **Turkey** [(17) Turna *et al.*, 2010].

(Huvé & Huvé, 1964; Meinesz, 1972; Boudouresque & Perret-Boudouresque 1987; Boudouresque *et al.*, 2024), Spain (Ballesteros, 1992; Ribera *et al.*, 1997; Cerrato *et al.*, 2023), Italy (Rindi *et al.*, 2002; Furnari *et al.*, 1999; Cossu & Gazale 1996; Sfriso (2010), Slovenia (Digenis *et al.*, 2024), and Croatia (Buosi, 2015). Recently, the increasing frequency of the typical stage in the Mediterranean has been related to environmental conditions similar to those of tropical areas, suggesting that the presence of this stage of *P. capitatus* may be a result of global climate change (Turna *et al.*, 2010; Boudouresque *et al.*, 2024).

Although *P. capitatus* has been found in several areas of the Mediterranean Sea, a genetic characterisation of the specimens found in this basin has never been performed, and consequently, a molecular comparison with the Atlantic species has not been possible.

The aims of this study were as follows: i) to report a population of *P. capitatus* in the Ionian Sea (central Mediterranean), showing the typical stage during the seasons of the year when this form had never been documented before; ii) to examine the possible role of this species as a climatic bioindicator; and iii) to molecularly compare the Mediterranean taxon and tropical species.

Materials and Methods

Sampling area

This study was conducted along the southern coast of the Magnisi Peninsula (37.151741 °N, 15.229357 °E). This area is located in the municipality of Priolo-Gargallo (Syracuse, eastern Sicily, Ionian Sea) (Fig. 2), and it is subject to various anthropogenic stresses related mainly to the discharge of high levels of industrial wastewater, especially in the major petrochemical industrial area. The surface water temperature generally varies between a minimum of 15 °C in winter (February) and a maximum of approximately 28 °C in summer (August) (CMEMS, 2021).

Sampling and identification

From 2020 to 2022, visual census (VC) activities were conducted near the Magnisi Peninsula in an extensive meadow of *P. capitatus* mixed with *Cymodocea nodosa* (Ucria) Ascherson, *Halophila stipulacea* (Forsskål) Ascherson, and *Posidonia oceanica* (L.) Delile by snorkelling. The community was found on sandy substrates at depths of 2–4 m. The VC was executed by obtaining photos via an Olympus Tg-6 underwater camera in January,

Fig. 2**:** The sampling site, which corresponds to the 11th circle depicted in Figure 1, is located along the southeastern coast of Sicily (Ionian Sea).

July, and November. During the VC activities, the seawater temperature was measured via an Aqualung i200C dive computer.

During every VC activity, twenty specimens of *P. capitatus* were randomly collected and preserved in 3% buffered formalin in seawater for identification at the Laboratory of Phycology of the University of Catania. Ten specimens were held in the Herbarium (CAT-section algae) of the Department of Biological, Geological, and Environmental Sciences of the University of Catania (Italy), whereas the other ten specimens were preserved in silica gel and used for molecular analysis.

