Mediterranean Marine Science

Polyclinum constellatum (Tunicata, Ascidiacea), an emerging non-indigenous species of the Mediterranean Sea: integrated taxonomy and the importance of reliable DNA barcode data

FEDERICA MONTESANTO, GIOVANNI CHIMIENTI, CARMELA GISSI, FRANCESCO MASTROTOTARO

Research Article

Polyclinum constellatum (Tunicata, Ascidiacea), an emerging non-indigenous species of the Mediterranean Sea: integrated taxonomy and the importance of reliable DNA barcode data

Federica MONTESANTO[1,2], Giovanni CHIMIENTI[1,2], Carmela GISSI[2,3,4] and Francesco MASTROTOTARO[1,2]

[1] Department of Biology, University of Bari "Aldo Moro", Via E. Orabona, 4, 70125, Bari, Italy

[2] CoNISMa, Piazzale Flaminio 9, 00197, Roma, Italy

[3] Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Italy

[4] Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies (IBIOM), CNR, Via Amendola 165/A, 70126 Bari, Italy

Corresponding author: Federica MONTESANTO; federica.montesanto@uniba.it

Contributing Editor: Xavier TURON

Abstract

Polyclinum constellatum is a colonial ascidian with a pantropical distribution, recently introduced into the Eastern Mediterranean Sea, indeed it was reported along Egyptian and Turkish coasts in 2016 and 2018, respectively. In the present study we report its presence along the coasts of Greece and Italy (Eastern and Central Mediterranean Sea, respectively), using an integrated approach combining morphological and molecular analysis. Colonies of *P. constellatum* were collected from artificial substrata in the harbour of Taranto (Ionian Sea) in November 2018 and in the marina of Heraklion (Crete, Aegean Sea) in October 2019. Remarkably, several colonies observed and collected in the Heraklion marina appeared as two or more masses joined at their base or fused together, often with different colour morpho-types. Here we provide their detailed morphological description and molecular characterization using a long fragment of the mitochondrial COI sequence as a DNA barcode. Furthermore, we present and discuss a comparative table of the main morphological features of all species of the genus *Polyclinum* known to date and an accurate analysis of the reliability of the *Polyclinum* COI sequence currently available. Our study proves that *P. constellatum* is further spreading in the Eastern Mediterranean Sea and has already reached the Central Mediterranean Sea. Moreover, the present study reveals the presence of erroneously assigned *Polyclinum* COI sequences in public databases and a possible synonymy between the species *P. constellatum*, *Polyclinum indicum* Sebastian, 1954 (accepted as *Polyclinum sebastiani* Brunetti, 2007) and *Polyclinum madrasensis* Sebastian, 1952. Overall, our data provide a useful tool for the accurate and reliable identification of this expanding species in other non-investigated areas and suggest the most likely vector of introduction of this non-indigenous species in the investigated localities.

Keywords: Ascidian; Non-Indigenous Species; COI; DNA barcoding; Mediterranean Sea.

Introduction

Ascidians (Tunicata, Ascidiacea) are one of the most frequent and successful taxa introduced in artificial habitats (Zhan *et al.,* 2015). Indeed, rapid growth, a long reproductive span period, high resistance to stress and reproduction by asexual fragmentation, which occurs in many colonial species (Aldred & Clare, 2014), make this taxon the "perfect" invader (Lambert, 2007). Moreover, ascidians can be transported over long distances (Clarke Murray *et al.,* 2011) as biofouling species attached to the hulls of boats or as lecithotrophic larvae in ballast waters. Marine infrastructure, such as piles, pontoons, and aquaculture facilities, provide new free substrata for the settling of Non-Indigenous Species (NIS), acting as regional corridors and increasing their spread potential at both a local and on the global level (Airoldi *et al.,* 2015; López-Legentil *et al.*, 2015). Massive overgrowth of non-indigenous ascidians has been documented causing severe impacts on marine infrastructure, aquaculture, and native benthic communities (Lambert, 2007; Aldred & Clare, 2014; Mastrototaro *et al*., 2015; Montesanto *et al.*, 2021).

The number of NIS identified in the Mediterranean Sea is currently increasing (Servello *et al.*, 2019). This is particularly true for ascidians (Ragkousis *et al.*, 2020; Orfanidis *et al.*, 2021), for which the lack of taxonomists, the often-ambiguous taxonomy, the presence of many cryptic and rare species (Petović *et al.*, 2018; Mastrototaro *et al*., 2019a, 2020a, b; Santin *et al*., 2021) and the consequent frequent misidentification of samples are among the main limiting factors for the identification of

Medit. Mar. Sci., 23/1 2022, 69-83

69

ascidian NIS (Izquierdo-Muñoz *et al.*, 2009). In this context, an integrated approach combining at least morphological and molecular data has often been proved to be essential for solving taxonomical issues (Mastrototaro *et al.*, 2019b, 2020b; Rocha *et al.*, 2019; Montesanto *et al.*, 2021; Salonna *et al.*, 2021).

Another important concern is that several ascidian species are considered cryptogenic, i.e., of obscure or uncertain origin, hence it cannot be proven whether they are native or have been introduced into a certain area, leading to a possible underestimation of the overall number of NIS (Carlton, 1996).

In the Central Mediterranean Sea, the Gulf of Taranto (Italy, Ionian Sea) is a well-known NIS hotspot (Mastrototaro *et al.,* 2003; Gravili *et al.,* 2010; Zenetos *et al.,* 2010; Occhipinti-Ambrogi *et al.,* 2011; Marchini *et al.,* 2013; Servello *et al.,* 2019) including many ascidian species (Mastrototaro & Brunetti, 2006; Mastrototaro *et al.,* 2008, 2019b). In the Eastern Mediterranean Sea, Greek waters are especially susceptible to biological invasions because of their crossroad position between the Western Mediterranean and the Black and Red Seas, but also due to the high level of maritime traffic and the presence of several human activities. Recent studies (Ulman *et al.,* 2017; 2019) highlighted the occurrence of several NIS in Heraklion marina (Crete, Aegean Sea), including ascidians (Ulman *et al.*, 2017, 2019; Ragkousis *et al.*, 2020).

The colonial ascidian *Polyclinum constellatum* Savigny, 1816 was first described based on specimens collected from Mauritius Island (South Africa, Indian Ocean). Nowadays, this species is considered as a cryptogenic species (Dias *et al.,* 2013), with a pantropical distribution characterized by a wide range of occurrences including the following biogeographic regions: Tropical Western Atlantic (Van Name, 1945; Millar, 1958; Monniot, 1972; Monniot, 1983; Goodbody, 1984; Rocha & Costa, 2005; Rocha *et al.*, 2010; Carman *et al.*, 2011; Fortaleza & Lotufo, 2018), Western Indian Ocean (Millar, 1955; Monniot & Monniot, 1997a, 1997b; Shenkar, 2012; Meenakshi & Gomathy, 2017) and Indo-pacific region (Kott, 1963; Tokioka, 1967; Monniot & Monniot, 1976; Lambert, 2003). *Polyclinum constellatum* has also been recently reported along Egyptian and Turkish coasts of the Eastern Mediterranean Sea, where it is considered a NIS (Halim & Messeih, 2016; Aydin-Onen, 2018).

Here we report the two westernmost records of this species from two marinas, Taranto and Heraklion, confirming the spreading of this species into other localities of the Eastern and Central region of the Mediterranean basin. We provide a detailed morphological description of the collected specimens, together with a systematic review and a tabular key of the main features of all the known species belonging to the genus *Polyclinum.* Furthermore, we report the molecular characterization of the collected colonies, based on a fragment of about 860 bp of the mitochondrial Cytochrome Oxidase subunit I (COI) gene, used as a DNA barcode, as well as revealing the presence of erroneously assigned *Polyclinum* COI sequences in public nucleotide databases and a possible synonymy between *P. constellatum*, *Polyclinum indicum*

Sebastian, 1954 (accepted as *Polyclinum sebastiani,* Brunetti 2007) and *Polyclinum madrasensis* Sebastian, 1952. This study expands the knowledge of the composition and distribution of alien ascidian species in the Mediterranean Sea, underlining the importance of an integrated approach, combining molecular and morphological analyses, for the identification of NIS.

Material and Methods

Sampling

Colonies of *P. constellatum* were photographed and collected from anthropogenic substrata present in Taranto harbour (Gulf of Taranto, Mar Grande, Ionian Sea; November 2018) and in the marina of Heraklion (Aegean Sea, Greece; October 2019) (Fig. 1). Several colonies were observed, and ten colonies were collected from ropes and piers by SCUBA diving at a depth of 1-4 m from each sampling site. Sampled colonies were relaxed with menthol crystals in seawater for approximately four hours (until no contraction of the zooids was detected) and preserved in a 4% formaldehyde solution in seawater. A subsample of each colony was also preserved in 99% ethanol for DNA extraction.

Morphological analysis

Morphological analyses were carried out considering the external and internal characteristics of the colonies (e.g., shape, colour, zooid arrangement, presence of sand within or only on the tunic surface) and the features of the zooids (e.g., presence of intermediate papillae in the pharynx, number of rows of stigmata, number of anal lobes) *sensu* Kott (1963). Moreover, the morphological features of collected specimens were compared to those belonging to the 47 *Polyclinum* species known thus far (Table 1).

COI analyses

Total DNA of the colonies of *P. constellatum* collected in Taranto (labelled P1) and Heraklion (labelled K7, K11, K12, K19, and K34) was extracted from 6-7 zooids of each subsample preserved in 99% ethanol. The DNA extraction was performed by using a modified CTAB method for sample P1 (Taranto) (Hirose & Hirose, 2009) and the Qiagen Blood & Tissue DNA kit for the samples collected in Heraklion marina. A 860 bp fragment of COI was amplified with the primer pair dinF/Nux1R or with a nested-PCR strategy where a first PCR with dinF/Nux1R was followed by a nested PCR with the cat1F/ux1R primer pair (Salonna *et al.,* 2021). Amplifications were carried out with the high fidelity PrimeStar HS polymerase (Takara Bio Inc.) or the DreamTaq polymerase (Thermo Fisher Scientific).

