Looks can be deceiving: *Didemnum pseudovexillum* sp. nov. (Ascidiacea) in European harbours

Turon X[1], Casso M[1,3], Pascual M[3], Viard F[2]

[1]Centre for Advanced Studies of Blanes (CEAB, CSIC), Blanes, Catalonia, Spain

[2] Sorbonne Université, CNRS, Laboratory AD2M (UMR7144), Station Biologique de Roscoff, Roscoff, France.

[3]Department of Genetics, Microbiology and Statistics, and IRBio, University of Barcelona, Barcelona, Spain

Corresponding author: X. Turon; e-mail: xturon@ceab.csic.es;

ORCID:

XT: 0000-0002-9229-5541

FV: 0000-0001-5603-9527

MP: 0000-0002-6189-0612

Abstract

A strongly divergent lineage, putatively a new cryptic species, of colonial ascidian was first detected as an anomalous sample in a population genomics study of the well-known worldwide invasive species *Didemnum vexillum* Kott, 2002. This putative new taxon, found in a marina in Roscoff, France, is indistinguishable from *Didemnum vexillum* in external aspect and coexists with it in syntopy. However, morphological characters such as spicules and larvae allow a clear-cut distinction. In accordance with the preliminary results based on genome-wide analyses, morphological traits and mitochondrial sequences of the Cytochrome Oxidase I gene both support the establishment of a new species *Didemnum pseudovexillum* sp. nov. Previous unidentified sequences in public databases showed that the new species is also present in NW Mediterranean marinas. *Didemnum pseudovexillum* sp. nov. is assigned for the time being a cryptogenic species status, although its presently known disjoint distribution across two biogeographic regions and its presence in ports are suggestive of an introduced species. Further studies should be performed to ascertain its current distribution and putative natural range and settle its native vs. non-native status. This finding casts doubts on previous reports of *Didemnum vexillum* and also calls for caution when performing fast field surveys of non-indigenous species such as Rapid Assessment Surveys (RAS) or BioBlitz surveys, based solely on external characters.

Keywords: ascidian, cryptogenic species, artificial substrate, biofouling, rapid assessment survey, Didemnidae

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Introduction

Taxonomy is at the heart of all biological studies (Bortolus 2008), and this holds particularly true in the study of introduced, non-indigenous species (NIS). Typically, an introduced species appears in a short time frame in a variety of geographic locations, often far away from its natural distribution range, as a result of human-mediated transport. In its introduction range, it is often identified by different specialists. If this happens in a group of difficult taxonomy and with few specialists, there are risks of misidentifications, repeated descriptions of new species, and overall failure of taxonomy to cope with a wide-scale perspective (Carlton 1999; Ojaveer et al. 2014).

Ascidians are a group of marine invertebrates which is paradigmatic in this respect. They are difficult to identify morphologically due to few diagnostic characters, which are often difficult to observe. In addition, morphological, chemical, and genetic variation within species suggest that many formally recognized species are in fact species-complexes (e.g., López-Legentil and Turon 2005; Bock et al. 2012; Teske et al. 2011). The problem is further complicated by declining taxonomic expertise (a global problem not limited to ascidians, Giangrande 2003). On the other hand, this group includes numerous and important introduced species (Lambert 2007; Shenkar and Swalla 2011, Zhan et al. 2015) with large-scale distributions, which has originated diverse taxonomic problems, as the long list of synonymies of some cosmopolitan species testifies (e.g., *Botrylllus schlosseri* (Pallas, 1766), *Botrylloides leachii* (Savigny, 1816), see Kott 1985).

When species had been well described, molecular barcoding can facilitate the correct identification of introduced species (Comtet et al. 2015), including cryptic introductions of widely-distributed ascidians (e.g., Turon et al. 2003; Bishop et al. 2013; Ordóñez et al. 2016). Population-based genetic studies (e.g., population genetics, phylogeography) have also unveiled that even well-known introduced species had more variability than previously thought, revealing divergent lineages, and putative cryptic species (i.e., species not distinguishable with morphological traits) (Pante et al. 2015a). Indeed, cryptic speciation has proved to be widespread, and in some cases the taxonomy has been resolved, such as in the case of the model “species” *Ciona intestinalis* (Linnaeus, 1767) (Brunetti et al. 2015; Malfant et al. 2018), while in other instances genetic clades remain to be formally named (e.g., *Diplosoma listerianum* (Milne Edwards, 1841), Perez-Portela et al. 2013, *Botryllus schlosseri,* López-Legentil et al. 2006; Bock et al. 2012; Griggio et al. 2014).

