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Spread of the non-indigenous ascidian *Aplidium accarensense* (Millar, 1953) in the Eastern Mediterranean Sea: morphological and molecular tools for an accurate identification

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Abstract

The aplousobranch ascidian *Aplidium accarensense* (Millar, 1953) was first described on the western coast of Africa, where it is considered native. Afterwards, this species was introduced along south-American Atlantic coasts, where it affected local shellfish farms through a massive colonization of both natural and artificial substrata. *Aplidium accarensense* has been recently reported along Catalan coasts and in the Tyrrhenian Seas (Western Mediterranean) where it represents a non-indigenous species, only recorded in harbours and aquaculture farms thus far. These Mediterranean records support the hypothesis that *A. accarensense* is currently expanding within the basin, representing a potential invasive species. In this study, several colonies of *A. accarensense* were found for the first time on artificial substrata within the semi-enclosed basin of the Mar Piccolo of Taranto (Italy, Ionian Sea), in the Eastern Mediterranean. Here we provide an updated description of *A. accarensense* combining both morphological and molecular approaches, in order to allow an accurate and reliable identification of this expanding species. Comparing the morphology of the specimens collected from Taranto with the previous descriptions, a slight intra-specific variability has been noticed. Therefore, we provide detailed comparisons of the specimens found in Taranto with all the other *A. accarensense* sampled in other areas of the world, in order to highlight the intra-species variability. The correct identification of a potentially-dangerous species such as *A. accarensense*, represents a needed step for environmental monitoring purposes and for implementing management strategies to mitigate the effects of non-indigenous species on natural ecosystems and human activities.

Keywords: Ascidiacea; NIS; Mediterranean Sea; COI; integrative taxonomy.

Introduction

Ascidians are considered one of the major fouling pests, due to the fact that several species can rapidly grow in large and even massive colonies, becoming invasive on both artificial and natural substrata thanks to their ability to resist a wide range of stresses (Lambert, 2002, 2007). Harbours and aquaculture facilities have been already identified as the principal gateways for the spread of invasive taxa including ascidians, which can be easily transported via shipping or attached to aquaculture products (López-Legentil *et al.*, 2015; Marchini *et al.*, 2013; Mastrototaro *et al.*, 2019). In fact, ascidians are one of the most successful animal taxa among the biofouling species that affect shellfish aquaculture and one of the major threats to this economic activity. The costs of fouling removal from different kind of surface

(e.g. nets, cages, ropes) and the possible suffocation phenomena among the farmed species caused by the fouling overgrowth (Lambert, 2007) are the main reasons for the increase in management costs in the aquaculture industry. Furthermore, man-made structures represent a further elective settlement site from which non-indigenous species can spread to neighbouring natural habitats, smothering and displacing autochthonous species, as well as possibly leading in alterations of the indigenous communities (Adams *et al.*, 2011; Carman & Grunden, 2010; Mastrototaro *et al.*, 2015). The increase in aquaculture facilities in the last 20 years has enhanced the spread of Non-Indigenous Species (NIS), providing new transfers and new surfaces (e.g. shells, ropes, nets, cages) for the spread of sessile species, including ascidians (Lambert, 2007). Considering that most of the aquaculture structures are usually suspended, predation can be lower than

that occurring on natural substrata, thereby anthropogenic surfaces can represent a refuge for biofouling organisms, among which many NIS can be present (Rocha *et al.*, 2009). Negative effects of biofouling in aquaculture include direct threats to cultured species, impacts on infrastructures (e.g. underwater structures such as cages, netting and pontoons), as well as associated environmental impacts on wild communities and local ecosystems. The number of NIS recorded in the Mediterranean Sea is rapidly increasing due to growing maritime traffics, the effects of the removal of natural barriers and commercial human activities, including aquaculture. On the other hand, the high number of NIS is also related to increased scientific efforts, such as the recent reassessments of the alien or cryptogenic status of some exotic species (Schlick-Steiner *et al.*, 2007; Vandepas *et al.*, 2015; Zenetos *et al.*, 2017; Servello *et al.*, 2019), as well as to the development of taxonomic approaches based on integrated morphological, ecological and molecular studies (Ojaveer *et al.*, 2014; Mastrototaro *et al.*, 2019, 2020). This is particularly true for the ascidians, whose complex taxonomy is one of the major limiting factors in the early detection of non-indigenous species (Izquierdo-Muñoz *et al.*, 2009; Ojaveer *et al.*, 2014).