Amplifications with PrimeStar HS polymerase were

70

Medit. Mar. Sci., 23/1 2022, 69-83
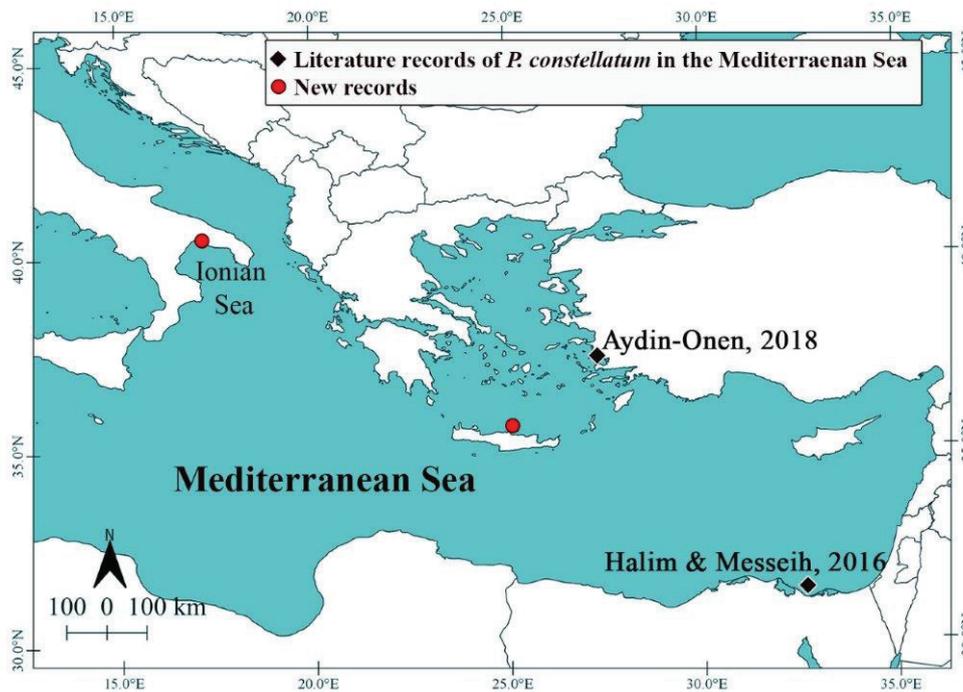
Fig. 1: Map of the Mediterranean Sea showing the literature records (black rhombuses) of *P. constellatum* and the new findings (red dots).

performed in a final reaction volume of 25 μl containing: 1X reaction buffer with 1 mM final concentration of $MgCl_2$ (Takara Bio Inc.), 0.2 mM of each dNTP, 0.3 μM of each primer and 1.25 Units of PrimeStar HS (Takara Bio Inc.). Amplification conditions were: 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 46°C or 50°C, extension for 1 min 30 s at 72°C; a final elongation step of 5 min at 72°C.

PCRs with the DreamTaq polymerase were performed in a final volume of 25 μl containing: 1X reaction buffer with 2 mM final concentration of $MgCl_2$ (Thermo Fisher Scientific), 0.2 mM of each dNTP, 0.4 μM of each of the two primers, and 1.25 Units of DreamTaq polymerase (Thermo Fisher Scientific). The amplification conditions were as follows: an initial denaturation for 3 min at 95°C, then 34 amplification cycles (denaturation for 30 s at 95°C; annealing for 30 s at 46-50°C; extension for 1 min 30 s at 72°C) followed by a final elongation step of 5 min at 72°C.

The obtained amplicons were purified with the DNA Clean&Concentrator kit (Zymo Research) and directly sequenced according to the Sanger method by Microsynth AG (Switzerland). The sequence quality check, comparisons and alignment were carried out with Geneious ver. 5.5.7.2 (Kearse *et al.,* 2012). The sequences obtained were deposited in the GenBank database (see Accession numbers MT873559 and OL597608). For comparative analyses, homologous sequences of the genus *Polyclinum* were searched for in the non-redundant nucleotide database (nr-nt db, on 21st September 2021) of the NCBI (National Center for Biotechnology Information) by Entrez text search, and by BLASTn (Altschul *et al.,* 1990) using our *P. constellatum* sequences as the query.

Uncorrected pairwise distances were calculated with PAUP 4.0a (Swofford, 2002), while a Maximum Likelihood (ML) phylogenetic tree of the genus *Polyclinum* ge-

nus was reconstructed with the online software PHYML v3.0 (http://www.atgc-montpellier.fr/phyml-sms/) (Guindon & Gascuel, 2003), which also includes the automatic model selection algorithm SMS (Smart Model Selection). The best-fit substitution model was selected using the Akaike Information Criterion (AIC). Bootstrap values, indicating node reliability, were based on 100 replicates. The sequence dataset used for this phylogenetic reconstruction is reported in Supplementary Table S1 and was extracted from the phylogenetic dataset published in Tabudravu *et al.* (2019). It includes representative species of the Polyclinidae family plus *Eudistoma* and *Pseudodistoma* species chosen as outgroups for their morphological similarities with Polyclinidae.

Results

Morphological analyses

The colonies collected in Taranto harbour and Heraklion marina were all morphologically identified as *P. constellatum* based on the following features: colonies without sand in/outside, zooids arranged in systems, post-abdomen (without vascular stolon) shorter than the thorax and abdomen combined, pharynx with 16-18 rows of stigmata, more than 15 stigmata per row, and a 6-lobed anus. These characteristics are in accordance with the key of *Polyclinum* species edited by Kott (1963) and they are also reported in the description of the species made by Van Name (1945).

Medit. Mar. Sci., 23/1 2022, 69-83

71

Table 1. Key to species belonging to the genus *Polyclinum*. In bold are reported the *P. indicum, P. madrasensis* and *P. constellatum* species. Dash indicates unavailable data.

1	2	3	4	5	6	Species
C	P	10	-	-	C, E	*P. reticulatum* Sluiter, 1915
B	P	10	10	A	-	*P. circulatum* Jensen, 1980
E	P	12–13	12–14	-	S	*P. fungosum* Herdman, 1886
C	P	11–13	12	-	C	*P. lagena* Monniot F. & Monniot C., 2006
C, E	P	11–15	14	A	C, E	*P. isipingense* Sluiter, 1898
E	P	11	15	P	S	*P. arenosum* (Sluiter, 1898)
E	P	15	13–15	A	C, E	*P. incrustatum* Michaelsen, 1930
E	-	14–16	18–20	P	-	*P. corbis* Kott, 2003
C	P	10–16	-	A	C	*P. aurantium* Milne-Edwards, 1841
B	P	12–14	10-12	A	C	*P. maeandrium* Sluiter, 1915
B	A	12	12–15	A	C	*P. molle* Rocha & Costa, 2005
C	P	14–16	16	A	C	*P. nudum* Kott, 1992
E	P	12–13	16–17	P	C, E	*P. marsupiale* Kott, 1963
C	P	12–13	18–19	-	-	*P. laxum* Van Name, 1945
E, B	P	11–14	10–12	A	S	*P. tingens* Monniot F., 2012
E	P	13	12	-	C, E	*P. tenuatum* Kott, 1992
E	P	11–13	13–15	P	-	*P. ramosum* Parker-Nance, 2003
C, B	P	8	20-25	P	C	*P. pute* Monniot C. & Monniot F., 1987
B	P	11–15	16	A	C, E, S	*P. neptunium* Hartmeyer, 1912
C, E	-	15–18	18–20	A	C, E, S	*P. sluiteri* Brewin 1956
E	P	8–10	18	-	S	*P. pedicellatum* Monniot F., 2012
C, B	A	13–18	14	-	-	*P. campi* Fortaleza & Lotufo, 2018
C	P	15–18	-	A	S	*P. festum* Hartmeyer, 1905
C	A	16–18	14–17	-	-	*P. cerebrale* Michaelsen, 1924
E	P	16	15	P	C, E	*P. terranum* Kott, 1992
C	P	8-12	20	A	C, S	*P. saturnium* Savigny, 1816
C	-	16	17	A	C, E, S	*P. michaelseni* Brewin, 1956
E	P	14–16	18–20	P	C, E	*P. tsutsuii* Tokioka, 1954
E	P	12–18	22	A	C	*P. psammiferum* Hartmeyer, 1911
C	P	18–19	19–20	-	C, E, S	*P. novaezelandiae* Brewin, 1958
C	P	18–20	22–24	A	S	10*P. orbitum* Kott, 1992
C	**-**	**13–15**	**14–16**	**-**	**-**	***P. indicum* Sebastian, 1954 (accepted as *P. sebastiani* Brunetti, 2007)**
-	P	14–15	7–8	-	C, E	*P. isiacum* Savigny, 1816
-	-	10	10–12	-	C, E	*P. tralaticia* Sluiter, 1913
-	-	9	14–16	-	-	*P. meridianum* Sluiter, 1900
-	P	12	15	-	-	*P. sibiricum* Redikorzev, 1907
C	P	12	20–22	P	C, E, S	*P. sundaicum* (Sluiter, 1909)
A	A	8–9	-	-	S	*P. minutum* Herdman 1886
A	P	10	6–8	-	-	*P. crater* Sluiter, 1909
A	P	11–14	8–6	A	C, E	*P. vasculosum* Pizon, 1908
A	A	12–13	19	A	-	*P. glabrum* Sluiter, 1895
A	P	11–12	20	P	-	*P. sacceum* Monniot F. & Monniot C., 2006
A	**P**	**13**	**13**	**-**	**C**	***P. madrasensis* (Sebastian, 1952)**
A	P	13–17	13–14	-	C, E, S	*P. planum* (Ritter & Forsyth, 1917)
A	**P**	**16–18**	**16–18**	**A**	**C**	***P. constellatum* Savigny, 1816**
A	P	16–17	23–25	A	C, E	*P. johnsoni* Monniot C. & Monniot F., 1989
-	P	17–18	-	-	-	*P. hesperium* Savigny, 1816

1 Presence of sand: C = coated, E = embedded, B = at the base, A = absent; 2 Systems of zooids: P = present, A= absent; 3 Number of rows of stigmata; 4 Number of stigmata per half-row; 5 Incubatory pouch: P = present, A = absent; 6 Shape of the colony: C = cushion, E = encrusting, S = stalked, pedunculated, lobed.