Survey methods to detect introduced marine species (reviewed in Campbell et al. 2007, Kakkonen et al. 2019) include non-destructive visual surveys such as rapid assessment surveys (RAS, e.g., Cohen et al. 2005, Bishop et al. 2015, Nall et al. 2015), photographic methods (e.g., Grey 2009), or BioBlitz surveys (e.g., Cohen et al. 2011). Often, there is no time, money, or expertise for sampling followed by in-depth accurate morphological or molecular analyses of the specimens found. Thus, these surveys often rely on external characteristics

such as general aspect and pigmentation, without morphological or molecular confirmation on voucher specimens. External characters are too variable in many ascidians, especially colonial species, to be deemed reliable, as demonstrated recently in surveys of *Botrylloides* spp in Europe (Viard et al. 2019). Indeed, taxonomic issues such as misidentifications or lack of resolution at low taxonomic levels are common problems of all survey methods (Campbell et al. 2007).

Paramount among ascidian NIS is the case of *Didemnum vexillum* Kott, 2002, a global invader in temperate waters. This species has a highly convoluted identification story, including several misidentifications in different areas and two descriptions as new species (reviewed in Lambert 2009). Eventually, genetic analyses proved that all populations so far recorded were conspecific (Stefaniak et al. 2009) and the name *Didemnum vexillum* (wrongly described as a native species in New Zealand, Kott 2002, see Lambert 2009) was adopted.

Didemnum vexillum is a species in principle easily identified based on external morphological characters, particularly when abundant on artificial substrates where it often smothers other organisms. In a population genomics study of *Didemnum vexillum* (Casso et al. 2019), using Genotyping-By-Sequencing methods, we routinely obtained samples from diverse localities (marinas or aquaculture facilities) around the world. Unexpectedly, inclusion in the analyses of specimens sampled in one location of the NE Atlantic (Roscoff-Bloscon marina, English Channel, France) resulted in a drop of more than 90% in the number of polymorphic loci shared among all samples, an outcome usually due to the mixing of several divergent species (Pante et al. 2015b). These preliminary results thus suggested that these specimens belong to a highly divergent lineage. This prompted a re-examination of these samples and further collections at the same marina, which uncovered the existence of a new species, “vexillum”-like in appearance and living in syntopy with “true” *Didemnum vexillum*, which is described in this paper.

MATERIAL AND METHODS

Morphological observation

We examined 17 colonies of *Didemnum* spp collected in Bloscon Marina, Roscoff, France (48º 41.95’ N, 3º 57.93’ W, Fig. 1) the 27th April 2015 and preserved in absolute ethanol. We also analysed five colonies from the same marina sampled the 29th June 2018, from each of which a fragment was preserved in formalin and a second fragment in absolute ethanol.

Morphological observation concentrated on the main features of colonies and zooids. Spicules were isolated from the tunic by dissolving tunic fragments in bleach (sodium hypochlorite, 35‰ concentration) in an oven at 80ºC. For scanning electron microscopy (SEM), the isolated spicules were then

dehydrated in a graded alcohol series, sputter coated with gold and observed in a Hitachi TM3000 microscope.

DNA extraction and amplification

We analysed six of the colonies collected in the sampling of April 2015 (hereafter colonies 1-6) and four of the colonies collected in June 2018 (colonies 7-10).

A fragment of about 590 bp of the cytochrome oxidase I (COI) mitochondrial gene was amplified and sequenced using primers designed by Stefaniak et al. (2009). For six colonies (1-6), the DNAs had been previously used to build the genomic libraries for GBS analyses and was obtained from a single thorax for each colony using a whole genome amplification (WGA) procedure as detailed in Casso et al. (2019). COI amplification was carried out in 20 μL final volume including 0.4μL of each primer (10 mM), 1μL $MgCl_2$ (25mM), 0.5 μL dNTPs (1mM), 0.2 μL of Tq polymerase corresponding to 1U (GoTaq, Promega), 4μL 5X buffer (GoTaq, Promega) and 1μL of DNA at a concentration of 50ng/μL. PCR started with an initial denaturation at 94ºC for 5 min, followed by 35 cycles of a denaturation step at 94ºC for 1 min, an annealing step at 50ºC for 1 min, and an elongation step at 72ºC for 1min, and a final elongation step at 72ºC for 7 min. The amplified DNA was purified with Exo-SAP (0.2U/μl Exonuclease and 0.2U/μl Shrimp Phosphatase) at a proportion of 1:2 (ExoSap:PCR product). The sequences for both strands were obtained at the Scientific and Technical Services of the University of Barcelona. For the other four colonies (7-10), five thoraces were pooled per colony and extracted using the REDExtract-N-Amp Tissue kit (Sigma-Aldrich), following manufacturer's recommendations. PCR amplification was done in 20 μL total reaction volume with 10 μL of REDExtract-N-Amp PCR reaction mix (Sigma-Aldrich), 0.8 μL (10 mM) of each primer, 6.4 μL of ultra-pure water (Sigma-Aldrich) and 2 μL of DNA at a concentration of ca. 5 ng/μL. PCR conditions were set as before. Sequencing was carried out (both strands) at Macrogen facilities (Netherlands). The resulting sequences were assembled, edited and aligned in BioEdit v.7.2.6 (Hall 1999).