Aplidium accarens (Millar, 1953), family Polyclinidae, was first recorded by Millar (1953) in the locality of Accra (Ghana, then called the Gold Coast, western Africa), hence the name *accarens*. Nowadays, its geographical distribution includes also Brazilian and Venezuelan

coasts (Rodrigues *et al.*, 1998 as *Aplidium* sp.; Rocha *et al.*, 2005, 2010; Bouzon *et al.*, 2012), where it was possibly introduced, as hypothesized by Rocha *et al.* (2009) (Fig. 1A). In particular, *A. accarens* was recorded in a Venezuelan marine protected area, with large colonies covering other species and representing a threat to the autochthonous diversity (Rocha *et al.*, 2010). It is also reported as threat for shellfish farms in southern Brazil (Rocha & Bonnet, 2009; Rocha *et al.*, 2009).

In 2015, *A. accarens* was recorded for the first time in the western Mediterranean Sea, found in 23 of the 32 harbours along the Catalan coasts (López-Legentil *et al.*, 2015). Afterwards, it was reported affecting mussel farms within the Ebro Delta (Casso *et al.*, 2018). Personal observations from X. Turon also indicated that this species is becoming abundant on artificial substrata within the Fusaro Lagoon in the Gulf of Naples (Italy, Tyrrhenian Sea) (see Appendix 2 of López-Legentil *et al.*, 2015; X. Turon pers. comm.) (Fig. 1B), thus alarmingly expanding its presence in the Mediterranean Sea. Our paper reports the first record of *A. accarens* in the Ionian Sea, within an area characterized by the presence of several human activities including aquaculture farms. We also provide a detailed morphological description of the species and its molecular characterization with an extended COI (Cytochrome Oxidase subunit I) sequence of about 830 bp. Moreover, an update on the currently known distribution of this potential pest species is provided, together with a detailed comparison of all morphological features that

