ePublishing

ΕΘΝΙΚΟ ΚΕΝΤΡΟ ΤΕΚΜΗΡΙΩΣΗΣ
EKT
NATIONAL DOCUMENTATION CENTRE

Mediterranean Marine Science

Vol 20, No 1 (2019)

Hitch-hikers of the sea: concurrent morphological and molecular identification of Symplegma brakenhielmi (Tunicata: Ascidiacea) in the western Mediterranean Sea

FRANCESCO MASTROTOTARO, FEDERICA MONTESANTO, MARIKA SALONNA, FLAVIA GRIECO, EGIGDIO TRAINITO, GIOVANNI CHIMIENTI, CARMELA GISSI

doi: 10.12681/mms.19390

To cite this article:

MASTROTOTARO, F., MONTESANTO, F., SALONNA, M., GRIECO, F., TRAINITO, E., CHIMIENTI, G., & GISSI, C. (2019). 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. https://doi.org/10.12681/mms.19390

https://epublishing.ekt.gr | **e-Publisher:** EKT | **Downloaded at:** 19/01/2025 01:18:32

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.19390

Hitch-hikers of the sea: concurrent morphological and molecular identification of *Symplegma brakenhielmi* (Tunicata: Ascidiacea) in the western Mediterranean Sea

Francesco MASTROTOTARO[1,2], Federica MONTESANTO [1,2], Marika SALONNA[3], Flavia GRIECO[1], Egidio TRAINITO[4], Giovanni CHIMIENTI[1,2] and Carmela GISSI[3,5]

[1] Department of Biology, University of Bari Aldo Moro, Via Orabona 4, 70125, Bari, Italy
[2] CoNISMa, Piazzale Flaminio 9, 00197, Roma, Italy
[3] Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari "A. Moro", Via E. Orabona, 4, 70125 Bari, Italy
[4] Villaggio I Fari, Loiri Porto San Paolo, 07020 Olbia Tempio, Italy
[5] IBIOM, Istituto di Biomembrane, Bioenergetica e Biotecnologie Molecolari, CNR, Via Giovanni Amendola 165/A - 70126 Bari, Italy

Corresponding author: federica.montesanto@uniba.it

Handling Editor: Xavier TURON

Received: 22 December 2018; Accepted: 26 February 2019; Published on line: 30 April 2019

Abstract

We report here one of the first records of the non-indigenous colonial ascidian *Symplegma brakenhielmi* in the western Mediterranean Sea. Colonies of this invasive species were collected in 2014 and 2018 along the North-eastern Sardinia coasts (Olbia, Italy). Further colonies were observed in 2016 in the Mar Piccolo basin (Gulf of Taranto, Italy). Both areas are strongly influenced by anthropogenic activities such as commercial shipping and aquaculture and these human-mediated pathways are the most likely vectors of introduction. In both areas, the colonies present two different color phenotypes, the yellow and the red type, with the yellow coloration never previously found in the Mediterranean Sea. Morphological and DNA barcode analyses of the collected specimens show that both these color types belong to the same species. Phylogenetic and species delimitation analyses based on the DNA barcode confirm our identification as *S. brakenhielmi,* but also indicate a surprisingly high similarity with published sequences of two other species, including the co-generic species *Symplegma rubra* Monniot, 1972. Morphological and molecular examination of a large number of samples of these species is needed in the near future to clarify this issue.

Keywords: *Symplegma brakenhielmi;* non-indigenous; NIS; ascidian; Mediterranean; COI; DNA barcode.

Introduction

The lack of ascidian-specialized taxonomists and the consequent frequent misidentification of samples have been among the major limiting factors in the ability to detect non-indigenous species of ascidians (Izquierdo Muñoz *et al.,* 2009). Systematic knowledge, particularly with respect to accurate species identification, is crucial to clarify causes and consequences of ascidian invasions in marine ecosystems. Defining clear identification keys, correctly revising the taxonomic nomenclature, as well as combining morphological and molecular evidence, are the main components of an integrated approach aimed at solving the problem of ambiguous taxonomy in ascidians (Zhan *et al.,* 2015).

Symplegma brakenhielmi is a colonial ascidian, first recorded in the Gulf of Mexico by Michaelsen in 1904, who named it *Diandrocarpa brakenhielmi*. Subsequently, in 1961, Tokioka described a new species from Melanesia, *Symplegma oceania*. Based on the similarity of morphological characters, Kott 1985 considered these two nominal species to be conspecific, retaining the name *S. oceania*. Later, considering the nomenclature rules (ICZN, 1985), Monniot & Monniot 1997 named it as *Symplegma brakenhielmi* (Michaelsen, 1904). This species has a pantropical distribution, having been recorded in Mexico (Michaelsen, 1904), the Western Indian Ocean (Kott, 1964, 1985; Michaelsen, 1904), Melanesia (Kott, 1981; Tokioka, 1961, 1967), Indonesia (Sluiter, 1904), Thailand (Tokioka, 1967), Hong Kong (Kott & Goodbody, 1980), South Korea (Rho & Park, 1998), China (Tokioka, 1967), Sri Lanka (Herdman, 1906), Madagascar (Plante & Vasseur, 1966; Vasseur, 1967), the Arabian Gulf (Monniot & Monniot 1997), California (Lambert & Lambert, 1998), Hawaii (Abbott *et al.,* 1997), Brazil (Rocha, 1991; Rodrigues & Rocha, 1993; Rocha & Barros de Faria, 2005; Ro-

cha & Kremer, 2005) and Venezuela (Rocha *et al.* 2010). Based on current knowledge, the recorded presence of this species in the Mediterranean Sea has been limited to the eastern basin, along the coasts of Turkey, Israel and Lebanon. More specifically, Bitar & Kouli-Bitar (2001), reported the first occurrence of *S. brakenhielmi* in the Mediterranean Sea, reviewing the list of macrozoobenthos species along the Lebanese coast. Furthermore, Çinar *et al.* (2006) recorded several reddish colonies on an artificial substratum during a survey on the presence of alien species along the Turkish Levantine coast. Further colonies have been reported more recently from the same area (Ulman, 2016; Ulman *et al.,* 2017; Halim & Messeih, 2016; Gerovasileiou *et al.,* 2017; Servello *et al.,* 2019). Shenkar & Loya (2009) found this species along the Israel coasts, also reporting only reddish colonies. A recent record of *S. brakenhielmi* in Sicilian waters (Palermo, Italy) was reported by Ulman *et al.* (2017), but without providing any images or detail about its identification. Moreover, possible misidentifications of the species as *Symplegma viride* Herdman, 1886 have been reported in the Mediterranean Sea (Izquierdo Muñoz *et al.,* 2009), supporting the idea that inaccurate specific identification can limit the ability to detect non-indigenous ascidians.