72

Medit. Mar. Sci., 23/1 2022, 69-83

Systematics

Polyclinum constellatum Savigny, 1816

Polyclinum constellatum Savigny, 1816:189; Van Name, 1945:68; Gravier, 1955:620; Millar, 1955:176; 1958:498; 1962:62; 1975:257; Tokioka, 1961:104; 1967:53; Almeida Rodrigues, 1962:194; Rodrigues Da Costa, 1969:192; Vasseur, 1970:212; Monniot C. & Monniot F., 1976:358; Monniot F., 1972:958.

Material examined

Taranto (40.478966°N, 17.225649°E): ten colonies collected in Taranto harbour at 1 m depth, separately preserved in 4%formalin in seawater and 99% ethanol. One colony was examined from both the morphological and molecular point of view (P1), and for the others only the morphological characteristics were checked.

Heraklion (35.343300°N, 25.136614°E): ten colonies collected within the marina at 3-4 m depth, separately preserved both in 4%formalin and 99% in ethanol. Five colonies were examined from both the morphological and molecular point of view (K7, K11, K12, K34). The remaining five colonies were only checked for morphological characteristics.

Two colonies were deposited in the collection of the Zoological Museum of the University of Bari (MUZAC): one collected in Heraklion (MUZAC: 6661 preserved in 4% formalin and MUZAC: 6662 in 99% ethanol) and the other in Taranto (MUZAC: 6663 preserved in 4% formalin and MUZAC: 6664 in 99% ethanol).

Colonies

The colonies collected in Taranto show brown-orange colour (Fig. 2A-B; colony P1), while colonies collected from Heraklion show different colours from red-orange (colony K11) to purplish black (colony K12) or greenish-blue (colony K7) (Figs. 2C-D, 4A-C). Some of these different coloured colonies appear to be joined or almost-completely fused together (Fig. 4A-C; colonies K11 and K12). Most of the colonies are massive and almost rounded in shape, up to 10 cm in height and 20 cm in width. They are characterized by a thick firm tunic without sand embedded, with zooids located only around the outer edge (Fig. 2D). Systems of zooids are arranged along the common cloacal openings consisting of small conical projections of the outer tunic (Fig. 2A-C). The red-orange colonies and the dark ones appear greyish and black, respectively, after preservation in 4% formalin.

Zooids

The zooids are 7-9 mm in length, reaching almost 12-15 mm with the vascular stolon (Fig. 3A). The thorax is longer than the abdomen. The post-abdomen is slightly
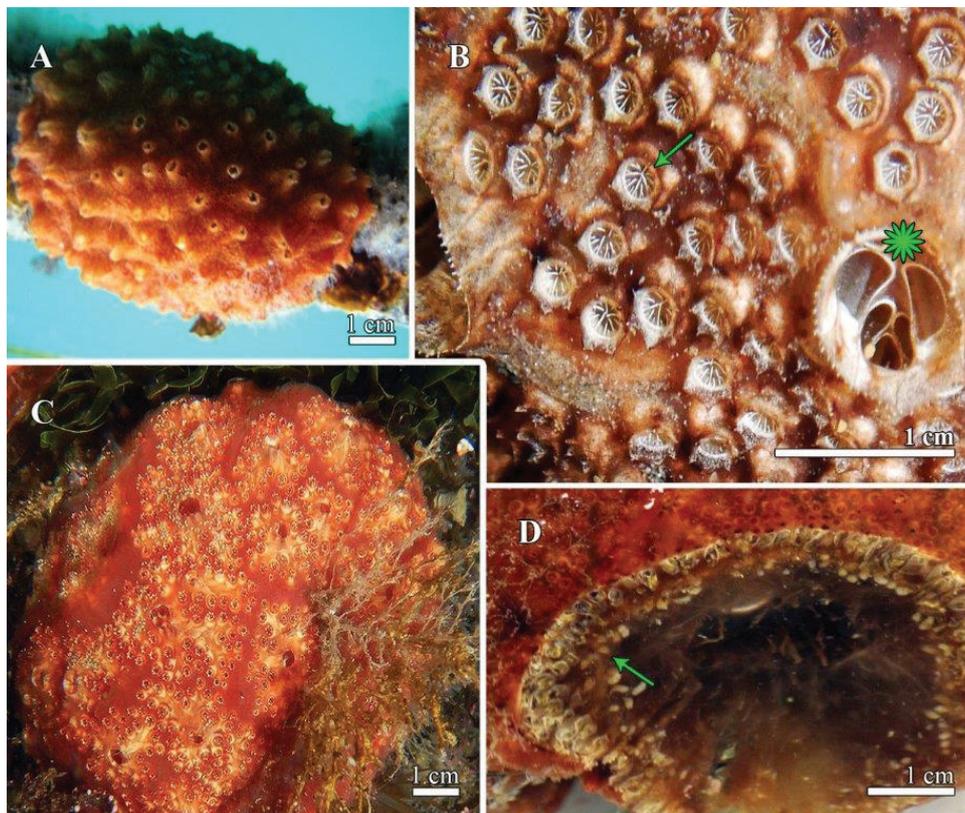
Fig. 2: A) Orange colony of *Polyclinum constellatum* from Taranto harbour (colony P1); B) Magnification of the oral (arrow pointing put the oral tentacles of different size) and cloacal aperture (asterisk); C) *P. constellatum* collected in Heraklion (colony K19) with zooids arranged in systems around the cloacal apertures; D) Section of the colony showing the zooids located only around the outer edge (arrow).

*Medit. Mar. Sci., 23/1 2022, 69-83*

73

longer than the abdomen and has a very long vascular stolon at its end (Fig. 3A-B). The branchial siphon ends with six pointed lobes and shows four orders of oral tentacles arranged regularly (Fig. 2B). The atrial siphon consists of a wide aperture with a long pointed atrial languet at its edge (Fig. 3A-B). The pharynx is characterized by 16–18 rows of stigmata, with about 16-18 stigmata per half-row (counted at the level of the 7th row) (Fig. 3B). The branchial transverse vessels show minute papillae not corresponding to the number of stigmata, but usually fewer in number (Fig. 3E). The dorsal lamina consists of narrow pointed languets (Fig. 3D). Six longitudinal muscles are visible near the branchial siphon (Fig. 3C) running towards the thorax on each side of the zooids. Up to 10 embryos can be incubated in the atrial cavity (Fig. 3B).

Alimentary canal

The abdomen contains the alimentary canal with a funnel-shaped oesophagus, followed by a smooth stomach and a twisted gut loop which lies almost horizontally (Fig. 3B). The rectum is long, extending to the middle of the thorax, ending in a six-lobed anus at the level of the 9th row of stigmata (Fig. 3B).

Post-abdomen

The sac-like post-abdomen is connected to the abdomen by a narrow stalk and contains the gonads and the heart (Fig. 3B). The testis consists of a cluster of follicles joined by ducts to the ovary lying among the male follicles, while the gonoducts end close to the anus (Fig. 3B). The
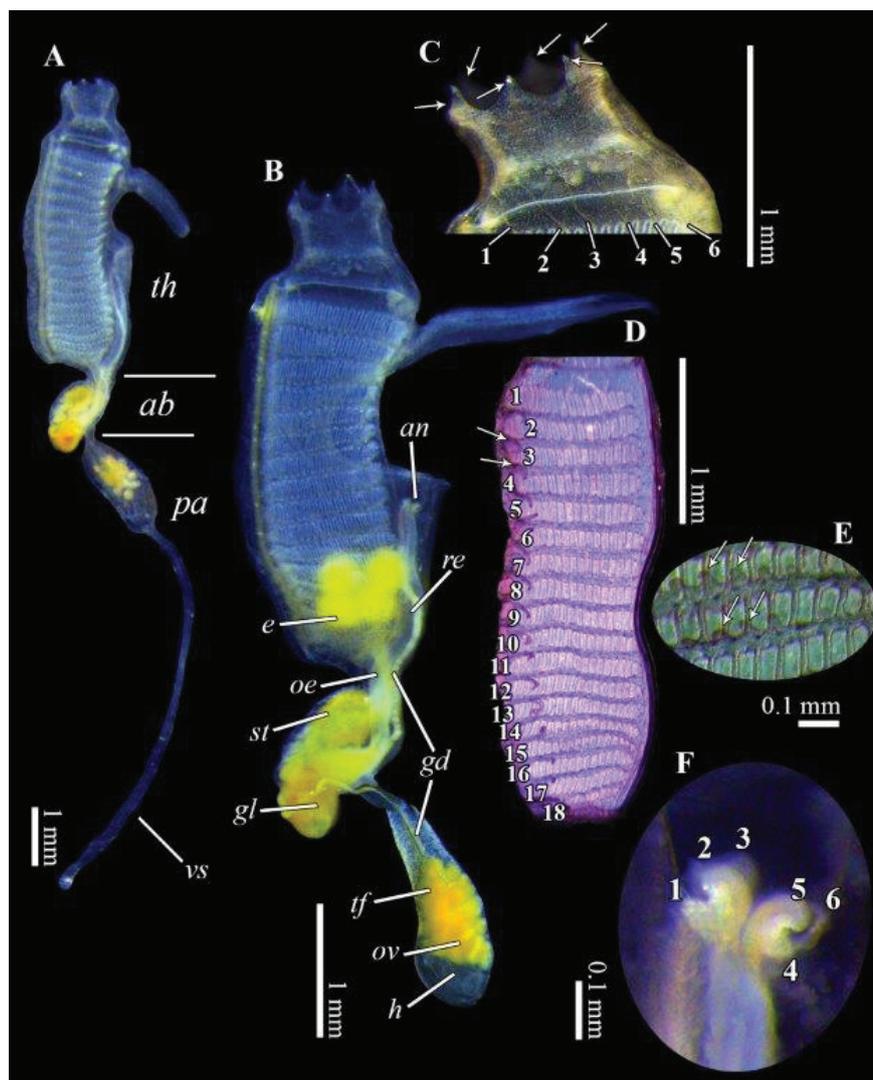