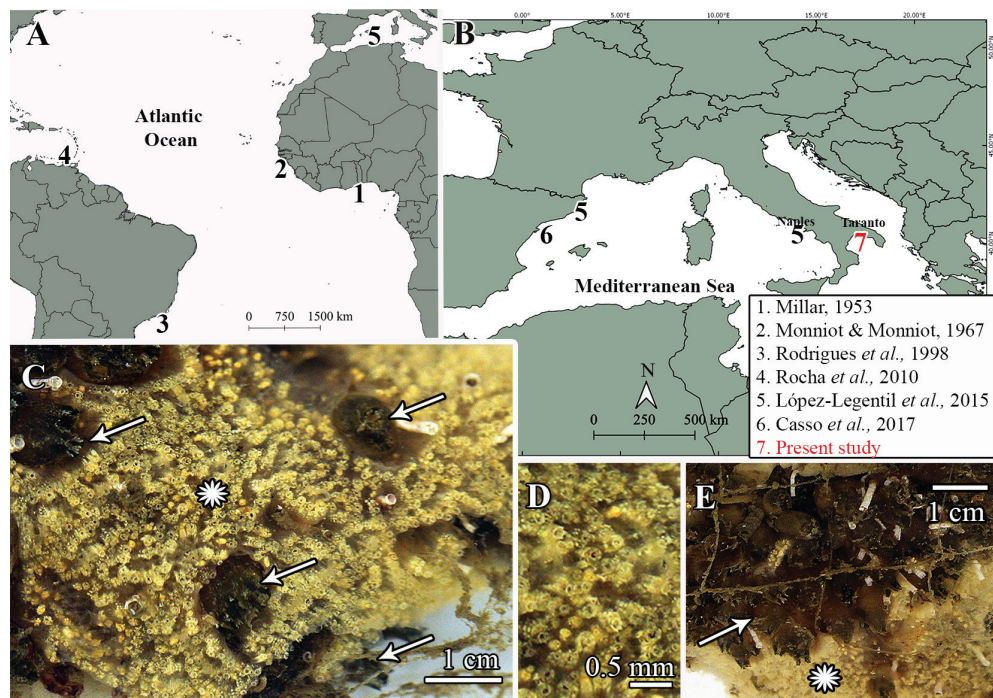


Fig. 1: A) Global occurrence of *A. accarens* (records numbered in temporal order); B) Detail of the records of *A. accarens* in the Mediterranean Sea; C) Colony of *A. accarens* (asterisk) collected in Mar Piccolo of Taranto (Ionian Sea) lying next to *Polyandrocarpa zorriventis* (arrows); D) Magnification of the yellow zooids embedded in the common transparent tunic; E) Magnification of the zooids of *P. zorriventis* (arrow) and *A. accarens* (asterisk).

have been reported so far for this species, in order to facilitate its identification in other areas.

Material and Methods

Study area

The study area is located in the Mar Piccolo of Taranto (North-western Ionian Sea, Italy), a semi-enclosed basin where a peculiar benthic community, dominated by filter- and suspension-feeders (such as sponges, polychaetes, molluscs and ascidians), coexists with a suite of anthropogenic activities and impacts, including high level of urbanization, heavy industries, intense maritime traffic and a commercial harbour as well as mussel and fish farms (Bracchi *et al.*, 2016; Tursi *et al.*, 2018) (Fig. 1B). During November 2018, a colony of *A. accarens* was collected from the submerged area of a pontoon in the Mar Piccolo basin (40.47605° N; 17.25192° E), at a depth of 2-5 m. In February 2020 and 2021, further colonies were observed and collected manually from different kind of artificial substrata close to the surface (e.g. submerged areas of pontoons, ropes) within the same basin. The sampled artificial substrates were almost totally covered by other non-indigenous ascidians such as *Polysandrocarpa zorritensis* Hoshino & Tokioka, 1967 and *Distaplia bermudensis* Van Name, 1902, as well as other ascidian species belonging to the families Clavelinidae, Didemnidae and the subfamily Botryllinae (Brunetti & Mastrototaro, 2017).

Sampling

A colony of *A. accarens* was photographed and collected by SCUBA diving in November 2018, while 50 and 70 colonies were collected in February 2020 and 2021, respectively. A subsample from a colony collected during each sampling (November 2018, February 2020 and February 2021) was directly preserved in 99% ethanol for molecular analysis, while the rest of the living colonies were relaxed with menthol crystals and preserved in 4% formalin solution in seawater for morphological investigations.

Molecular analyses

Total DNA was extracted from one of the subsamples of *A. accarens* preserved in ethanol collected in November 2018 (labelled P16), using a modified CTAB method (Hirose & Hirose, 2009). The extraction was performed from 2-3 zooids isolated from the common tunic. A fragment of the mitochondrial COI gene of about 830 bp was amplified using the primer pair dinF/Nux1R (Brunetti *et al.*, 2017). PCRs were performed with the 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° C, extension for 1 min 30 s at 72° C, and a final elongation step of 5 min at 72° C. After cleaning with an Amicon Ultra-0.5 mL centrifugal filter device (NMWL of 100kDa, Millipore), amplicons were directly sequenced according to the Sanger method at Microsynth AG (Switzerland). Sequence quality check was also carried out visually with Geneious ver. 5.5.7 (<http://www.geneious.com>; Kears *et al.*, 2012). The obtained COI sequence was compared to the public NCBI nucleotide non-redundant database (nt-nr db, 10th June 2020, www.ncbi.nlm.nih.gov/nucleotide) using the online Basic Local Alignment Search Tool (BlastN) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul *et al.*, 1990) The sequence was deposited at ENA (European Nucleotide Archive) with Accession Number MT873555.