This study reports the presence of *S. brakenhielmi* in the western and central Mediterranean Sea, in particular along the North-eastern coasts of Sardinia (Olbia, Western Tyrrhenian Sea) and in the Mar Piccolo basin (Gulf of Taranto, Northern Ionian Sea). Both finding areas are strongly influenced by human activities and pressures (Bracchi *et al*., 2016; Tursi *et al*., 2018), such as high maritime traffic, commercial shipping and import/export of living marine species for aquaculture, considered the most important pathways for the introduction of the alien species. Therefore, a hypothesis on the most likely vector of introduction of this non-indigenous species in the western Mediterranean basin is suggested. In both areas the colonies of *S. brakenhielmi* present two different colorations, red and yellow, with the yellow coloration reported here for the first time in the Mediterranean Sea.

A brief review of the genus *Symplegma* and a detailed morphological descriptions of the red and yellow colonies collected are provided, together with their molecular characterization based on the mitochondrial COI gene (Cytocrome Oxidase subunit I) (Hebert *et al.,* 2003) This study represents a contribution to the knowledge of the composition and distribution of alien species in the Mediterranean Sea, and it underlines the importance of a synergistic approach between morphological and molecular analyses, especially in the identification and study of non-indigenous species.

Material and Methods

Survey site

Red and yellow colonies of S. *brakenhielmi* were collected in the North-eastern Sardinian Sea, along the "Lido del Sole" beach (40.91444° N; 9.56611° E) near Olbia (Italy), at a depth of 3-5 m (Fig. 1) in July 2014 and November 2018. This area is mainly characterized by the presence of the seagrass *Cymodocea nodosa* (Ucria) Ascherson, 1870, the bivalve *Pinna nobilis* Linnaeus, 1758, the large solitary ascidians *Phallusia mammillata* (Cuvier, 1815), *Microcosmus* spp. and *Halocynthia papillosa* (Linnaeus, 1767), as well as colonial ascidians belonging to the genus *Clavelina*.

Further red and yellow colonies were observed in August 2016 in the Mar Piccolo basin (Gulf of Taranto, South Adriatic, Italy) (40.47707° N; 17.24627° E). The colonies were attached to glass bottles and other marine litter on a detritic bottom, at a depth of 7 m (Fig. 3), in a military area where sampling is forbidden.

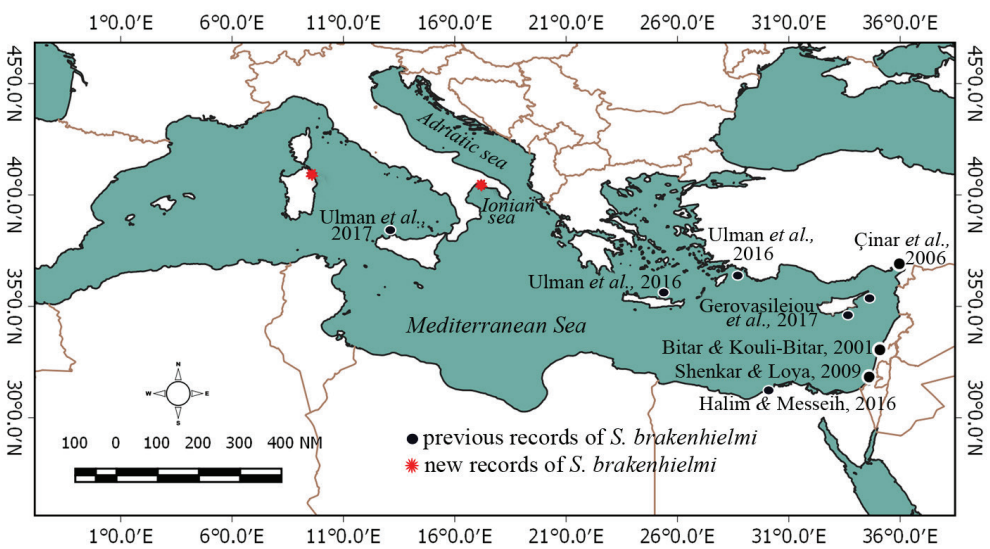

Fig. 1: Map of the Mediterranean Sea showing the previous records of *S. brakenhielmi* (black dots) and the two new records (red asterisks).

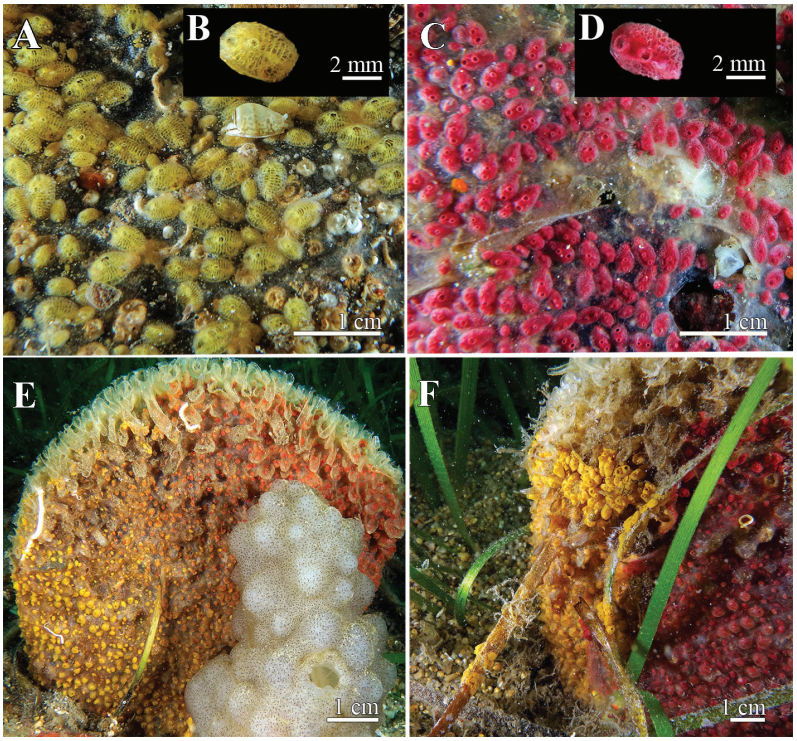

Fig. 2: A) Living yellow colonies of *Symplegma brakenhielmi* sampled in Sardinia (Italy); B) Magnification of a yellow zooids showing the reticulated pattern; C) Living red colonies of *Symplegma brakenhielmi* sampled in Sardinia (Italy); D) Magnification of a red zooid showing the reticulated pattern; E) Detail of the yellow and red colonies on valves of *Pinna nobilis* and leaves of *Cymodocea nodosa.*