Fig. 3: A) Whole zooid of *Polyclinum constellatum*, showing a clear division into thorax, abdomen and post-abdomen with a long vascular stolon. *ab*, abdomen; *pa*, post-abdomen; *th*, thorax; *vs*, vascular stolon; B) Zooid with evident pharynx, rectum, anus and four embryos incubated in the atrial cavity. The funnel-shaped oesophagus, the smooth stomach and the twisted gut loop are visible in the abdomen. The post-abdomen shows the heart at its terminal end, as well as several rounded testicular follicles and the ovary, with the gonoducts running parallel to the rectum. *an*, anus; *e*, embryos; *gd*, gonoducts; *gl*, gut loop; *oe*, oesophagus; *ov*, ovary; *h*, heart; *r*, rectum; *st*, stomach; *tf*, testicular follicles; C) Magnification of the oral siphon with six pointed lobes (arrows) and six longitudinal muscle bands (indicated with numbers 1-6); D) Branchial sac with 18 rows of stigmata and narrow languets of the dorsal lamina (arrows); E) Magnification of the pharynx, with minute papillae (arrows) at the level of the transverse vessels; F) Magnification of the six-lobed anus (lobes indicated with numbers 1-6).

74

Medit. Mar. Sci., 23/1 2022, 69-83

heart is positioned at the end of the post-abdomen (Fig. 3B), followed by a long narrow vascular stolon (Fig. 3A).
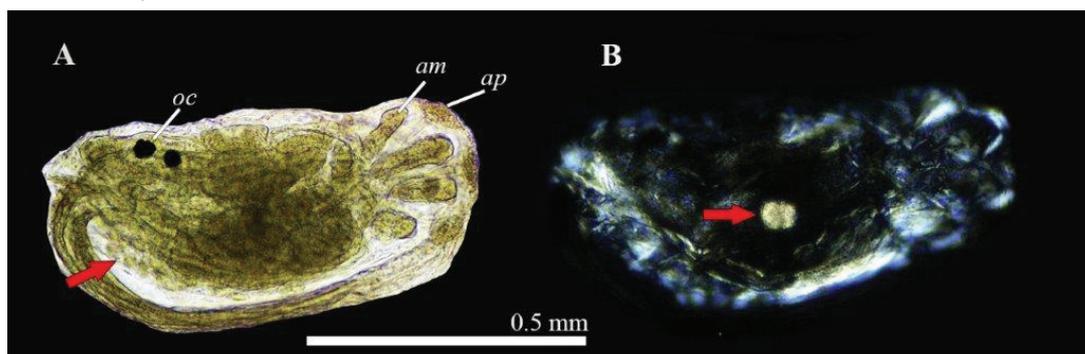
Larva

Several embryos (from 4 up to 15) are incubated in the peribranchial cavity. Fully developed larvae are approximately 0.5 mm in trunk length, and they have one or two calcite crystals usually placed in the middle of the body or close to the ocellus (Fig. 5A-B). They have three adhesive papillae on a sagittal plane and four pairs of long ectodermal ampullae. Furthermore, a group of small vesicles is usually present on the ventral side of the larvae (Fig. 5A).
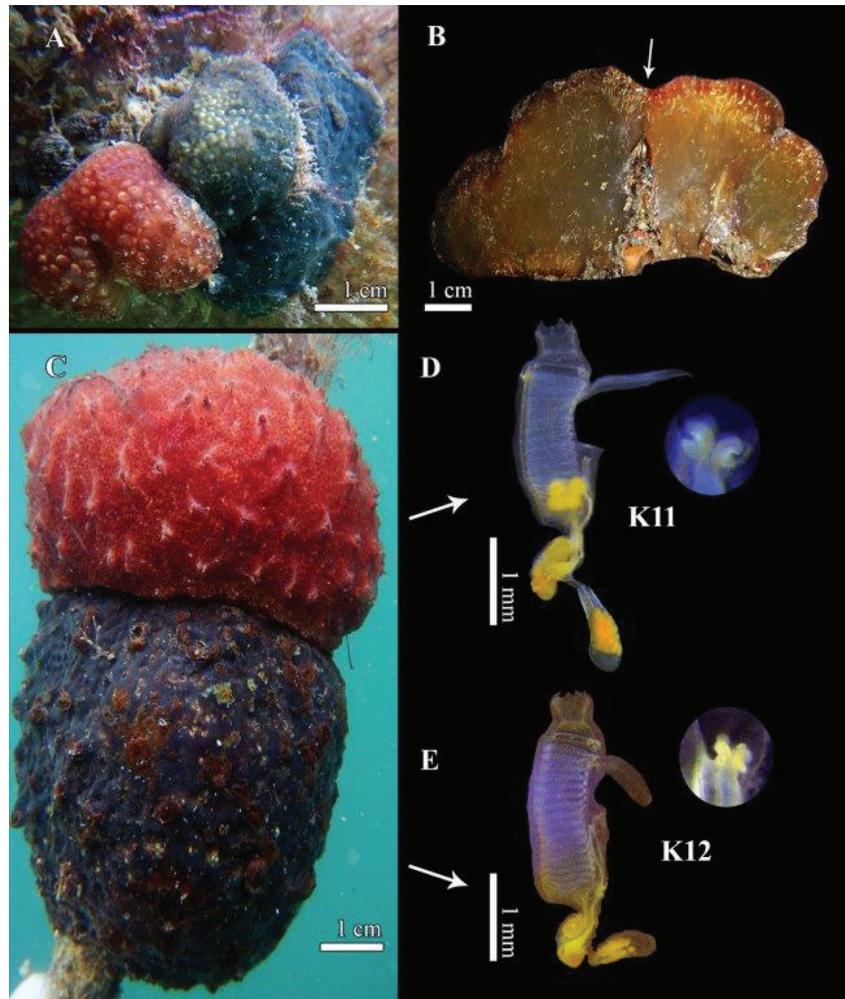
Fig. 4: A, C) Colonies of *Polyclinum constellatum* with different colours photographed and collected in the Heraklion marina (Crete) (A: colony K11 and C: colony K12); B) Transversal section of the colonies, joined only at the surface layer (upper white arrow); D) Zooid extracted from the red-orange colony (K11), with magnification of the 6-lobed anus; E) Zooid extracted from the dark blue colony (K12) with magnification of the 6-lobed anus. Both K11 and K12 have the same COI haplotype (sequence AC number: MT873559).

Fig. 5: A) Larva of *P. constellatum*, showing the ocellus, four long narrow ampullae, three adhesive papillae and a group of a few small ventral vesicles (red arrow). *am*, ampullae; *ap*, adhesive papillae; *oc*, ocellus; B) Larva of *P. constellatum*, red arrow pointing out the calcite crystal in the middle of the body.

COI sequence comparisons

The obtained COI sequences were 811-816 bp long and correspond to two haplotypes differing in only 5 nucleotides (i.e., 4 synonymous substitutions at the third codon position and one non-synonymous substitution at the first codon position). One haplotype (named P1) is shared between the Taranto and five Heraklion colonies, while the other haplotype (named K7) was only found in the blue-greenish K7 colony from Heraklion. Moreover, the COI sequences obtained from colonies with different morphotypes showed 100% identity, including the red-orange (K11) and dark blue (K12) Heraklion colonies joined together (Fig. 4A-E).

A total of 33 *Polyclinum* sequences belonging to 7 different species were found in the nt-nr database by text and similarity search (NCBI, 21st Sept 2021). As detailed in Table 2, many of these sequences show multiple unexpected indels, terminal regions lacking COI similarity and/or containing long homopolymers, or no significant similarity to ascidians. Six of these sequences are also described in the entry as pseudogenes or "unverified" sequences, meaning that the GenBank staff were unable to verify their accuracy and/or annotations (Table 2). All these elements are clues of low-quality sequences, or possible PCR contamination. Indeed, our BlastN analyses show that three of these *Polyclinum* sequences belong to Lepidoptera and two others do not have significant similarity to any nr-nt sequence, so they cannot by unambiguously assigned to a species (Table 2). Table 3 reports the mean inter- and intra-species pairwise uncorrected distances, calculated only for the confirmed *Polyclinum* sequences and excluding the above-mentioned non-homologous or low-quality terminal regions. It is noteworthy that the mean distance between *P. constellatum* and *P. indicum* sequences is only 1.34% ± 0.65% and all comparisons between *P. constellatum, P. indicum,* and *P. madrasensis* show a mean uncorrected difference < 1.34%. In addition, our K7 haplotype is identical to a short *P. constellatum* sequence from Puerto Rico (see MT637964 in Table 2), and both P1 and K7 haplotypes have a mean distance from *indicum/constellatum/madrasensis* ranging from 0.09 to 1.25% (Table 3) Based only on these data, our specimens could be equally assigned to *P. constellatum,* to *P. indicum,* or *P. madrasensis.* However, our morphological results clearly show that both our haplotypes belong to *P. constellatum.* It should be noted that all public COI sequences, including the few published ones (Table 2), lack a morphological description of the samples. Only for two *P. indicum* sequences (MH235542 and MH367293), do the Authors report that they were originally identified as belonging to *Polyclinum saturnium* through conventional morphological analyses and then re-assigned to *P. indicum* based on similarity to the public COI (Khan *et al.*, 2020). A ML phylogenetic reconstruction of Polyclinidae representatives (Fig. 6) shows the monophyly of the genus *Polyclinum,* but all nodes inside the genus remain unresolved, except for a statistically significant clade containing all *P. constellatum* sequences except P1 (the position of the P1 haplotype is unresolved). Moreover, our results suggest taxonomic mis-assignments of the *P. indicum* and *P. madrasensis* public COI sequences.