DNA isolation and sequencing

DNA was extracted following the protocol of Allen *et al.* (2006), and 1% polyvinylpyrrolidone (PVP, w/v, Mr. 10,000) was added to the extraction buffer to remove the released phenolic compounds. For three *Penicillus* specimens, we amplified the *tufA*, *rbcL,* and 18S rDNA regions via primers from Händeler *et al.* (2010), Verbruggen *et al.* (2009), and Kooistra (2002), respectively (Table 1). To amplify the 18S rDNA region, in contrast to the methods used by Verbruggen *et al.* (2009) and Kooistra (2002), we combined SS09HF and ITS2. Amplification reactions of 20 µL were performed using 2 µL of Buffer 10X (Thermo ScientificTM), 0.2 mM dNTPs, 10 µM of each primer, 1 unit of Taq Polymerase (DreamTaq, Thermo ScientificTM), and 2 µL of template DNA. The amplification reactions were performed in a thermocycler (Eppendorf Mastercyler Nexus, Hamburg, Germany) with the following profile: 2 min at 92 °C (denaturation); 35 cycles of denaturation for 35 s at 94 °C, annealing for 1 min at 44 °C (for *tufA*), 48 °C (for *rbc*L), or 43 °C (for 18S rDNA); extension for 1 min at 72 °C; and a final extension for 10 min at 72 °C. The PCR products were cleaned via the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced along both directions with the PCR primers. To identify the relatedness of our specimens with the *Penicillus* genus and the phylogenetic neighbourhood, we performed a BLASTn search with the obtained sequences to retrieve the most similar sequences from the GenBank database to be added to our set for phylogenetic analysis. All the sequences were aligned with the MUSCLE algorithm of MEGA 11 software (Tamura *et al.*, 2021), which was also employed for the downstream phylogenetic analyses. We used sequences of *Udotea*, *Penicillus*, *Rhipocephalus*, and *Chlorodesmis* obtained from the GenBank database (Lam & Zechman, 2006; Verbruggen *et al.*, 2009; Lagourgue *et al.,* 2018). A maximum likelihood analysis was conducted with a Jukes–Cantor model and 1,000 bootstrap replicates for branch support.

Results

During the visual census activities, we observed extensive populations of *P. capitatus* of about 400 square metres on sandy substrates, characterised mainly by *P. oceanica*, *Cymodocea nodosa,* and *Halophila stipulacea* meadows, together with other macrophytes such as *Caulerpa cylindracea* Sonder, *Caulerpa taxifolia* v. *distichophylla* (Sonder) Verlaque, Huisman *et* Procaccini, *Dasycladus vermicularis* (Scopoli) Krasser, and *Cystoseira foeniculacea* (Linnaeus) Greville.

The typical stage of *P. capitatus* was detected in each of the sampling months: in January, together with the *Espera* stage, when the seawater temperature was 16 °C (Fig. 3a-b); in July, when the seawater temperature was 27 °C (Fig. 3c-d); and in November, when the seawater temperature was 20 °C (Fig. 3e-f).

Table 1. Oligonucleotide primers used for amplification and sequencing of the three loci used in this study.

Marker name	5'-3' primer sequence	Source
rbcL-7F	CCAMAAACWGAAACWAAAGC	Lam and Zechman (2006); Verbruggen *et al.* (2009)
rbcL-791 R	GGNAYACCNAAWTCTTTIGC	
rbcL-1391R	TCTTTCCAAACTTCACAAGC	
rbcL-712F	CATTAYTTAAATGCWACWGC	
HtufAF	ATGATWACNGGHGCNGCWCAAATGG	Händeler *et al.* (2010)
HtufAR	TTGTTCKAACATAAAATTGWGGTC	
TW3F	GCAAGTCTGGTGCCAGCATCT	Kooistra (2002); Verbruggen *et al.* (2009)
SS17HR	CCTTGTTACGACTTCTCCTTCCTC	
SS09HF	GGTGAAATTCTTGGATTTRYGRAAGAC	
ITS2	GCTACGTCCTTCATCGACGC	