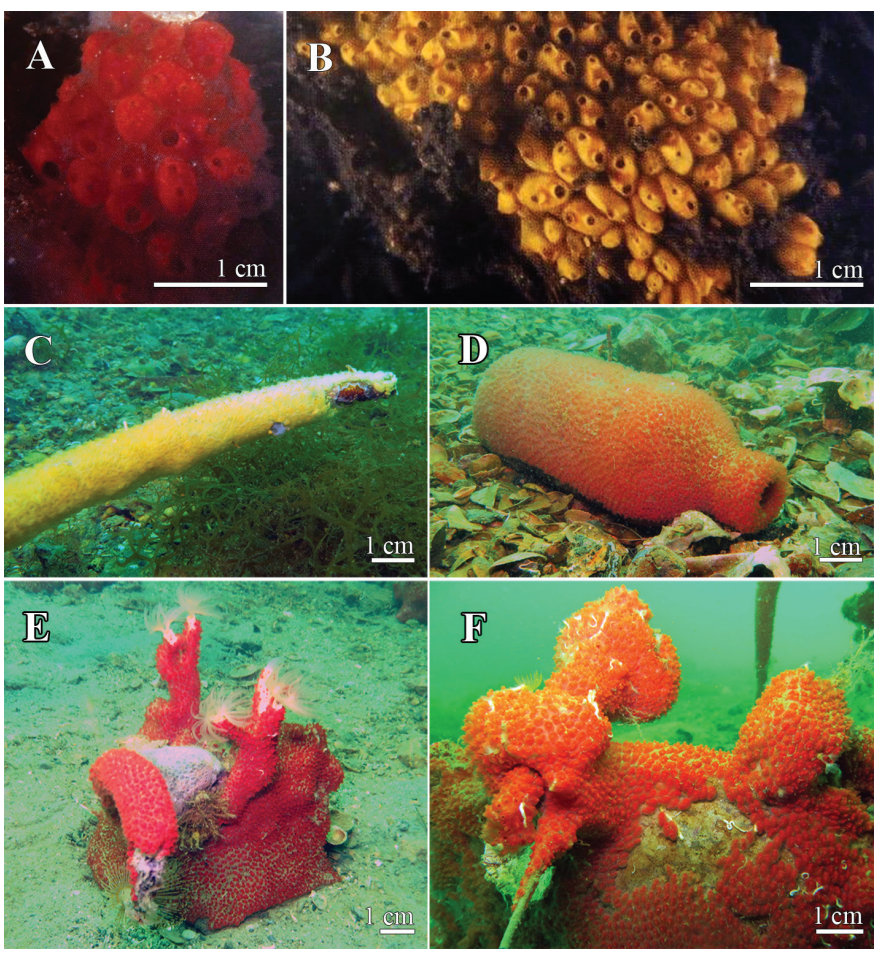

Fig. 3: A-B) Red and yellow colonies of *Symplegma brakenhielmi* photographed along North-eastern coasts of Sardinia (Olbia, Italy) in 2003 (pictures from Trainito, 2005, reported as *Styelidae* sp.); C-F) Red and yellow colonies *S. brakenhielmi* observed in the Gulf of Taranto (Italy).

Sampling

A total of ten yellow and red colonies were manually collected by SCUBA diving in Sardinia waters. Colonies were settled on different biotic and abiotic substrata, such as leaves of *C. nodosa*, shells of *P. nobilis* and rocks (Fig. 2). The sampled colonies were narcotized with menthol crystals in seawater and preserved in a 4% formaldehyde solution in seawater. Moreover, subsamples of two red and two yellow colonies were preserved in 99% ethanol and in RNA later for molecular investigation.

Molecular analyses

Total DNA was extracted from the four colonies in ethanol or in RNA later (two yellow and two red) using a modified CTAB method (Hirose & Hirose, 2009). In particular, the DNA was extracted from 2-3 zooids isolated from each colony. Molecular characterization was based on the amplification and sequencing of a fragment of the mitochondrial COI (Cytochrome Oxidase subunit I) gene. In particular, the analysed COI fragment is about 850 bp and contains the 650 bp-long barcode region commonly amplified with Folmer's primers (Folmer *et al.*, 1994). The 850 bp -long amplicon was obtained using the primer pair dinF/Nux1R (Brunetti *et al.*, 2017).

PCRs were performed with high fidelity PrimeStar HS DNA polymerase (Takara Bio Inc.) in a 25 µl reaction volume containing: 1X reaction buffer with 1 mM final concentration of MgCl2 (Takara Bio Inc.), 0.2 mM of each dNTP, 0.3 µM of each primer and 1.25 Units of PrimeStar HS DNA polymerase (Takara Bio Inc.). Amplification conditions were: 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 50 or 48°C (depending on the sample), extension for 1 min 30 s at 72°C; a final elongation step of 5 min at 72°C. After cleaning with the Amicon Ultra-0.5 mL centrifugal filter devices (NMWL of 100kDa, Millipore), amplicons were directly sequenced according to the Sanger method at Eurofins Genomics (Ebersberg, Germany). Sequence quality check and assembly were carried out with Geneious ver. 5.5.7 (http://www.geneious.com; Kearse *et al.*, 2012). Sequences, all identical, were deposited at ENA (European Nucleotide Archive) with Accession Number LS992554.

The obtained COI sequences were compared to the public NCBI nucleotide non-redundant database (nt-nr db, 10th May 2018, www.ncbi.nlm.nih.gov/nuccore) using the online Basic Local Alignment Search Tool (BlastN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.*, 1990). Phylogenetic reconstructions and species delimitation analyses were carried out on all available COI sequences of the subfamily Polyzoinae (nt-nr db, 10th May 2018). One representative COI sequence of Botryllus *schlosseri* (Pallas, 1766) (Griggio *et al.*, 2014), another Styelidae closely related to Polyzoinae phylogenetically was used as an outgroup. Sequences were aligned using MUSCLE (Edgar, 2004) maintaining codon information, and the alignment was manually checked in order to delete regions corresponding to the primers used in amplicon production, often erroneously saved in the public COI sequences. The final alignment, available on request, is 838 nt long and consists of 442 ungapped sites and 19 sequences. The list of analysed sequences is reported in Table 1, together with additional data.

Pairwise identity percentages between sequences were calculated using Geneious ver. 5.5.7 (http://www.geneious.com, Kearse *et al.*, 2012).

Species delimitation analyses were carried out with the Automatic Barcode Gap Discovery method, ABGD, a method that sorts sequences into OTUs (Operational Taxonomic Units), corresponding to hypothetical species, based on the statistical inference of the "barcode gap", i.e., the gap between intra- and inter-specific diversity (Puillandre *et al.*, 2012). ABGD analyses were performed on the web-based interface (http://wwwabi.snv.jussieu.fr/public/abgd/) using the default values for both the minimum relative gap width (X=1.5) and for the scanned range of prior intraspecific divergence (Pmin – Pmax: 0.001 – 0.1). Three COI alignments, differing in the number of aligned sites and sequences, were tested, i.e., the whole alignment including the B. *schlosseri* outgroup, and all the 838 gapped and ungapped sites (alnBS_838); the two alignments including only the 442 ungapped sites, with (alnBS_442) or without the B. *schlosseri* outgroup (aln_442). These alignments were analysed in order to verify possible bias on the barcode gap calculation due to missing data (i.e., gapped sites) or to the presence of the B. *schlosseri* outgroup. For each analysed alignment, pairwise distances were calculated according to the three models of nucleotide evolution available in ABGD: Jukes-Cantor (JC, Jukes & Cantor, 1969), Kimura 2 parameter (K2P, Kimura, 1980) and uncorrected p-distances (p-dist). These analyses allowed us to exclude possible bias of the selected evolutionary model on the OTU delimitation. Therefore, a total of 12 ABGD analyses were performed.