Taxonomic remarks

The Polyclinidae family includes colonial species with a lobed oral siphon, zooids arranged in systems with common cloacal openings, as well as the gonads and heart in the post-abdomen. The genus *Polyclinum* is characterized by a twisted gut loop, almost horizontal, and a pharynx with simple branchial papillae. *Polyclinum constellatum* is the type species of the genus.

The only species belonging to the genus *Polyclinum* known thus far in the Mediterranean Sea is *P. aurantium* Milne-Edwards, 1841 (Brunetti & Mastrototaro, 2017). This species differs from *P. constellatum* mainly in having one or few zooid systems per colony and the presence of sand grains in the tunic. This is also confirmed by Van Name (1945), Tokioka (1961) and Fortaleza & Lotufo (2018) who report that *P. constellatum* colonies are usually free of sand, and whether some sand is present on the surface or not, it does not pervade the interior of the colony.

The World Register of Marine Species (WoRMS Editorial Board, 2022) reports 47 species belonging to the genus *Polyclinum*. Among these species, *P. macrophyllum* Michaelsen, 1919, *P. complanatum* Herdman, 1899, and *P. hospitale* Sluiter, 1909 cannot be considered valid species. In detail, *P. macrophyllum* cannot be separated from *P. vasculosum* (*sensu* Kott, 1992). *P. complanatum* is not properly assigned, indeed it has a long abdomen different from that characteristic of Polyclinidae species, and it could be a species of *Pseudodistoma* (Kott, 1992). Finally, the correct authorship of *P. hospitale* should be Sluiter, 1895, and it is probably an *Aplidium* species (Kott, 1992). Three valid species are not yet present in WoRMS: *P. minutum* Herdman, 1886, *P. tralaticia* Sluiter, 1913, and *P. ramosum* Parker-Nance, 2003. *P. tralaticia* is considered valid by Kott (1992) and Parker-Nance (2003) albeit erroneously reported as *P. tralatica. P. ramosum*, described as a new species by Parker-Nance (2003), has sand embedded in the tunic, branchial papillae one third of the length of the stigmata and a bilobed anus. *P. minutum* has a branchial sac with only 8-9 rows of stigmata.

Considering the above-mentioned considerations, Table 1 reports a taxonomic key for 47 *Polyclinum* species, listed according to their main features and completed with authorship.

Among the 47 accepted species of *Polyclinum*, only 9 are characterized by the absence of sand embedded in or coating the colonies. Moreover, most of the species characterized by the absence of sand outside or throughout the tunic have a lower number of rows of stigmata compared to those of *P. constellatum*, apart from for *P. planum* (Ritter & Forsyth, 1917) (13-14 rows of stigmata) and *P. johnsonii* Monniot & Monniot, 1989 (16-17 rows). However, *P. planum* can be distinguished from *P. constellatum* by the characteristic paddle-shaped colonies

76

Medit. Mar. Sci., 23/1 2022, 69-83

Table 2. COI sequences of the nt-nr database (NCBI, 21 September 2021) analysed in this study. Incorrectly assigned sequences are reported in bold, identified as not belonging to ascidians according to BlastN analyses. *haplotypes analysed in this study.

AC Number	Notes	Species and Description	bp	Locality	Reference
AY116606	Lepidoptera from BlastN *vs.* nr-nt	*Polyclinum aurantium, pseudogene*	669	France	Stach & Turbeville, 2002
JQ408691	No significant similarity in BlastN	*Polyclinum aurantium, pseudogene*	380	India	Jaffar Ali *et al.*, 2016
KF414702	Lepidoptera from BlastN *vs.* nr-nt	UNVERIFIED: *Polyclinum aurantium*	646	India	Jaffar Ali unpub
KF414711	Lepidoptera from BlastN *vs.* nr-nt	UNVERIFIED: *Polyclinum sp.* ASP-2013	644	India	Jaffar Ali unpub
KJ944391	Terminal region non-homologous to COI	UNVERIFIED: *Polyclinum indicum*	507	India	Ahmed & Jaffar Ali, 2015
KP072781		*Polyclinum indicum*	550	India	Jaffar Ali unpub
KT276223		*Polyclinum indicum*	565	India	Jaffar Ali unpub
KT345963		*Polyclinum indicum*	550	India	Jaffar Ali unpub
KX650772	Terminal region non-homologous to COI	*Polyclinum indicum*	532	India	Jaffar Ali unpub
KX650773		*Polyclinum indicum*	576	India	Jaffar Ali unpub
KX650774		*Polyclinum indicum*	551	India	Jaffar Ali unpub
KX650775	Terminal region non-homologous to COI	*Polyclinum indicum*	682	India	Jaffar Ali unpub
KX650776		*Polyclinum indicum*	580	India	Jaffar Ali unpub
KX650777		*Polyclinum indicum*	667	India	Jaffar Ali unpub
KX650778		*Polyclinum indicum*	600	India	Jaffar Ali unpub
KX650779		*Polyclinum indicum*	589	India	Jaffar Ali unpub
KX650780		*Polyclinum indicum*	600	India	Jaffar Ali unpub
KX650781		*Polyclinum indicum*	631	India	Jaffar Ali unpub
KX650782	Terminal region non-homologous to COI and with homopolymers	*Polyclinum indicum*	689	India	Jaffar Ali unpub
KX650783	Multiple indels	*Polyclinum indicum*	609	India	Jaffar Ali unpub
KX650784	Terminal region non-homologous to COI and with homopolymers	*Polyclinum indicum*	593	India	Jaffar Ali unpub
KX650785		*Polyclinum indicum*	610	India	Jaffar Ali unpub
KX650786		*Polyclinum indicum*	610	India	Jaffar Ali unpub
MH235542	Multiple indels	*Polyclinum indicum*	508	India	Khan *et al.*, 2020
MH367293		*Polyclinum indicum*	610	India	Khan *et al.*, 2020
MT637964		*Polyclinum constellatum*	593	Puerto Rico	Nydam, unpub
MT873559*		*Polyclinum constellatum, haplotype P1*	816	Italy	This study
OL597608*		*Polyclinum constellatum, haplotype K7*	811	Grece	This study
MW858363		*Polyclinum constellatum*	863	Florida, USA	Nydam, unpub
MW858364		*Polyclinum constellatum*	859	Florida, USA	Nydam, unpub
MW990087		*Polyclinum constellatum*	808	Abu Dhabi Emirates	Govindharaj *et al.*, unpub
KX650787	Multiple indels and terminal region non-homologous to COI	*Polyclinum saturnium*	502	India	Jaffar Ali unpub
JN107814	No significant similarity in BlastN	UNVERIFIED: *Polyclinum madrasensis*	641	India	Jaffar Ali unpub
KU220954	Multiple indels	*Polyclinum madrasensis*	531	India	Jaffar Ali unpub
KX138512	Multiple indels and terminal region non-homologous to COI	*Polyclinum sp.* HAJ-2016	600	India	Jaffar Ali unpub

with the zooids arranged in rows only along one side of the colony, while *P. johnsoni* has a higher number of stigmata per half-row (23-25).

Most of the remaining accepted species are characterized by having sand embedded in or coating the tunic. The main morphological characters of these species are detailed and summarized in Table 1. In particular, according to the COI comparisons, the sandy tunic species *P. indicum* Sebastian, 1952 and *P. saturnium* (Savigny, 1816) is grouped with *P. constellatum*. *P. indicum* is characterized by zooids having an enlarged languet with a small protuberance at its end (Sebastian, 1954) (Erulan & Ananthan, 2009). The specific name *P. indicum* is no longer accepted, since it was revised by Brunetti (2007), who proposed *P. sebastiani* as *nomen novum*. *P. saturnium* has colonies coated with sand, a denticulated atrial lip, only 8-12 rows of stigmata and a bilobed anus (Kott, 1992).

Lastly, the absence/presence of sand is not reported for *P. hesperium* Savigny, 1816, *P. meridianum* Sluiter 1900, *P. sibiricum* Redikorzev, 1907, or *P. tralaticia* (Sluiter, 1913). *P. hesperium* is a poorly described species collected by Savigny (1816) in the Suez gulf and then by Shenkar (2012) in the Red Sea, which is characterized by 18-20 row of stigmata. The *P. meridianum* description is only based on its original description (Sluiter, 1900), reporting colonies with a poorly developed tunic, a tri-lobed atrial languet and 9 rows of stigmata. *P. sibiricum* has systems of 8-10 zooids with 12 rows of stigmata and 15 stigmata per half-row (Redikorzev, 1907). *P. tralaticia* has 10 rows of stigmata with 10-12 stigmata per half-row and no branchial papillae on transverse vessels (Sluiter, 1913).

According to the key to species in Table 1, *P. constellatum* is close to the co-generic species *P. johnsoni*, *P. planum* and *P. hesperium*. However, *P. constellatum* has a slightly different number of stigmata per half-row compared to those of *P. planum* and *P. johnsoni* and less rows of stigmata compared to those of *P. hesperium*. Moreover, *P. johnsoni*, which has only been collected at a depth of 300 m is characterized by transverse vessels

bearing long papillae with an orange-yellow opaque pigment, as well as short wide dorsal languets (Monniot & Monniot, 1989), while *P. planum* is characterized by the pedunculated shape of the colony (Van Name, 1945).