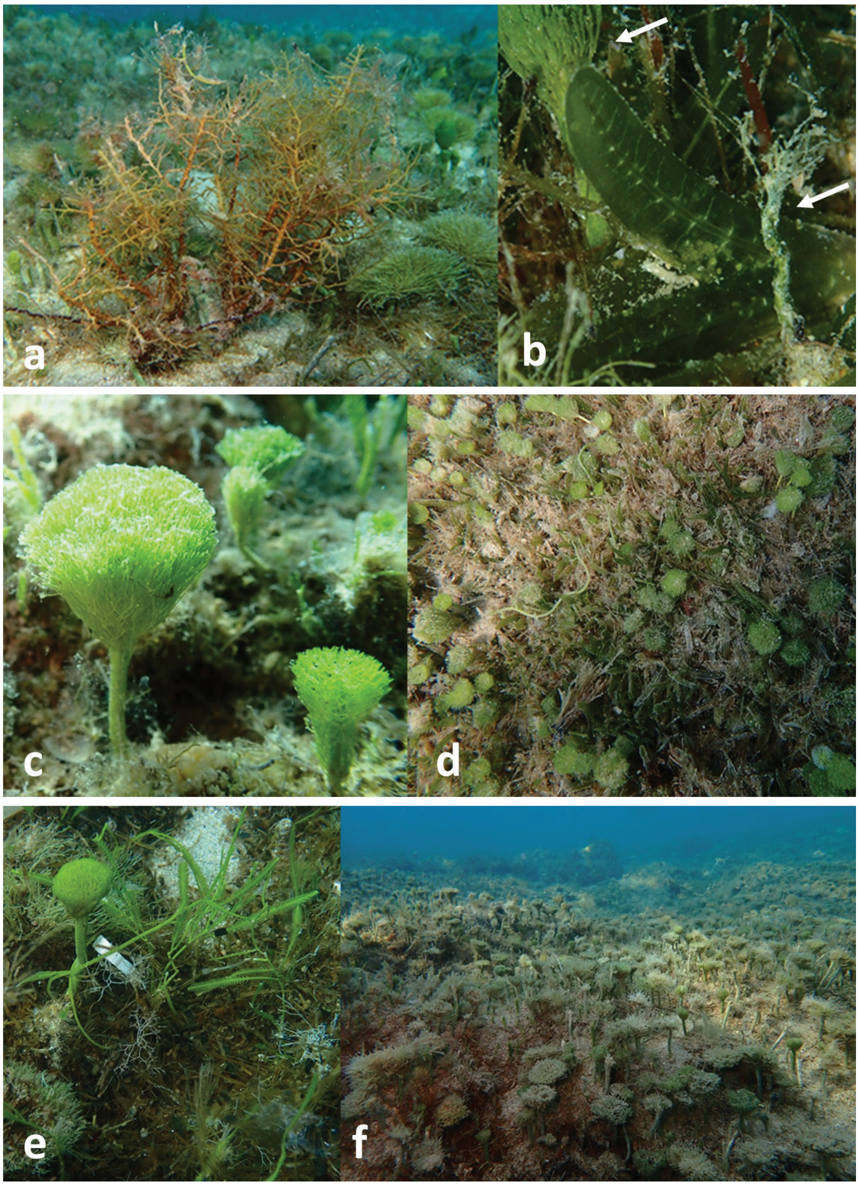

***Fig. 3*:** Photos obtained during visual census activities: a-b *P. capitatus* in January; b the arrows point to the *Espera* (on the right) and typical stages (on the left); c-d show the typical stage in July; e-f show the typical stage in November.

Fig. 4: Herbarium sheet showing the characteristic features of *P. capitatus* (B = base; S = stalk; C = capitulum).

Fig. 5: Siphon of the stalk with radial branches referred to as crampons or lobules (arrows).

Morphological observations

The collected thalli demonstrated the characteristic features of the typical stage of *P. capitatus* described by Turna *et al.* (2010) and Cormaci *et al.* (2014): a brush habit, which consists of interwoven coenocytic filaments forming the three distinct regions of the thallus (Fig. 4). The base consists of rhizoidal, descending syphons that are prostrate and mostly colourless, which are highly branched and can trap grains of sand. The stalk, which partially penetrates the *capitulum*, consists of a medullary part and a cortex. The first part consists of a tangle of ascending siphons longitudinally disposed of and provided by radial branches referred to as crampons or lobules (Fig. 5), which together constitute the cortex. The *capitulum* consists of single, dichotomously ramified syphons with a rounded apex.

Molecular analyses

A total of 9 new sequences were generated in this study for the collected specimens (NCBI submission ID 2804785), with 3 sequences for each genetic marker (*rbcL*, 1,161–1,198 bp; *tufA*, 680–698 bp; *18S rDNA*, 734–1397 bp). The final alignment of the three genetic markers used was 1,324 bp and 19 sequences for *rbcL*, 2,222 bp and 23 sequences for 18S rDNA, and 839 bp

Table 2. GenBank accession numbers of the marker sequences downloaded from the NCBI database used for phylogenetic analyses.