The phylogenetic reconstruction of Polyzoinae was performed on the alnBS_838 alignment according to the maximum likelihood (ML) method using online PHYML v3.0 software, which includes automatic model selection by SMS (Smart Model Selection) (Guindon & Gascuel, 2003) (http://www.atgc-montpellier.fr/phyml-sms/). The best-fit substitution model selected under the Akaike Information Criterion (AIC) was HKY85+I+G. The proportion of invariant sites (I) and the gamma shape parameter (alpha) for the 4 rate categories were estimated by the PHYML v3.0 software itself. Bootstrap values, indicating node reliability, were based on 100 replicates. B. *schlosseri* was used as an outgroup.

Results

All the collected yellow and red colonies were identified as *S. brakenhielmi* based on numerous morphological characters of the colony, zooids and mature larvae, as well as on COI sequence comparisons (see below). Morphological and molecular analyses on sampled colonies refer to 2014 and 2018, while the reanalysis of published photos

Table 1. COI sequences of the nt-nr database (NCBI, 10th May 2018) analysed in this study.

AC number in the nt-nr	Species	Isolate	Sampling locality	bp	% nt identity to our COI *	Note
LS992554	*Symplegma brakenhielmi*		Italy	834		This study
FJ528648	*Symplegma rubra*		Kenya	620	96.9	
KX138510	*Symplegma brakenhielmi*	DBTIC136	India	676	95.5	
KT276224	*Symplegma brakenhielmi*	DBTIC38	India	567	97.8	
KU360802	*Symplegma brakenhielmi*	ASC17	India	600	96	
KT276228	*Symplegma brakenhielmi*	DBTIC49	India	645	96.5	
KR815818	*Symplegma brakenhielmi*	DBTIC08	India	615	53.9	Erroneous species assignment
KR815819	*Symplegma brakenhielmi*	DBTIC12	India	543	26.0	Erroneous species assignment
FJ528651	*Stolonica socialis*	i69	Spain	297	96.6	To be confirmed
FJ528647	*Polyzoa opuntia*	i73	Argentin	628		
FJ528652	*Distomus variolosus*	i84	UK, Plymouth	676		
FJ528653	*Distomus variolosus*	i85	UK	679		
FJ528654	*Distomus variolosus*	i86	UK	678		
KF309623	*Distomus variolosus*	DS-5	Spain, Catalunya	586		
KF309643	*Polyandrocarpa zorritensis*	iSCR-3	Spain, Catalunya	586		
KY111429	*Polyandrocarpa zorritensis*	i29	USA	545		
KY111428	*Polyandrocarpa anguinea*	i28	USA	576		
KY111427	*Polyandrocarpa anguinea*	i27	USA	555		
KY111426	*Polyandrocarpa anguinea*	i26	USA	618		
KX138505	*Polyandrocarpa zorritensis*	DBTIC10	India	524		
KX138504	*Polyandrocarpa zorritensis*	DBTIC09	India	523		
FM177702	*Botryllus schlosseri*	VE	Italy	1548		

*: Identity to our COI sequence of 834 bp

points to the presence of this species along Sardinia coasts as early as 2003 (Trainito, 2005, reported as *Styelidae* sp., Fig. 3A-B). Since then, the species has been observed in Olbia constantly every year, with the period of greatest abundance between the end of autumn and the beginning of summer (Trainito, personal communication). Moreover, accurate analysis of the photos taken within the military area of Taranto allows the identification of both red and yellow colonies as *S. brakenhielmi* (Fig. 3C-F).

Symplegma brakenhielmi (Michaelsen, 1904)
Diandrocarpa brakenhielmi Michaelsen, 1904: 50-Mexico.
Symplegma viride: Van Name, 1945: 232-Tropical Atlantic Ocean.
Symplegma oceania Tokioka, 1961: 114-New Caledonia (Noumea Harbour);
Symplegma oceania: Kott, 1985:257. Pacific and Indian Oceans.

Material examined

Olbia (North-eastern Sardinian Sea, Italy), (40.91444° N; 9.56611° E), 3-5 m.

Four colonies (two of the red-type and two of the yellow-type), preserved both in 70% ethanol after 4% formalin, and in 99% ethanol, have been deposited in the collection of the Museum of the University of Bari (Code: MUZAC-6326; MUZAC-6327).

External appearance

Living individuals appeared as flat and encrusting colonies, red or yellow in color, up to 30 cm in diameter and about 2 mm thick. Colonies grew freely over any hard substrata, such as large solitary ascidians (e.g., *Phallusia mammillata*, *Microcosmus* spp.), bivalve shells (e.g., *P. nobilis*), seagrass leaves (e.g., *C. nodosa*) and rocks. Furthermore, in the sampling site, red and yellow colonies were adjacent on the same substratum (Fig. 2E-4F). Zooids were completely embedded in a transparent and jelly-like tunic, more or less crowded and not arranged in systems (Fig. 2A-B). They were different in size, up to