Discussion

Among the NIS spreading throughout the Mediterranean Sea, *P. constellatum* is confirmed to be an emerging one that is widening its latitudinal range of distribution, deserving proper attention soon. Integrated taxonomy is fundamental for prompt species identification. Here we provide a detailed morphological description associated to the sequencing of a reliable DNA barcode, consisting of a long COI fragment of about 860 bp, together with an overview of the morphology of the species belonging to the genus *Polyclinum*. For the DNA barcoding method, the building of a reliable reference database of DNA barcodes of known species is a fundamental step. Taxonomic expertise is required for the selection and species assignment of the specimens used in building a reliable reference database. Indeed, erroneous identification of the reference specimens will lead to incorrect species assignment whenever DNA barcodes generated from unidentified samples are compared to the reference database. An erroneous reference DNA barcode will therefore propagate the initial error. Here we provide example specimens for *P. constellatum* for which a DNA barcode has been correctly produced and associated to detailed specimen descriptions. This is particularly important for the genus *Polyclinum*, since we have observed incorrect taxonomic assignments and sequence anomalies/errors in several public COI sequences reported as belonging to *Polyclinum* species (Table 2), probably due to the intrinsic difficulties in the discrimination between congeneric species, PCR contamination, or errors in database sequence submission. Moreover, all the public *Polyclinum* sequences lack a morphological description of the specimens. In this case, our comparative analyses (Table 3; Fig. 6) suggest that the sequences related to *P. indicum*

Table 3. Pairwise uncorrected distances for intra- and inter-species comparisons within the genus *Polyclinum*. Square brackets contain the number of sequences analysed for each species. Values < 1.5 are in bold. K7 and P1 are the haplotypes analysed in this study, while the label "public *P. constellatum*" represents the sequences of *P. constellatum* present in the nt-nr database.

Percentage values	K7 (this study) *P. constellatum*	P1(this study) *P. constellatum*	public *P. constellatum* [4]	*P. indicum* [21]	*P. saturnium* [1]	*Polyclinum sp.* KX138512 [1]
K7, *P. constellatum* (this study)						
P1, *P. constellatum* (this study)	**0.62**					
public *P. constellatum* [4]	**0.09**	**0.69**	**0.17 a**			
P. indicum [21]	**1.25**	**0.94**	**1.34**	**1.30 a**		
P. saturnium [1]	8.95	8.27	8.97	8.47		
Polyclinum sp KX138512 [1]	4.02	3.48	4.19	4.02	8.69	
P. madrasensis [1]	**0.36**	**0.20**	**0.51**	**0.71**	6.56	3.53

a: intraspecies comparisons value
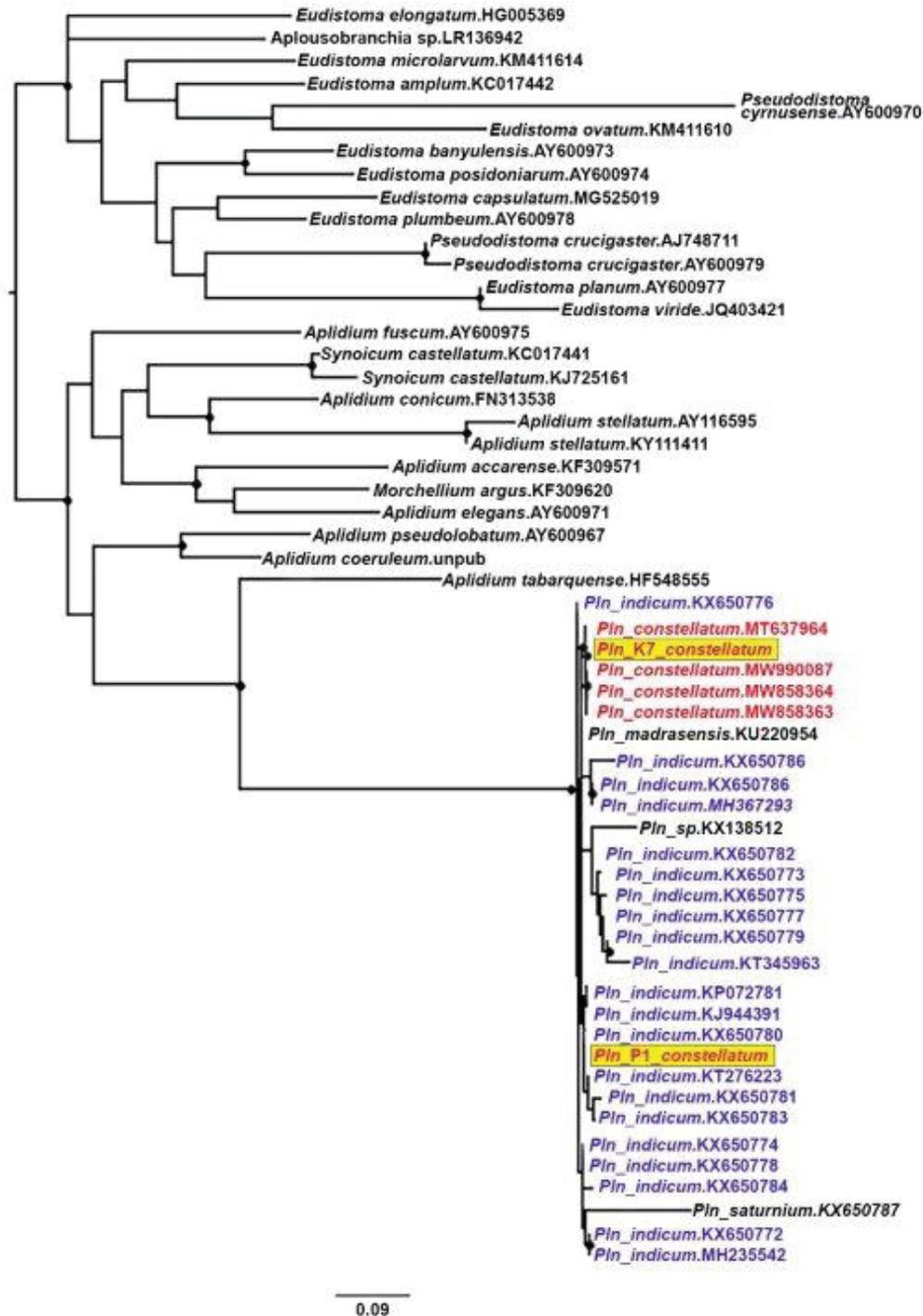
Fig. 6: ML phylogenetic tree of the genus *Polyclinum* (sequences abbreviation: Pln) based on COI nucleotide sequences (1560 aligned nucleotide sites; best-fit substitution model GTR+I+G; bootstrap on 100 replicates). *Eudistoma* and *Pseudodistoma* species were used as outgroups. The sequence list and species abbreviations are reported in Supplementary table S1. Black dots: bootstrap values ≥ 70 %; red: *P. constellatum* sequences; blue: *P. indicum* sequences; yellow background: our sequences.

and *P. madrasensis* are the result of specimen misidentification or of erroneous sequence submission to the public nucleotide database. However, a possible synonymy between *P. constellatum*, *P. indicum* and *P. madrasensis* should be further investigated, again using an integrated approach applied to accurately described specimens of all three species. It must be noted that the current documented distribution of *P. indicum* and *P. madrasensis* is limited to the coasts of India, from where *P. constellatum* has not been recorded to date. Indeed, *P. indicum* and *P. madra-*

sensis are somewhat similar to *P. constellatum*, but the description of some morphological elements is missing (Table 1) and no data are available on their intraspecific variability since the only morphological descriptions of *P. madrasensis* and *P. indicum* are those of Sebastian (1952 and 1954, respectively).

Our identification of the colonial ascidian *P. constellatum* through integrated taxonomy can also be useful for environmental monitoring purposes. Indeed, reference COI databases with accurately-identified species

are a critical requirement for metabarcoding applications, which are considered a promising tool for the management of NIS (Bucklin *et al.*, 2016; Nousias & Montesanto, 2021). Studies carried out on the same specimens and combining an accurate morphological description and DNA barcoding analyses are a prerequisite to allow the improvement in early detection capability of NIS. Such studies would also be of use for planning appropriate measures and strategies (e.g., boat removal from the water prior hull cleaning, restriction/control of the movement of aquaculture species) to predict, prevent and/or limit the secondary spread of non-indigenous species within the Mediterranean basin and beyond.

Our observation of different colonies almost or completely fused together suggests the hypothesis that *P. constellatum* might be able to form chimeric entities, as observed for other NIS such as *Didemnum vexillum* and *Diplosoma listerianum* (Casso *et al.,* 2019, Watts *et al.,* 2019; Bishop & Sommerfeldt, 1999; Sommerfeldt & Bishop, 1999). Indeed, chimerism represents an obvious advantage as it can improve colonisation success and resilience in colonial organisms, enhancing their invasive potential (Casso *et al.,* 2019). Aplousobranch ascidians, such as *P. constellatum*, have zooids not interlinked by blood vessels so allorecognition, the mechanism at the base of the chimerism and colony fusion, cannot be explained by migration of stem-cells from the zooids through the colony vascular system (Bishop & Sommerfeldt, 1999). Considering that the tunic contains a large number of motile cells, it is possible that stem cells can also be exchanged by the tunic matrix (Bishop & Sommerfeldt, 1999). This might be taken in consideration for those groups which are characterized by an extensive production of tunic matrix, such as the Polyclinidae. However, this hypothesis needs to be experimentally verified on many specimens.