Taxa	Locality	GenBank accession number
Udotea cyathiformis	Panama	AF416403
Penicillus pyriformis	Panama	AF416410.1
Penicillus pyriformis	Panama	AF416409.1
Udotea looensis	Florida	AF416397.1
Penicillus dumetosus	Panama	AF416407.1
Penicillus pyriformis	Bermuda	AF416408.1
Penicillus dumetosus	Panama	AF416406.1
Chlorodesmis caespitosa	Panama	AF416400.1
Chlorodesmis caespitosa	Panama	AF416399.1
Udotea orientalis	South Africa	AF416395.1
Penicillus nodulosus	Australia	AF416398.1

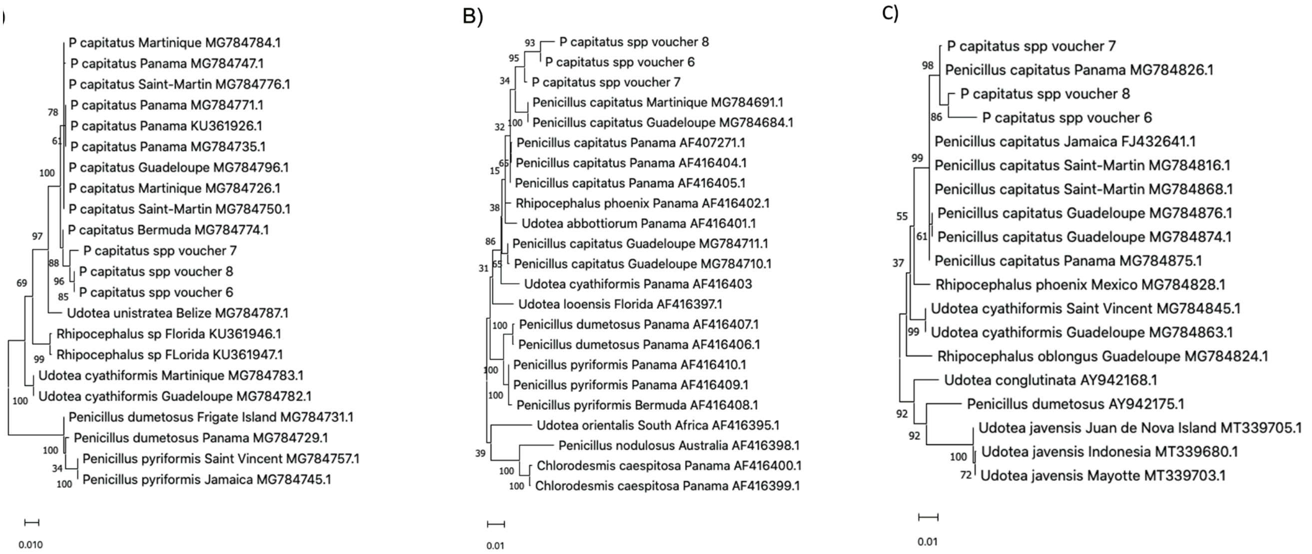

Fig. 6: Phylogenetic trees inferred from the *tufa (A)*, *rbcL (B),* and 18S rDNA *(C)* regions. ML values are indicated on the branches. Only values above 60 were considered.

and 22 sequences for *tufA*. The final alignments included the sequences obtained from GenBank, as reported in Table 2. The phylogenetic analysis results confirmed that the collected Mediterranean specimens belonged to the *Penicillus capitatus* species. In fact, all the vouchers analysed in this study were clustered together with other *P. capitatus* sequences, with the 18S rDNA marker including *P. capitatus* from Panama within the Mediterranean cluster (Fig. 6).