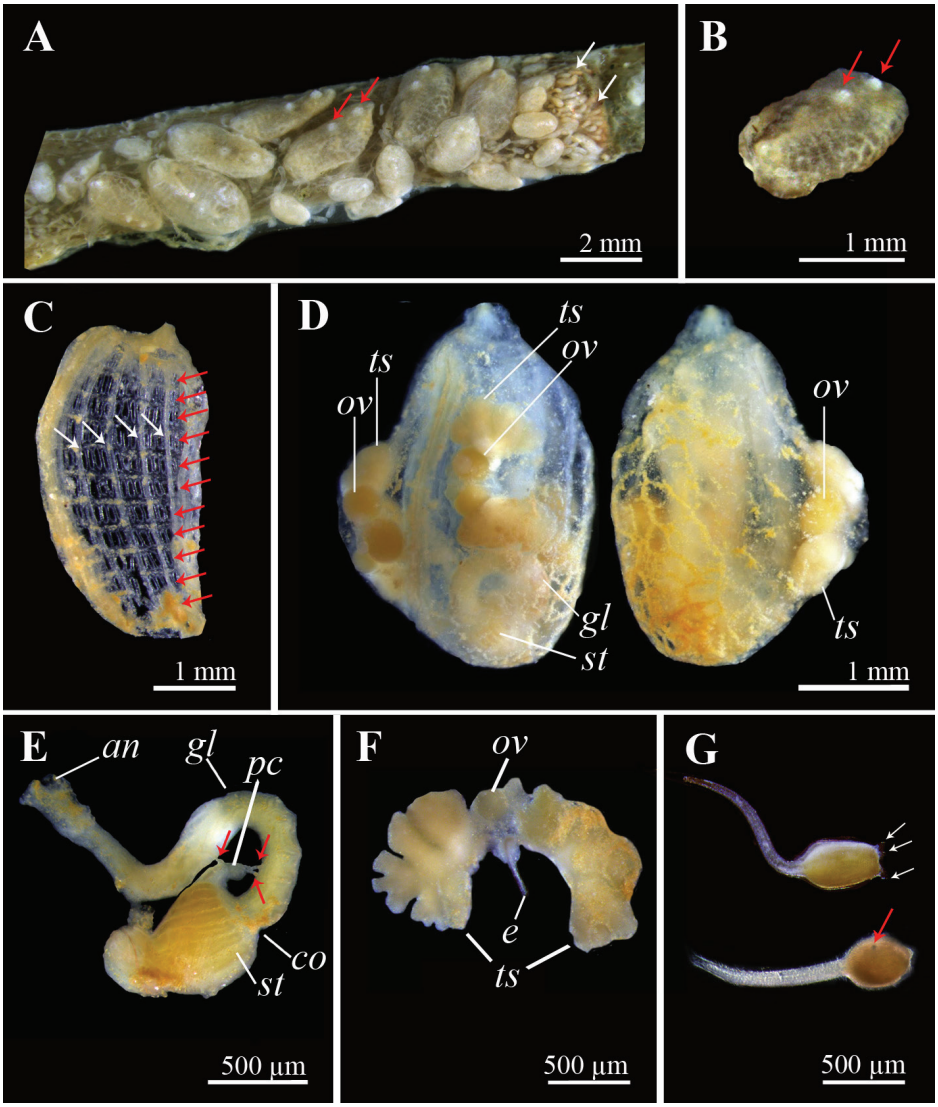

Fig. 4: A) Colony of *Symplegma brakenhielmi* (after preservation in a solution of 4% formalin) showing the zooids prostate and embedded in the transparent test (red arrows indicate the siphons while white indicate basal stolon and ampullae); B) Detail of a preserved zooid showing the reticulated pattern (red arrows indicate the siphons); C) Branchial sac (red arrows indicate 11 stigmata rows); D) Ventral and dorsal view of a whole zooid with mature gonads on both sides of the body. *ov*, ovary; *ts*, testis; *gl*, gut loop; *st*, stomach; E) Digestive tract, showing the stomach with about 8 folds on each side and a curved pyloric caecum (red arrows point out two intestinal connectives, one ramified). *st,* stomach; *co*, constriction; *gl*, gut loop; *pc*, pyloric caecum; *an*, anus; F) Gonads consisting of a small ovary with one egg and two deeply divided pyriform male follicles with their ducts joined at the base of the ovary forming a narrow vas deferens. *ov*, ovary; *ts*, testis; *e*, vas deferens; G) Tad-pole larvae with white arrows indicate the three adhesive organs tri-radially arranged with a red arrow indicating the photolith.

4 mm in length, with the youngest individuals intercalated between adults. Basal stolons and spherical ampullae were evident between the zooids, becoming particularly abundant around the margins of each colony, where they were elongated and parallel to one another (Fig. 4A). The gelatinous tunic of red specimens was slightly tougher than that of yellow ones in preserved colonies. Both red and yellow fresh colonies became uniformly grey in formalin (Fig. 4A).

Zooids

Zooids were dorso-ventrally flattened with the oral and atrial apertures on the upper side of the body, the first one near the anterior end and the second in the middle (Fig. 4A, 4C). Both the apertures opened at the end of a short tubular siphon with a smooth rim. Atrial tentacles, fine and variable in number and length, were arranged around the oral siphon. Both in preserved and *in vivo* colonies, reddish brown (in red colonies) or yellowish (in yellow colonies) spots were evident on the body wall of zooids, resulting in a reticulated pattern (Fig. 2B-C; Fig. 4A-B). Zooids of the colonies collected during November 2018 appeared smaller than those collected in July 2014.

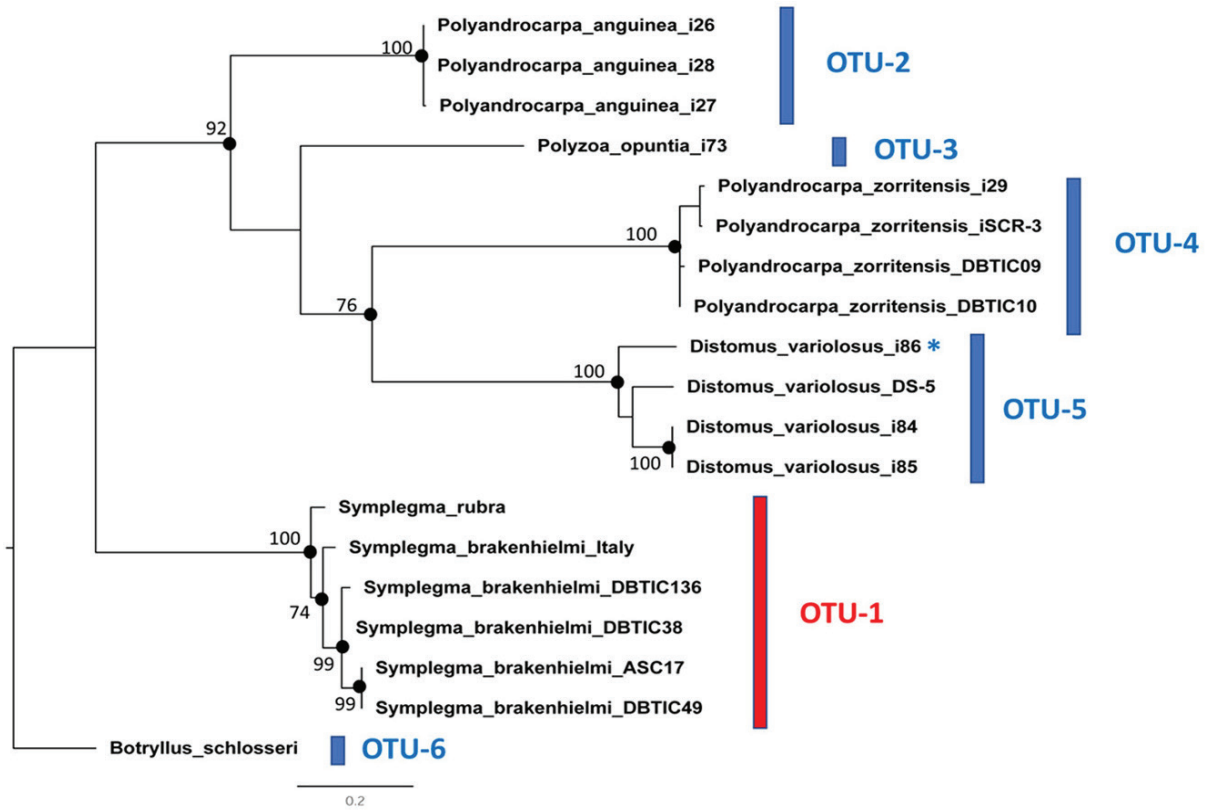

Fig. 5: Maximum Likelihood phylogenetic tree of Polyzoinae based on COI (PhyML: HKY85 + I + G model; bootstrap on 100 replicates). Solid boxes indicate putative species (OTU) identified by ABGD method. Asterisk indicates an additional OTU identified only in the analyses of the alnBS_838 dataset. Black dots indicate nodes with bootstrap support > 75%. *B. schlosseri* was used as the outgroup.