This study reports the first record of the NIS *P. constellatum* in Cretan waters (eastern Mediterranean Sea) and in the Ionian Sea (central Mediterranean Sea). Although we did not perform a sampling aimed at estimating the abundance of this species in the studied areas, several colonies were observed in both localities, close to aquaculture facilities, with mean densities of 0.50 ± 0.15 colonies per m^2, albeit only restricted to artificial substrate thus far. Further sampling will be required to investigate the potential occurrence of this species also on natural substrata in the nearby areas. Both areas are characterized by the presence of marinas influenced by recreational boating, commercial maritime traffic, aquaculture farms and many other anthropogenic impacts (Bracchi *et al.,* 2016; Tursi *et al*., 2018; Ulman *et al.,* 2019). Indeed, in the area of Taranto live seafood is imported from Greece in large amounts everyday (e.g., 10t/day of the mussel *Mytilus galloprovincialis* are estimated to be imported to Taranto from the Adriatic coast, Greece and Spain) (Cecere *et al.,* 2016). Furthermore, Cecere *et al.* (2016) report that boats licensed to go beyond 12 nautical miles from Taranto harbour often reach Greek islands, while recreational boating has been recognized as a significant vector of primary introduction and sec-

ondary spreading of NIS (Clarke Murray *et al.,* 2011). These vectors of introduction represent the most likely pathways through which *P. constellatum* could have spread between both investigated areas of the Eastern and Central Mediterranean Sea.

References

Ahmed, N.S., Jaffar Ali, H.A., 2015. In Silico Analysis of COI Gene Sequences of Two Colonial Ascidians *Polyclinum indicum* and *Didemnum candidum* from Gulf of Mannar. *Science and humanities*, 217.

Airoldi, L., Turon, X., Perkol-Finkel, S., Rius, M., 2015. Corridors for aliens but not for natives: effects of marine urban sprawl at a regional scale. *Diversity and Distributions,* 21, 755-768.

Aldred, N., Clare, A.S., 2014. Mini-review: impact and dynamics of surface fouling by solitary and compound ascidians. *Biofouling,* 30 (3), 259-270.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215 (3), 403-410.

Aydin-Onen, S., 2018. Distribution of ascidians with a new record of the non-indigenous species *Polyclinum constellatum* Savigny, 1816 from the Aegean coast of Turkey. *Turkish Journal of Fisheries and Aquatic Sciences*, 18 (9), 1077-1089.

Bishop, J.D., Sommerfeldt, A.D., 1999. Not like *Botryllus*: indiscriminate post–metamorphic fusion in a compound ascidian. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 266 (1416), 241-248.

Bracchi, V.A., Marchese, F., Savini, A., Chimienti, G., Mastrototaro, F. *et al*., 2016. Seafloor integrity of the Mar Piccolo Basin (Southern Italy): quantifying anthropogenic impact. *Journal of Maps,* 12, 1-11.

Brunetti, R., 2007. Nomenclatural Acts: homonymy in the Ascidiacea (Tunicata) and proposed nomina nova. *Zootaxa,* 1613, 67-68.

Brunetti, R., Mastrototaro, F., 2017. *Ascidiacea of the European Waters*. Edagricole - New Business Media II, Milano, 447 pp.

Bucklin, A., Lindeque, P.K., Rodriguez-Ezpeleta, N., Albaina,

80

Medit. Mar. Sci., 23/1 2022, 69-83

A., Lehtiniemi, M., 2016. Metabarcoding of marine zoo-plankton: prospects, progress and pitfalls. *Journal of Plankton Research*, 38 (3), 393-400.

Carlton, J.T., 1996. Biological Invasions and Cryptogenic Species. *Ecology,* 77 (6), 1653-1655.

Carman, M.R., Bullard, S.G., Rocha, R.M., Lambert, G., Dijkstra, J.A. *et al.,* 2011. Ascidians at the Pacific and Atlantic entrances to the Panama Canal. *Aquatic Invasions*, 6 (4), 371-380.

Casso, M., Tagliapietra, D., Turon, X., Pascual, M., 2019. High fusibility and chimera prevalence in an invasive colonial ascidian. *Scientific reports,* 9 (1), 1-10.

Cecere, E., Petrocelli, A., Belmonte, M., Portacci, G., Rubino, F., 2016. Activities and vectors responsible for the biological pollution in the Taranto Seas (Mediterranean Sea, southern Italy): a review. *Environmental Science and Pollution Research,* 23, 12797-12810.

Clarke Murray, C., Pakhomov, E.A., Therriault, T.W., 2011. Recreational boating: a large unregulated vector transporting marine invasive species. *Diversity and Distributions*, 17 (6), 1161-1172.

Dias, G.M., Rocha, R.M., Lotufo, T.M.C., Kremer, L.P., 2013. Fifty years of ascidian biodiversity research in São Sebastião, Brazil. *Journal of the Marine Biological Association of the United Kingdom,* 93 (1), 273-282.

Erulan, V., Ananthan, G., 2009. On the occurrence of a colonial ascidian, *Polyclinum indicum* (Sebastian, 1952) in Vellar Estuary, Parangipettai (Southeast Coast of India). *Middle-East Journal of Scientific Research,* 4 (4), 279-281.

Fortaleza, M.T.V., Lotufo, T.M.D.C., 2018. Polyclinidae (Ascidiacea: Aplousobranchia) from the Gulf of Mexico, collected during the" Hourglass" cruises (1965-1967). *Zootaxa*, 4422 (4), 519-536.

Goodbody, I., 1984. Ascidians from Caribbean shallow water localities. *Studies on the Fauna of Curaçao and other Caribbean Islands*, 67 (1), 62-75.

Gravili, C., Belmonte, G., Cecere, E., Denitto, F., Giangrande, A. *et al.,* 2010. Non-indigenous species (NIS) along the Apulian coast, Italy. *Chemistry & Ecology,* 26, 121-142.

Guindon, S., Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic biology*, 52 (5), 696-704.

Halim, Y., Messeih, M.A., 2016. Aliens in Egyptian waters. A checklist of ascidians of the Suez Canal and the adjacent Mediterranean waters. *The Egyptian Journal of Aquatic Research*, 42 (4), 449-457.

Hirose, M., Hirose, E., 2009. DNA barcoding in photosymbiotic species of *Diplosoma* (Ascidiacea: Didemnidae), with the description of a new species from the southern Ryukyus, Japan. *Zoological Science,* 26 (8), 564-8.

Izquierdo Muñoz, A., Diaz Valdes, M., Ramos-Esplà, A.A., 2009. Recent non-indigenous ascidians in the Mediterranean Sea. *Aquatic Invasions*, 4, 59-64.

Jaffar Ali, A., Akram, S.A., Arshan, K.M., 2016. New distributional data on ascidian fauna (Tunicata: Ascidiacea) from Mandapam coast, Gulf of Mannar, India. *Biodiversity data journal*, 4, e7855.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M. *et al.,* 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28 (12), 1647-9.

Khan, K.B., Praba, L., Jaffar Ali, H.A., 2020. Biological identification of ascidians from Vizhinjam Bay, southwest Coast of India using CO1 gene sequences. *Mitochondrial DNA Part A*, 31 (5), 209-217.

Kott, P., 1963. The ascidians of Australia. IV. Aplousobranchiata Lahille: Polyclinidae Verril (continued). *Marine and Freshwater Research*, 14 (1), 70-118.

Kott, P., 1992. The Australian Ascidiacea Part 3, Aplousobranchia (2). *Memoirs of the Queensland Museum*, 32, 375-620.

Lambert, G., 2003. Marine biodiversity of Guam: the Ascidiacea. *Micronesica*, 35 (36), 588-597.

Lambert, G., 2007. Invasive sea squirts: a growing global problem. *Journal of Experimental Marine Biology and Ecology,* 342, 3-4.

López-Legentil, S., Legentil, M.L., Erwin, P.M., Turon, X., 2015. Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. *Biological Invasions,* 17 (6), 1623-1638.

Marchini, A., Ferrario, J., Occhipinti-Ambrogi, A., 2013. Recent additions to the alien marine biota along Italian coasts. *Rapport Commission Internationale Mer Méditeranée*, 40, 887.

Mastrototaro, F., Matarrese, A., D'Onghia, G., 2003. Occurrence of *Musculista senhousia* (Mollusca: Bivalvia) in the Taranto seas (eastern-central Mediterranean Sea). *Journal of Marine Biology Association of the United Kingdom,* 83, 1279-1280.

Mastrototaro, F., Brunetti, R., 2006. The non-indigenous ascidian *Distaplia bermudensis* in the Mediterranean: comparison with the native species *Distaplia magnilarva* and *Distaplia lucillae* sp. nov. *Journal of the Marine Biological Association of the United Kingdom*, 86, 181-185.

Mastrototaro, F., D'Onghia, G., Tursi, A., 2008. Spatial and seasonal distribution of ascidians in a semi-enclosed basin of the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom,* 88, 1053-1061.

Mastrototaro F., Chimienti G., Matarrese A., Gambi M.C., Giangrande A., 2015. Growth and population dynamics of the non-indigenous species *Branchiomma luctuosum* Grube (Annelida, Sabellidae) in the Ionian Sea (Mediterranean Sea). *Marine Ecology*, 36, 517-529.

Mastrototaro, F., Chimienti, G., Montesanto, F., Perry, A.L., García, S. *et al.*, 2019a. Finding of the macrophagous deep-sea ascidian *Dicopia antirrhinum* Monniot, 1972 (Chordata: Tunicata) in the Tyrrhenian Sea and updating of its distribution. *The European Zoological Journal*, 86 (1), 181-188.

Mastrototaro, F., Montesanto, F., Salonna, M., Grieco, F., Trainito, E. *et al.*, 2019b. Hitch-hikers of the sea: concurrent morphological and molecular identification of *Symplegma brakenhielmi* (Tunicata: Ascidiacea) in the western Mediterranean Sea. *Mediterranean Marine Science*, 20, (1), 197-207.