Results

The sampled colonies were identified as *A. accarens* based on morphological characters of the colonies, as well as of zooids and larvae (Millar, 1953; Monniot & Monniot, 1967; Monniot, 1969). The relative COI sequence (826 bp) was 100% identical to 24 sequences of *A. accarens* (586–587 bp, 69% coverage) obtained from specimens morphologically described as *A. accarens* (Appendix 2 of López-Legentil *et al.*, 2015). No other highly similar (>90% identity) sequences belonging to different species were available in the nt-nr database, indicating the absence in this database of taxonomically mis-assigned sequences of *A. accarens*.

Systematics

Aplidium accarens (Millar, 1953)

Amaroucium accarens Millar, 1953

Amaroucium accarens: Monniot C. & Monniot F., 1967

Amaroucium accarens: Monniot F., 1969

New record

November 2018, February 2020 and February 2021: Mar Piccolo of Taranto, Ionian Sea, Mediterranean Sea.

Previous records in the Mediterranean Sea

2012–2013: harbours of the Catalan coast (North-eastern Iberian Peninsula, Balearic Sea) (López-Legentil *et al.*, 2015)

2013: artificial substrata (pilings of a bridge) at Fusaro Lagoon (Naples, Tyrrhenian Sea) (Turon, X. pers. comm. in López-Legentil *et al.*, 2015)

2015–2016: aquaculture facilities at Ebro Delta (Spain, Balearic Sea) (Casso *et al.*, 2018)

Material examined

About 70 colonies were observed and collected during 2018, 2020 and 2021 in the Mar Piccolo of Taranto (40.47605°N; 17.25192°E), 2–5 m depth.

Two colonies of *A. accarens* collected from Mar Piccolo of Taranto preserved both in 99% ethanol and 4% formalin (Code: MUZAC–6658 and MUZAC–6659, respectively) have been deposited in the collection of the Zoological Museum of the University of Bari.

External appearance

Encrusting, yellowish and slightly cushion-shaped colonies of about 2 cm thick. The tunic is transparent and without sand embedded (Fig. 1C-E). Colonies grow freely over the artificial hard substrata intercalated between

other colonial ascidians (e.g. *Polyandrocarpa zorritensis*, *Clavelina* spp.). Bright yellow zooids are visible and completely embedded in the jelly-like tunic, almost crowded and not arranged in systems (Fig. 1C-E). Colonies and zooids became grey after preservation in formalin.

Zooids

Zooids are different in size (up to 10 mm in length), with the post-abdomen often very long (up to 6 mm) (Fig. 2A). Zooids have a lobed oral siphon with 6 small pointed lobes (Fig. 2B) and an atrial siphon with a short simple languet which arises from the upper rim, at the level of the first row of stigmata (Fig. 2B). The thorax is longer than the abdomen and the pharynx has 7-10 rows of stigmata, with about 10-11 stigmata per half row (counted in the middle part of the pharynx) (Fig. 2C).