Branchial sac

The branchial sac was crossed by 10-11 rows of stigmata in both yellow and red colonies (Fig. 4C). Four longitudinal vessels on each side ran through the inner part of the branchial sac (Fig. 4C). The dorsal lamina was arranged in two parallel smooth edged blades.

Alimentary canal

The digestive system occupied the posterior end of the left side of the body. The stomach was pyriform, with 13 to 16 parallel folds. On the distal end its wall presented a blind diverticulum, the pyloric caecum (*pc*), which was stout and curved. Two connections linked the caecum with the intestinal loop; the first joined the base of the caecum and the descending branch of the gut loop, while the other one was branched, resulting in three connections that joined the curve of the caecum with the ascending limb (Fig. 4E). A notable constriction is placed between the stomach and the intestine (Fig. 4E). The anus, which showed a smooth margin, opened into the atrial cavity (Fig. 4E).

Gonads

The gonads, one on each side, were attached to the inner wall of the body. The right one was placed on the middle of the body, while the left one was located slightly anteriorly, in the middle of the range between the anterior end and the gut loop (Fig. 4F). Each gonad consisted of an ovary with a variable number of eggs (up to 3-4), and a testis divided into two pyriform male follicles sometimes lobed at their distal end, one anterior and the other posterior to the ovary. Male and female gonads were both mature at the same time. The deferens ducts came together in a single vas deferens which opened into the peribranchial cavity (Fig. 4F). Gonads and larvae were only recorded from the colonies collected in July 2014.

Larvae

S. brakenhielmi showed an ovoviviparous mode in reproduction, with larvae development occurring in the parental atrial cavity before release. Up to 4 larvae were occasionally found in the peribranchial cavity and were characterized by different development stages. Tadpole larvae were approximately 1.5 mm in total length, with the trunk measuring about 500 µm (Fig. 4G). They were orange in color, like the mature eggs. The trunk had three unstalked adhesive organs, tri-radially arranged, and a single sense organ, the photolith (Fig. 4G).

COI sequence comparisons

The 834-bp long COI sequences obtained from the two yellow and the two red type colonies were identical, thus demonstrating that these colonies belong to the same species. The comparison to the nt-nr db (10th May 2018) showed that our 834-bp sequence is 96.5±0.99 % identical to the four shortest (567-676 bp) COI sequences of *S. brakenhielmi* and, surprisingly, it is also 96.9 % identical to a 620-bp long COI sequence of the congeneric *S. rubra* (Table 1). Phylogenetic and species delimitation analyses were performed within the Polyzoinae in order to clarify the significance of the molecular similarity between *S. brakenhielmi* and *S. rubra*. The COI phylogenetic reconstruction (Fig. 5) strongly supported the clustering of all specimens of the two *Symplegma* species in a monophyletic clade (100% bootstrap) and identified monophyletic clades for three of the other four analysed species (*i.e., Polyandrocarpa anguinea*, *Polyandrocarpa zorritensis* and *Distomus variolosus*). All 12 ABGD species delimitation analyses (carried out on the three different alignments - alnBS_838, alnBS_442 and aln_442 - using the JC, K-2P and p-dist evolutionary distances) highlighted the existence of a clear barcode gap and showed a perfect match between the initial and the recursive partitions for prior intraspecific divergences ranging from 0.1% to 5.99%. Remarkably, all ABGD analyses included *S. brakenhielmi* and *S. rubra* sequences in the same OTU, thus suggesting a possible synonymy between *S. brakenhielmi* and *S. rubra* (Fig. 5). Apart from the case of *Symplegma*, each ABGD-identified OTU corresponded to a distinct species, as labeled by the sequence's author based on morphological identification (Fig. 5), with the only exception being the creation of a singleton OTU for *Distomus variolosus* "i86" just in the analyses of the alnBS_838 alignment (data not shown). This suggested that the ABGD species delimitation results are mainly correct, in spite of the analysis of a dataset consisting of few sequences per species and only one *S. rubra* representative. The only available COI of *S. rubra* (FJ528648) was published without a morphological description of the relative specimen (Perez-Portela *et al.,* 2009), but the Authors confirmed that this specimen had the typical red line encircling the siphons used as species diagnostic character for *S. rubra* (X. Turon, personal communication). Nevertheless, the possible synonymy between *S. brakenhielmi* and *S. rubra* must be further investigated through the concomitant molecular and morphological analyses of additional samples of the two species.

Unexpectedly, our *S. brakenhielmi* sequence was also 96.6 % identical to a very short COI sequence of *Stolonica socialis* (297 bp) (Table 1). The usage of *Symplegma*-specific primers to obtain this *S. socialis* COI (Perez-Portela *et al.,* 2009) raised doubts as to the source of this sequence, which therefore also needs to be confirmed by amplification and sequencing of additional *S. socialis* specimens. Indeed, we are not aware of ascidians belonging to different genera showing such a high level of similarity in COI barcode fragments.

The nt-nr db also contained two COI sequences of *S. brakenhielmi* (KR815818-19) having only 26-54% identity to our sequence (Table 1) but with highest and statistically significant similarity to aplousobranch and arthropod COIs according to BlastN results. These two sequences, both unpublished, are clearly the result of errors in db sequence submission or specimen identification.

Discussion

Within the Styelidae family, the Polyzoinae subfamily is represented by colonial ascidians with zooids never arranged in systems, characterized by extensive color polymorphism, including pomegranate purple, orange-red, pale lemon, greenish lemon or sulfur yellow (Kott, 1985). In their recent monograph on European Ascidians, Brunetti & Mastrototaro (2017) report a key to genera of the subfamily Polyzoinae. According to this key, the assignment of the genus *Symplegma* was here based on the following characteristics: gonads all hermaphrodite with a single pair of male follicles, placed one on each side of the body wall lining the atrial cavity; branchial sac without folds and with four internal longitudinal vessels. Kott has published two different keys to species of the genera *Symplegma* (Kott, 1964, 1985). In the first key, Kott distinguished the four species *Symplegma connectans* Tokioka 1949, *Symplegma reptans* Oka 1927, *Symplegma viride* Herdman 1886 and *S. oceania* by the presence and shape of the pyloric caecum, as well as the number of its connections with the intestine loop and the number of rows of stigmata (Kott, 1964). Based on this first key, *S. connectans* is characterized by zooids without a pyloric caecum, but with a connection that links the stomach directly to the gut loop. *S. reptans* has a rounded pyloric caecum with 1 or 2 intestinal connections, and only up to ten rows of stigmata. *S. viride* is characterized by a branchial sac with more than 10 rows of stigmata and a curved pyloric caecum connected through 1 or 2 intestinal connections to the gut loop. *S. oceania* has a pyloric caecum linked to the gut loop by three intestinal connectives. In the second key, the same author (Kott, 1985), only described the three species *Symplegma arenosa* Kott 1972, *S. reptans,* and *S. oceania*, that can be distinguished by the presence and shape of the pyloric caecum, the position of the zooids and the number of stomach folds. According to this more recent key, *S. arenosa* is characterized by upright zooids, while those of *S. reptans* and *S. oceania* are prostrate and embedded in the common tunic. Moreover *S. reptans* does not show more than 8 stomach folds, and a short and straight pyloric caecum, while *S. oceania* has more than 8 folds and with a long and curved pyloric caecum. Subsequently, Monniot & Monniot 1997, as already mentioned, considering the nomenclature rules (ICZN, 1985), recognised *S. oceania* as a junior synonym of *S. brakenhielmi* (Michaelsen, 1904). Moreover, two other species of the genus were described: *S. rubra* and *Symplegma bahraini* Monniot & Monniot 1997. Although these latter two species are very similar to S. *brakenhielmi,* they can be distinguished from *S. brakenhielmi* because