Mastrototaro, F., Aguilar, R., Alvarez, H., Blanco, J., García, S. *et al.*, 2020a. Mesophotic rocks dominated by *Diazona violacea*: a Mediterranean codified habitat. *The European Zoological Journal*, 87 (1), 688-695.

Mastrototaro, F., Montesanto, F., Salonna, M., Viard, F., Chimienti, G. *et al.*, 2020b. An integrative taxonomic framework

Medit. Mar. Sci., 23/1 2022, 69-83

81

for the study of the genus *Ciona* (Ascidiacea) and description of a new species, *Ciona intermedia*. *Zoological Journal of the Linnean Society,* 190 (4), 1193-1216.

Meenakshi, V.K., Gomathy, S., 2017. Habitat Destruction and Ascidian Biodiversity Loss-An Assessment. *Inernational Journal of Life Sciences*, 5 (1), 55-62.

Millar, R.H., 1955. On a collection of ascidians from South Africa. *Proceedings of the Zoological Society of London,* Vol. 125 (1), 169-221.

Millar, R.H., 1958. Some ascidians from Brazil. *Journal of Natural History*, 1 (8), 497-514.

Monniot, F., 1972. Ascidies aplousobranches des Bermudes. Polyclinidae et Polycitoridae. *Bulletin du Muséum National d'Histoire Naturelle*, 82, 949-962.

Monniot, F., 1983. Ascidies littorales de Guadeloupe III**.** Polyclinidae. *Bulletin du Muséum National d'Histoire Naturelle*, 4, 413-422.

Monniot, C., Monniot, F., 1976. Ascidies de la côte du Mozambique. *Revue de Zoologie africaine*, 90 (2), 357-392.

Monniot, C., Monniot, F., 1989. Ascidians collected around the Galapagos islands using the Johnson-Sea-Link research submersible. *Proceedings of the Biological Society of Washington,* 102 (1), 14-32

Monniot, C., Monniot, F., 1997a. Records of ascidians from Bahrain, Arabian Gulf with three new species. *Journal of Natural History,* 31, 1623-1643.

Monniot, F., Monniot, C., 1997b. Ascidians collected in Tanzania. *Journal of East African Natural History*, 86 (1), 1-35.

Montesanto, F., Chimienti, G., Gissi, C., Mastrototaro, F., 2021. Spread of the non-indigenous ascidian *Aplidium accarense* (Millar, 1953) in the Eastern Mediterranean Sea: morphological and molecular tools for an accurate identification. *Mediterranean Marine Science*, 22 (2), 246-254.

Nousias, O., Montesanto, F., 2021. Metagenomic profiling of host-associated bacteria from 8 datasets of the red alga *Porphyra purpurea* with MetaPhlAn3. *Marine Genomics*, 100866.

Occhipinti-Ambrogi, A., Marchini, A., Cantone, G., Castelli, A., Chimenz, C. *et al.*, 2011. Alien species along the Italian coasts: an overview. *Biological Invasions,* 13, 215-237.

Orfanidis, S., Alvito, A., Azzurro, E., Badreddine, A., Souissi, J.B. *et al.*, 2021. New Alien Mediterranean Biodiversity Records (March 2021). *Mediterranean Marine Science*, 22 (1), 180-198.

Parker-Nance, S., 2003. *Aplousobranch ascidians (Tunicata: Ascidiacea) from southern Africa*. PhD thesis, University of Port Elizabeth, South Africa, 199 pp.

Petović, S., Grieco, F., Macic, V., Montesanto, F., Mastrototaro, F., 2018. New data on *Aplidium tabarquense* (Tunicata: Ascidiacea) distribution in the Adriatic Sea. *Turkish Journal of Zoology*, 42 (1), 146-151.

Ragkousis, M., Abdelali, N., Azzurro, E., Badreddine, A., Bariche, M. *et al.*, 2020. New Alien Mediterranean Biodiversity Records (October 2020). *Mediterranean Marine Science*, 21 (3), 631-652.

Redikorzev, V., 1907. Die ascidien der russischen Polar expedition 1900-1903. *Zoologischer Anzeiger*, 31 (17-18), 521-525.

Rocha, R.M., Costa, L.V., 2005. Ascídias (Urochordata: Ascidiacea) do Arraial do Cabo, Rio de Janeiro, Brasil. *Iheringia.*

Série Zoologia, 95 (1), 57-64.

Rocha, R.M., Guerra-Castro, E., Lira, C., Pauls, S.M., Hernández, I. *et al*., 2010. Inventory of ascidians (Tunicata, Ascidiacea) from the National Park La Restinga, Isla Margarita, Venezuela. *Biota Neotropica,* 10 (1), 209-218.

Rocha, R.M., Salonna, M., Griggio, F., Ekins, M., Lambert, G. *et al.*, 2019. The power of combined molecular and morphological analyses for the genus *Botrylloides*: identification of a potentially global invasive ascidian and description of a new species. *Systematics and Biodiversity*, 17 (5), 509-526.

Salonna, M., Gasparini, F., Huchon, D., Montesanto, F., Haddas-Sasson, M. *et al.*, 2021. An elongated COI fragment to discriminate botryllid species and as an improved ascidian DNA barcode. *Scientific reports,* 11 (1), 1-19.

Santin, A., Aguilar, R., Akyol, O., Begburs, C., Benoit, L. *et al*., 2021. New records of rare species in the Mediterranean Sea (March 2021*). Mediterranean Marine Science*, 22 (1), 199-217.

Savigny, J., 1816. *Mémoires sur les animaux sans vertèbres*. *Savigny, J.-C., 1816. Seconde Partie.*

Panckoucke C.L.F, Paris, 239 pp.

Sebastian, V.O., 1952. A new species of synascidian from Madras. *Current Science,* 21, 316-317.

Sebastian, V.O., 1954. On *Polyclinum indicum*, a new ascidian from the Madras coast of India. *Journal of the Washington Academy of Sciences*, 44 (1), 18-23.

Servello, G., Andaloro, F., Azzurro, E., Castriota, L., Catra, M. *et al.,* 2019. Marine alien species in Italy: a contribution to the implementation of descriptor D2 of the Marine Strategy Framework Directive*. Mediterranean Marine Science,* 20 (1), 1-48.

Shenkar, N., 2012. Ascidian (Chordata, Ascidiacea) diversity in the Red Sea. *Marine Biodiversity*, 42 (4), 459-469.

Sluiter, C.P., 1900. Tunicaten aus dem stillen Ocean. Ergebnisse einer Reise nach dem Pacific. *Zoologische Jahrbücher*, 13, 1-35.

Sluiter, C.P., 1913. Ascidien von den Aru-Inseln. *Senckenbergischen Naturforschenden Gesellschaft* 3 (1), 63-78.

Sommerfeldt, D., Bishop, J.D., 1999. Random amplified polymorphic DNA (RAPD) analysis reveals extensive natural chimerism in a marine protochordate. *Molecular Ecology,* 8 (5), 885-890.

Stach, T., Turbeville, J.M., 2002. Phylogeny of Tunicata inferred from molecular and morphological characters. *Molecular phylogenetics and evolution*, 25 (3), 408-428.

Swofford, D.L. 2002. PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates, Sunderland, Massachusetts, 130 pp.

Tabudravu, J.N., Pellissier, L., Smith, A.J., Subko, K., Autréau, C. *et al.*, 2019. LC-HRMS-database screening metrics for rapid prioritization of samples to accelerate the discovery of structurally new natural products. *Journal of natural products,* 82 (2), 211-220.

Tokioka, T., 1961. Ascidians collected during the Melanesia Expedition 1, Ascidians presented by Dr R.L.A. Catala of the Aquarium of Noumea. *Publications of the Seto Marine Biological Laboratory,* 134, 104-138.

Tokioka, T., 1967. Pacific Tunicata of the United States National Museum. *US National Museum Bulletin,* 251, 1-247.

Tursi, A., Corbelli, V., Cipriano, G., Capasso, G., Velardo, R.

et al., 2018. Mega-litter and remediation: the case of Mar Piccolo of Taranto (Ionian Sea). *Rendiconti Lincei. Scienze Fisiche e Naturali*, 29 (4), 817-824.

Ulman, A., Ferrario, J., Occhipinti-Ambrogi, A., Arvanitidis, C., Bandi, A. *et al.,* 2017. A massive update of non-indigenous species records in Mediterranean marinas. *PeerJ*, 5, e3954.

Ulman, A., Ferrario, J., Forcada, A., Arvanitidis, C., Occhipinti-Ambrogi, A. *et al.,* 2019. A hitchhiker's guide to Mediterranean marina travel for alien species. *Journal of Environmental Management,* 241, 328-339.

Van Name, W.G., 1945. *The North and South American ascidians.* Bulletin of the American Museum of Natural History, New York, 462 pp.

Watts, A.M., Hopkins, G.A., Goldstien, S.J., 2019. Chimerism and population dieback alter genetic inference related to invasion pathways and connectivity of biofouling populations on artificial substrata. *Ecology and evolution,* 9 (6), 3089-3104.

WoRMS Editorial Board, 2022. *World Register of Marine Species.* https://www.marinespecies.org at VLIZ (Accessed 19 January 2022)

Zenetos, A., Gofas, S., Verlaque, M., Çinar, M., Garcia Raso, J. *et al.,* 2010. Alien species in the Mediterranean Sea by 2010. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part I. Spatial distribution. *Mediterranean Marine Science,* 11 (2), 381-493.

Zhan, A., Briski, E., Bock, D.G., Ghabooli, S., MacIsaac, H.J., 2015. Ascidians as models for studying invasion success. *Marine Biology,* 162 (12), 2449-2470.

Supplementary Data

The following supplementary information is available online for the article:

Table S1: Polyclinidae COI sequences, other than *Polyclinum* species, present in the phylogenetic tree in Figure 6. The dataset is a subset of the one analysed in Tabudravu *et al.*, 2019.