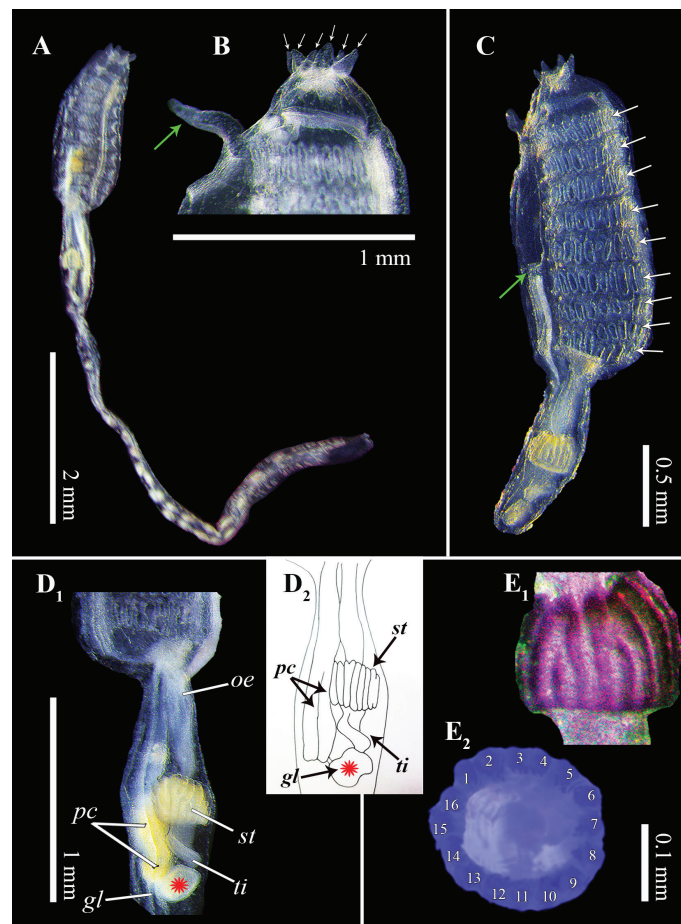


Fig. 2: Zooid of *A. accarens* from Taranto (Ionian Sea). A) Whole zooid; B) Magnification of the 6-lobed oral siphon (white arrows) and the simple languet (green arrow); C) Thorax and abdomen with 9 rows of stigmata (white arrows) and anus (green arrow) opening halfway through the thorax at the level of the 6th row of stigmata; D₁) Alimentary canal with the yellow longitudinally folded stomach with the gut loop characterized by the presence of a dilatation (red asterisk) of the twisted intestine (*ti*) and the rectum dividing into two proximal caeca. *gl*, gut loop; *oe*, oesophagus; *pc*, proximal caeca; *st*, stomach; D₂) Schematic drawing of the alimentary canal, gut loop with a dilatation (red asterisk) and enlarged in two proximal caeca; *gl*, gut loop; *pc*, proximal caeca; *st*, stomach; E₁) Stomach with longitudinal folds; E₂) Transversal section of the stomach with 16 folds.

Alimentary canal

The abdomen region is shorter than the thorax and marked by a constriction. The abdomen contains the alimentary canal with a plicated stomach (Fig. 2D₁-D₂), wider than long, with about 16 longitudinal folds sometime broken (Fig. 2E₁-E₂). After the stomach the intestine is twisted and subsequently forms a dilatation of the gut loop (Fig. 2D₁-D₂). The rectum begins with a narrow and very short tract that bifurcates into two proximal caeca. The rectum ends with an anus that opens halfway through the thorax (at the level of the beginning of the 6th row of stigmata) (Fig. 2C-D). There is no constriction between the abdomen and the post-abdomen regions (Fig. 2A-3B).

Gonads and larvae

The ovary appears yellow-brown in colour and it is composed of few oocytes placed anterior to the testis, which consists of several rounded follicles arranged in a double line, quite distant from the gut loop (Fig. 3B-C). Several zooids have 2-3 mature larvae in their peribranch-

chial cavity on the right side, with the most well developed larvae placed anteriorly (Fig. 3B). Fully developed larvae are 0.4-0.5 mm in trunk length with a well-developed tail and visible ocellus. At the anterior end of the trunk there are three adhesive organs (Fig. 3E) and several spherical ectodermal ampullae arranged in 4 groups of 5-8. The ampullae are placed between the adhesive organs, as well as dorsally and ventrally to them (Fig. 3D-E).