they never present male and female mature gonads at the same time. In addition, *S. rubra* showed a characteristic pigmented ring linking both siphons, while S. *bahraini* showed a lighter area encircling the siphons and two larval sense organs (Kott, 2004; Monniot, 2002; Monniot & Monniot, 1997).

Based on our morphological analyses, it is possible to assign both our red and yellow type colonies to *S. brakenhielmi*. Indeed, our colonies are characterized by prostrate zooids embedded in the common test, more than 8 stomach folds, a long curved caecum with two intestinal connectives from the pyloric caecum, one simple and one divided in two (Kott, 1964, 1985), gonads mature at the same time, the lack of a pigmented ring or lighter area linking both siphons, and only one larval sense organ.

This study also confirms that *S. brakenhielmi* is characterized by color variability with at least two color types: the yellow and the red type. This is the first record of *S. brakenhielmi* in the western and central Mediterranean Sea but also the first report of yellow-type colonies in the Mediterranean Sea, since only reddish colonies had previously been found along the coasts of Turkey, Israel and Lebanon (Bitar & Kouli-Bitar, 2001; Çinar *et al.,* 2006, Shenkar & Loya, 2009; Ulman, 2016; Ulman *et al.,* 2017). So far, *S. brakenhielmi* yellow colonies have been found in south and central America (Lotufo, 2002; Goodbody, 2003), while red colonies have been recorded from the Indian Ocean, Melanesia and the eastern Mediterranean Sea (Tamilselvi *et al.,* 2011; Tokioka, 1961; Çinar *et al.,* 2006; Shenkar & Loya, 2009).

The possible synonymy between *S. brakenhielmi* and *S. rubra,* highlighted by the ABGD species delimitation analyses, is not supported by the current morphological description of these two species. Therefore, it needs to be verified through the analysis of a larger number of COI sequences and other non-genetic characters, such as morphological and ecological traits, in both species. It is also crucial to carry out all these analyses on exactly the same specimens, in order to exclude sampling bias or specimen misidentification.

Our study highlights the importance of a synergistic approach between morphological and molecular studies. In particular, each published nucleotide sequence should be linked, in the relative paper or in the database entry, to the morphological description of the specimen and/or to the taxonomic key used for the species assignment. Only in this way will it be possible to reduce the degree of uncertainty due to a potential rough specific identification, and to unambiguously associate a nucleotide sequence to specific morphological characters. This approach is even more important for non-indigenous species, since it will allow management policies to be promptly implemented. Target 5 of the EU Biodiversity Strategy (COM EU, 2011) states that European countries are committed to identify Invasive Alien Species and their pathways by 2020, in order to develop management strategies able to control and prevent the introduction and establishment of new invasive species. Ascidians are one of the main bio-fouling taxa (Gewing *et al.,* 2017) and important indicators of invasions. In fact, the short-lived non-feeding larvae do not usually disperse very far (Svane & Young, 1989) so a new finding in distant localities is usually symptomatic of human-mediated transport. The two localities where S. *brakenhielmi* has been found (Olbia and Taranto) are both strongly affected by anthropogenic activities, such as industrial activities, intense naval traffic and large mussel farms (Cardellicchio *et al.,* 1991; Bracchi *et al*., 2016; Marchini *et al.,* 2016; Tursi *et al*., 2018). Indeed, the areas surrounding Olbia and Taranto are considered Italian hotspots of alien species introduction (Cecere *et al.,* 2016; Marchini *et al.,* 2013; Mastrototaro *et al.,* 2008). In particular, *S. brakenhielmi* could be considered an established species on North-Eastern Sardinian coasts, having been recorded every year since 2003, with gonad maturation during the summer (Trainito, 2005). Recent studies carried out in these two areas also show that the alien species pool depends on the importation of mussels for breeding and that even the movement of vessels from these harbors can be an important invasion pathway for further spread of these aquaculture hitch-hikers (Cecere *et al.,* 2016; Marchini *et al.,* 2016). The proximity of the finding's localities to commercial harbors and aquaculture activities suggests that mollusk importation together with naval traffic could be the most likely vector of introduction, as well as of the future spread, of the invasive ascidian *S. brakenhielmi*.

Acknowledgements

This work was partially funded by PRIN-2015 (MIUR, Italy, grant number: 2015NSFHXF) to C.G. and by the Molecular Biodiversity Laboratory of the Italian node of Lifewatch (CNR). We thank Matteo Montagna for suggestions in sequence analyses.

The authors declare that they have no conflict of interest.

References

Abbott, D., Newbwerry, A., Morris, K., 1997. *Section 6B: Ascidians (Urochordata). Reef and Shore Fauna of Hawaii.* Bishop Museum Special Publications, Honolulu, 64 pp.

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), 403-10.

Bitar, G., Kouli-Bitar, S., 2001. Nouvelles données sur la faune et la flore benthiques de la cote Libanaise. Migration Lessepsienne. *Thalassia Salentina,* 25, 71-74.

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., Manni, L., Mastrototaro, F., Gissi, C., Gasparini, F., 2017. Fixation, description and DNA barcode of a neotype for *Botryllus schlosseri* (Pallas, 1766) (Tunicata, Ascidiacea). *Zootaxa,* 4353(1), 29-50.

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

Cardellicchio, N., Annicchiarico, C., Leone, G., Marra, C., Paterno, P. *et al.,* 1991. I mari di Taranto: problematiche di impatto ambientale. *Atti della Società italiana di Ecologia,* 12, 769-775.

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.

Çinar, M.E., Bilecenoglu, M., Ozturk, B., Can, A., 2006. New records of alien species on the Levantine coast of Turkey. *Aquatic Invasions,* 1, 84-90.

COM EU, 2011. Our life insurance, our natural capital: an EU biodiversity strategy to 2020. Communication from the Commission to the European Parliament, the Council, the Economic and Social Committee and the Committee of the Regions*. Official Journal of the European Union,* 244, 1-16.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics,* 5 (113), 1-19.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology,* 3(5), 294-299.