Discussion

Our morphological and molecular analysis data confirmed that the studied specimens corresponded to *P. capitatus* observed in the western Atlantic, the area where the genus *Penicillus* reached its maximum diversity (Guiry & Guiry, 2023). On the basis of the available data, it is not possible to formulate concrete hypotheses regarding the origin of this species in the Mediterranean. However, it is possible that for *P. capitatus,* the same scenarios assumed by Rindi *et al.* (2020) for *Halimeda tuna* (J.Ellis *et* Solander) J.V. Lamouroux and by Varela-Álvarez *et al.* (2015) for *Caulerpa prolifera* (Forsskål) J.V. Lamouroux are replicated. These authors suggested two main hypotheses regarding the origin of *H. tuna* and *C. prolifera* in this basin. Both assumptions refer to the period of closure of the Strait of Gibraltar (Miocene, ca. 5–6 Mya), which led to the occurrence of the Messinian salinity crisis in the Mediterranean, with the formation of separate hypersaline basins. *H. tuna* (Rindi *et al.*, 2020) and *C. prolifera* (Varela-Álvarez *et al.*, 2015) may have survived during the Messinian crisis in one of those basins, or they may have gone extinct during this period and subsequently recolonised the Mediterranean from Atlantic areas with the reopening of the Strait of Gibraltar. Therefore, both of these hypotheses could be possible for *P. capitatus*. In addition, more recent Mediterranean east–west disconnection and environmental heterogeneity hypothesised earlier (Orfanidis & Breeman, 1999) and verified recently (Konstantinidis *et al.,* 2022) date back to the transition from the Pleistocene to the Holocene and the postglacial era.

As mentioned above, *P. capitatus* exhibits two morphological stages. To date, in the Mediterranean, the typical stage is associated with the warmest months of the year, from late summer to the beginning of autumn, suggesting that only during this period does this species encounter the optimal conditions to reach the adult stage (Turna *et al.*, 2010; Boudouresque *et al.*, 2024). However, our observation of this stage in the coldest months could also indicate a rise in the seawater temperature, which enabled this form to develop in winter. Nevertheless, the study area exhibits a particular coastline conformation because it is an enclosed bay with shallow water, scarce wave motion, and low water exchange (Cormaci *et al.*, 1985). Therefore, the general increase in the seawater temperature might be locally amplified here. Moreover, another factor that may influence the local seawater temperature is the thermal effect linked to cooling water spills from petrochemical factories and industries in neighbouring areas. Additionally, in Malta (Bilecenoglu *et al.*, 2013), patches characterised by *P. capitatus* were found in bays where thermal effluents from electrical power stations and intense aquaculture activity were discharged. The occurrence of *P. capitatus* is also related to the presence of brackish water (Collado-Vides *et al.*, 2011). Previously, Cormaci *et al.* (1985) highlighted that the polluting discharges from neighbouring areas, such as Augusta (Syracuse), were characterised by freshwater features or a lower salinity. Therefore, the presence of large populations of *P. capitatus* could also be related to the relatively low salinity caused by industrial effluents. These impacts, together with the global rise in the seawater temperature, might involve variations in phytobenthic assemblages, allowing the establishment of warm-water and nonindigenous species. Indeed, *P. capitatus* was found in association with the alien seagrasses *H. stipulacea*, *C. cylindracea,* and *C. taxifolia* var. *distichophylla*. This pattern, according to Sangil *et al.* (2010) and Picciotto *et al.* (2016), may reproduce a warm-water association, suggesting a shift and reorganisation of Mediterranean macrophytobenthic assemblages due to global change (Mannino *et al.*, 2019). Thus, the finding of the typical stage of *P. capitatus* during a longer period of the year could represent the first evidence of changes in macrophytobenthic assemblages. This may promote the establishment of warm-affinity species, endangering native species that are less competitive under these new environmental conditions, as already noted by Boudouresque *et al.* (2024).

In conclusion, the observation of the typical stage of *P. capitatus* in the Mediterranean Sea during cold seasons could indicate a general increase in the seawater temperature in this basin. Therefore, this species could be considered a climatic bioindicator. Furthermore, owing to its presence at sites with industrial wastewater effluents, *P. capitatus* may be employed as an indicator species of environmental and ecosystem alterations.

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