Remarks

The colonies found in the Mar Piccolo of Taranto showed some morphological differences from previous descriptions of the species (Table 1). In particular, our description is in accordance with López-Legentil *et al.* (2015), except for the absence of zooids systems and for some slight differences (Table 1). Small differences were also found with respect to the descriptions by other authors. In detail, in his first description Millar (1953) reports smaller zooids with a slightly larger number of uninterrupted stomach folds (17-20) (Fig. 3A). Monniot

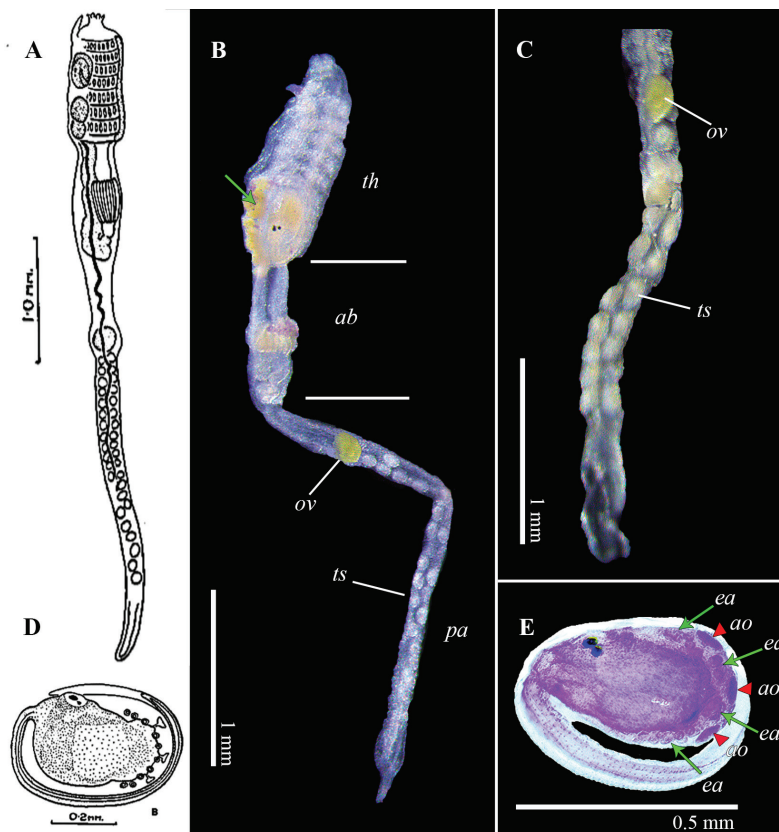


Fig. 3: Sexual apparatus and larvae of *A. accarensis*. A) Zooid from original description (Millar, 1953); B) Zooid showing the gonads in the post-abdomen and several larvae in the peribranchial cavity, one mature (green arrow). *ab*, abdomen; *ov*, ovary; *pa*, post-abdomen; *th*, thorax; *ts*, testis. C) Magnification of the post-abdomen showing the ovary and the male follicles arranged in a double line. *ov*, ovary; *ts*, testis. D) Drawing of the larva from the original description of Millar, (1953); E) Larva with three adhesive organs (red triangles) and ectodermal ampullae (green arrows) arranged in 4 groups. *ao*, adhesive organs; *ea*, ectodermal ampullae.

Table 1. Comparison of the main features reported for the species *A. accarensis*. In bold, the most variable characters highlighted in the previous description compared to our observations.