Gerovasileiou, V., Akel, E., Akyol, O., Alongi, G., Azevedo, F. *et al.,* 2017. New Mediterranean Biodiversity Records (July, 2017). *Mediterranean Marine Science,* 18 (2), 355-384.

Gewing, M.T., López-Legentil, S., Shenkar, N., 2017. Anthropogenic factors influencing invasive ascidian establishment in natural environments. *Marine environmental research,* 131, 236-242.

Goodbody, I., 2003. The ascidian fauna of Port Royal, Jamaica I. Harbor and mangrove dwelling species. *Bulletin of Marine Science,* 73 (2), 457-476.

Griggio, F., Voskoboynik, A., Iannelli, F., Justy, F., Tilak, F. *et al.,* 2014. Ascidian mitogenomics: comparison of evolutionary rates in closely related taxa provides evidence of ongoing speciation events. *Genome Biology and Evolution,* 6, 591-605.

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.

Hebert, P.D., Cywinska, A., Ball, S.L., Dewaard, J.R., 2003. Biological identifications through DNA barcodes. *Proceeding of the Biological Science,* 270 (1521), 313-321.

Herdman, W.A., 1906. Report on the Tunicata collected by Prof. Herdman at Ceylon in 1902. *Report of the Ceylon Pearl Oyster Fisheries,* 5, 295-348.

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.

ICZN, 1985. *International Code of Zoological Nomenclature. 4th Edition.* The International Trust for Zoological Nomenclature, The Natural History Museum, London, 106 pp.

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

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. *Mammalian protein metabolism,* 3 (24), 22-126.

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.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution,* 16(2), 111-120.

Kott, P., 1964. *Stolidobranch and Phlebobranch ascidians of the Queensland coast*. St. Lucia University of Queensland Press, Brisbane, 151 pp.

Kott, P., 1972. The ascidians of South Australia II. Eastern Sector of the Great Australian Bight and Investigator Strait. *Transactions of the Royal Society of South Australia,* 96, 165-196.

Kott, P., 1981. The ascidians of the reef flat of Fiji. *Proceedings of the Linnean Society of New South Wales,* 105, 147-212.

Kott, P., 1985. *The Australian Ascidiacea part 1, Phlebobranchia and Stolidobranchia.* Memoirs of Queensland Museum, Brisbane, 440 pp.

Kott, P., 2004. Ascidiacea (Tunicata) in australian waters of the Timor and Arafura seas. *Beagle: Records of the Museums and Art Galleries of the Northern Territory, The,* 20, 37-81.

Kott, P., Goodbody, I., 1980. *The ascidians of Hong Kong.* p. 530-554. In: Proceedings of the first international marine biological workshop: the flora and fauna of Hong Kong and Southern China, Hong Kong, Hong Kong University Press, Hong Kong.

Lambert, C.C., Lambert, G., 1998. Non-indigenous ascidians in southern California harbors and marinas. *Marine Biology*, 130, 675-688.

Lotufo, T.M.D.C., 2002*. Ascidiacea (Chordata: Tunicata) do litoral tropical brasileiro.* Doctoral dissertation, Universidade de São Paulo, São Paulo, 184 pp.

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.

Marchini, A., Ferrario, J., Occhipinti-Ambrogi, A., 2016. The relative importance of aquaculture and shipping as vectors of introduction of marine alien species: the case of Olbia (Sardinia). *Rapport Commission Internationale Mer Méditeranée,* 41, 430.

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.

Michaelsen, W., 1904. *Revision des kompositen Styeliden oder Polyzoinen.* Mitteilungen aus dem naturhistorischen. Kommissionsverlag von Lucas Gräfe & Sillem, Hamburg, 230 pp.

Monniot, C., 1972. Ascidies stolidobranches des Bermudes. *Bulletin du Muséum National d'Histoire Naturelle,* 57, 617-643.

Monniot, C., 2002. Stolidobranch ascidians from the tropical western Indian Ocean. *Zoological Journal of the Linnean Society,* 135, 65-120.

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

Perez-Portela, R., Bishop, J.D., Davis, A.R., Turon, X., 2009. Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetic Evolution,* 50, 560-570.

Plante, R., Vasseur, P., 1966. Sur une collection d'ascidies de la region de Tulear (cote Sud-Ouest de Madagascar). *Annales de l'Université de Madagascar. Série A: Sciences de la Nature et Mathématiques,* (4), 143-152.

Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology,* 21 (8), 1864-1877.

Rho, B.J., Park, K., 1998. Taxonomy of ascidians from Geojedo Island in Korea. *Animal Systematics, Evolution and Diversity,* 14, 173-192.

Rocha, R.M., 1991. Replacement of the compound ascidian species in a southeastern Brazilian fouling community. *Boletim do Instituto Oceanogràfico,* 39, 141-153.

Rocha, R.M., Barros de Faria, S., 2005. Ascidians at Currais islands, Paranà, Brazil: taxonomy and distribution. *Biota Neotropica,* 5(2), 1-20.

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

Rocha, R.M., Kremer, L.P., 2005. Introduced ascidians in Paranaguà Bay, Paranà, southern Brazil. *Revista Brasileira de Zoologia,* 22(4), 1170-1184.

Rodrigues, S., Rocha, R., 1993. Littoral compound ascidians (Tunicata) from Sao-Sebastiao, Estado de Sao-Paulo, Brazil. *Proceedings of the Biological Society of Washington,* 106(4), 728-739.

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., Loya, Y., 2009. Non-indigenous ascidians (Chordata: Tunicata) along the Mediterranean coast of Israel. *Marine Biodiversity Records,* (2), 1-7.

Sluiter, C.P., 1904. *Die Tunicaten der Siboga-Expedition*. Die Socialen and Holosomen Ascidien. E.J. Brill, Leiden, 126 pp.

Svane, I., Young, C.M., 1989. The ecology and behaviour of ascidian larvae. *Oceanography Marine Biology,* 27, 45-90.

Tamilselvi, M., Sivakumar, V., Abdul Jaffar Ali, H., Thilaga, R.D., 2011. Distribution of alien tunicates (Ascidians) in Tuticorin coast, India. *World Journal of Zoology,* 6(2), 164-172.

Tokioka, T., 1949. Contributions to the Japanese ascidian fauna II. Notes on some ascidians collected chiefly along the coast of Kii Peninsula. *Publications of the Seto Marine Biological Laboratory,* 134, 39-64.

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.

Trainito, E., 2005*. Atlante di flora e fauna del Mediterraneo.* Il castello ed., Milano, 256 pp.

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., 2016. Alien Ascidian Taxonomy and Identification Training. In: *Cost Action Alien Challenge, 05-20 November 2016.* Universidad de Alicante, Alicante.

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.

Vasseur, P., 1967. Contribution a l'étude des ascidies de l'Ile Maurice (Archipel des Mascareignes, Ocean Indien). *Recueil des Travaux de la Station marine d'Endoume,* (6), 101-139.

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.