	Millar, 1953	Monniot & Monniot, 1967	Monniot, 1969	Rocha et al., 2005	López-Legentil et al., 2015	Present study
Locality	Western Atlantic Ocean (Accra, Gold Coast, Africa)	Western Atlantic Ocean (Cape Verde, Africa)	Western Atlantic Ocean (Dakar, Senegal, Africa)	Eastern Atlantic Ocean (Marinha do Arvoredo, Santa Catarina, Brasil)	Western Mediterranean Sea (Catalan coasts, Spain)	Eastern-central, Mediterranean Sea (Taranto, Italy)
Colony	no sand, flat and fleshy	encrusted with sand	massive, grey	light blue	yellowish, without sand	yellowish, without sand
Systems	-	-	not evident	circular, 6-7 zooids	circular or elongated	absent
Zoooid length	3 mm	5-7 mm	-	4-6 mm	2-10 mm	4-8 mm
Post abdomen	short	long	-	sometimes long	-	sometimes long
Oral lobes	6	6	6	6	6	6
Atrial languet	simple, short	simple	simple, short, sometimes absent	simple, short	simple, short	simple, short
Rows of stigmata	6-10 (usually 7)	10	8	7-10	6-9 (usually 7)	7-10
Stigmata in half row	10	12-15	-	5	12	9-11
Alimentary canal	stomach with uninterrupted folds	stomach with 12 uninterrupted folds, intestine enlarged in two caeca	stomach with 10-14 uninterrupted folds, twisted and then dilated intestine, rectum begins with two caeca	Stomach with 12-17 folds	Stomach with 17 folds, dilated intestine, rectum divided into two caeca, anus at the level of the 4th stigmata row	Stomach with 12-17 folds, sometimes interrupted, twisted and then dilated intestine, rectum divided into two caeca, anus at the level of the 6th stigmata row
Testis	longitudinal series	-	longitudinal series	28 lobes	-	longitudinal series, arranged in a double row
Ovary	one big brown oocyte anterior to testis	-	anterior to testis	5 oocytes	Few oocytes, most of the post-abdomen is occupied by densely packed male follicles	few brown oocytes anterior to testis
Larva	10 vesicles round each side	-	10 vesicles round each side	-	Several spherical ectodermal ampullae, arranged in 4 groups variable in number (>25), two between the papillae and one each dorsally and ventrally	Several spherical ectodermal ampullae, arranged in 4 groups of 5-8, two between the papillae and one each dorsally and ventrally

C. & Monniot F. (1967) report the presence of sand in the tunic, zooids with 12-15 stigmata in a half row and stomach consisting of uninterrupted folds. Monniot C. (1969) describes grey massive colonies with zooid systems not being apparent, uninterrupted stomach folds sometimes without the atrial languet. Colonies collected from South America by Rocha *et al.* (2005) were light blue in colour, with zooids arranged in circular systems and uninterrupted stomach folds. Despite these small morphological differences, Monniot C. & Monniot F. (1967), Monniot (1969) and López-Legentil *et al.* (2015) all indicate the presence of the two proximal caeca at the base of the rectum, and Monniot C. (1969) reports the twisted intestine and the dilatation after the stomach. These particular features of the alimentary canal are well evident in all the zooids analysed. The features of the larvae match almost all descriptions by the above-mentioned authors (see also Fig. 3D-E), as also shown in Table 1.

Discussion

In this study we update the distribution of the non-indigenous species *A. accarens* in the Eastern Mediterranean Sea. This species was not present in the Mar Piccolo basin until 2018 (F. Mastrototaro pers. obs.), then only one colony was observed and collected in November 2018, while numerous colonies were found to be extensively colonizing the artificial substrata of this basin in February 2020 and 2021. Further monitoring will be useful to point out whether *A. accarens* could be considered established in this area, as for the Fusaro Lagoon (López-Legentil *et al.*, 2015).

As already observed for other ascidian species (Rocha *et al.*, 2019; Monniot *et al.*, 2011), the taxonomic identification of *A. accarens* requires detailed analyses, both morphological and molecular, due to its complex morphological features and to the status of cryptogenic species (Rocha & Bonnet, 2009). The slight differences in the morphological features of *A. accarens* specimens recorded, indeed, highlight a certain intraspecific diversity, as the external appearance of this species could vary. For instance, blue and grey colonies have been recorded in Africa and South America, respectively (Monniot C. & Monniot F, 1967; Rocha *et al.*, 2005), while the specimens collected in the Mediterranean Sea show a yellow coloration (this study; López-Legentil *et al.*, 2015). In addition, the number of stomach folds and number of stigmata per half row can be slightly different (Millar, 1953; Monniot & Monniot, 1967; Rocha *et al.*, 2005) (Table 1). This study represents a detailed and updated guideline for the correct identification of *A. accarens*, allowing and facilitating its detection in the future. In addition to the universal barcoding primers developed by Folmer *et al.* (1994), the new primers here used, and able to amplify an extended COI sequence, can further help to perform population genetics studies on a global scale, aimed at understanding the genetic structure of the different *A. accarens* populations found along the African, American and European coasts. These new primers were set to facilitate

species discrimination and detection of cryptic/new species in Botryllinae, but they have also been successfully for the amplification of a wide range of ascidian species, including *A. accarens* (Salonna *et al.*, 2021).

The slight morphological variability (e.g. colour of the colonies, number of stomach folds and number of stigmata per half row) observed in the colonies collected from the Mediterranean Sea, along the coasts of both Spain and Italy, could be explained by considering the typical founder effect that can occur in new populations of NIS (Roman, 2006). Nevertheless, this hypothesis can be confirmed only through further samplings and morphological comparisons of the introduced populations found in South America and the Mediterranean Sea with the native African populations, together with the study of the COI haplotypes. The biology of this species is mostly unknown, indeed studies on its reproduction, growth rates or possible production of secondary metabolites are lacking. Our samplings revealed the presence of mature gonads and larvae during November and February in three different years, suggesting that the populations of *A. accarens* recorded in the Mar Piccolo basin are characterized by a long reproductive period, which may have helped its spread in the basin.

This species, which is native to the African coasts, is considered as introduced in South America (Rodrigues *et al.*, 1998; Rocha *et al.*, 2005; Rocha *et al.*, 2010; Guerra-Castro *et al.*, 2011) and along the coasts of Spain (López-Legentil *et al.*, 2015; Casso *et al.*, 2018), colonizing both natural and artificial substrata, and often affecting the shellfish farming (Casso *et al.*, 2018). The Mar Piccolo basin shares many features with the above-mentioned localities, being characterized by a suite of anthropogenic impacts, an industrial harbour, as well as large mussel and fish farms (Bracchi *et al.*, 2016). The finding of *A. accarens* further highlights the role of the Mar Piccolo basin as NIS hot-spot (Mastrototaro *et al.*, 2003, 2008, 2019; Mastrototaro & Brunetti, 2006; Longo *et al.*, 2007; Gravili *et al.*, 2010; Zenetos *et al.*, 2010; Occhipinti *et al.*, 2011; Servello *et al.*, 2019), stressing the importance of detailed taxonomic studies in high-risk areas for the introduction of NIS. These areas are often the starting point for the spread of NIS on a larger scale, representing the only possible chance to detect the introduction of potentially dangerous species and, eventually, avoid or mitigate their negative effects on both the natural community and the local economy. For this purpose, the comprehensive and timely identification of NIS is of critical importance to facilitate the development of cost-effective management strategies able to control and prevent their establishment and spread. In fact, the earlier an invasion is detected, the cheaper and quicker is the attempt to mitigate it (Hulme *et al.*, 2006, 2008), since the costs associated with removing biofouling are often high, difficult to quantify and ineffective once a species has become invasive (Fletcher & Westcott, 2013). *A. accarens* represents a potential risk for human activities and natural biodiversity, since it can easily be moved from place to place through shipping, farming facilities and mussel's commercialization. Thus, monitoring its distribution and

population features represent a necessary step to understand the potential spreading of this species in the Mediterranean Sea and to prevent its negative effects on basin scale.

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