Genetic analyses

To compare the obtained sequences with those already existing for the genus, we performed a search in the Barcode of Life Database (BOLD) at http://www.v3.boldsystems.org (accessed 20 Dec 2019). The query comprised all COI-5P sequences available in public databases with taxonomy = *Didemnum*. Sequences were recorded by species name and by Barcode Index Numbers (BINs, Ratnasingham and Hebert 2013). We aligned the sequences using the in-built BOLD aligner, eliminating sequences with contaminants and with stop codons.

The sequences were trimmed to a common length of 597 bp and collapsed into haplotypes using the online tool FaBox v.1.5 (Villesen 2007) at http://users-birc.au.dk/palle/php/fabox/index.php. The sequences obtained in the present study were added to the alignment, and a preliminary NJ tree was constructed using Mega7 software (Kumar et al. 2017). A perusal of this tree showed several inconsistencies among the downloaded sequences. For this reason and for ease of presentation of results, we selected a subset of sequences based on the following criteria: we deleted sequences without a species name when they did not fall close to our sequences in the tree, and for species or clades with many sequences, we randomly picked five haplotypes each. Finally, we deleted sequences that looked clearly divergent or misplaced in the trees and whose BLAST results suggested that they were erroneous sequences (possibly contaminations or errors in species identification).

The aligned sequences were then evaluated with the modelTest function of the R package *phangorn* (Schliep 2011) to select the best-fit evolutionary model of nucleotide substitution based on the Akaike Information Criterion (AIC). This model was then selected in a maximum likelihood tree search in Mega with default options and 1,000 bootstrap replicates. A sequence of *Diplosoma listerianum* was used as an outgroup. A species delimitation analysis was performed in this tree using three approaches, different in nature and properties, to ensure confidence in the outcome of the species delineation. We used first multi-rate Poisson Tree Processes (mPTP, Kapli et al. 2016) as implemented in the web-service available at http://mptp.h-its.org using the default values. We also ran an Automatic Gap Discovery analysis (ABGD, Puillandre et al. 2012) using the web-service (https://bioinfo.mnhn.fr/abi/public/abgd/) with simple distance and a relative gap width of one. We explored a range of prior intraspecific divergences between 0.01 and 0.1. Finally, we used the single threshold general mixed Yule coalescent model (GMYC) (Pons et al. 2006); the analysis was performed with the R library *splits* (Ezard et al. 2009), using an ultrametric tree built with Mega using the RelTime method (Tamura et al. 2012).

Results

All of the colonies collected in 2015 and two of those collected in 2018 belonged to the new species, while another three colonies sampled in 2018 were morphologically assignable to *Didemnum vexillum* based on spicules and zooid characteristics (no larvae present) following Lambert (2009) and Ordóñez et al. (2015). This morphological distinctiveness was also confirmed with sequence data (see below).

Description

Didemnum pseudovexillum sp. nov. Turon & Viard

Holotype: colony 8 Bloscon Marina, Roscoff, 29/06/2018. Paratypes: colonies 1 to 4, Bloscon Marina, Roscoff, 27/04/2015; colony 7, Bloscon Marina, Roscoff, 25/06/2018. Deposited at the Center of Resources for Animal Biodiversity (formerly Museum of Zoology) of the University of Barcelona, refs CRBA-90721 (holotype) and CRBA-90722 to CRBA-90726 (paratypes).

Etymology: the name *pseudovexillum* refers to the close external resemblance of this species to *Didemnum vexillum,* and thus calls for caution to avoid confusing the two species on the basis of external aspect.

The colonies are large and encrusting, and are highly abundant in the marina studied. When the available space is occupied, the colonies tend to generate uprising lobes giving them a tri-dimensional appearance. The colour is yellowish-orange, and the surface shows darker canals surrounding zones with zooidal apertures. Overall, the aspect is indistinguishable from *Didemnum vexillum* colonies found in close syntopy (exactly the same walls) in the marina studied (Fig. 2).

The colony surface has a whitish tinge due to the presence of spicules, with white rims corresponding to spicule accumulations in the oral siphons (Fig. 3A). The colony thickness reaches 2-3 mm. There is a thin distal tunic layer with more or less abundant spicules (never so abundant as to give this layer a coriaceous consistence) and a thick basal layer poor in spicules (Fig. 3B). In-between lie the thoraces of the zooids, whose abdomens are embedded in the upper part of the basal layer. The cavity of the colony runs between these two tunic layers, with the main canals penetrating the basal tunic between abdomens (Fig. 3B).

The spicules are generally between 20-30 μm in diameter, reaching up to 40 μm (Fig 4A-C). They have many somewhat bluntly tipped short rays, about 30 in the visible field, and ca. 10 in optical section. This is stark contrast with the spicules of *Didemnum vexillum* from the same locality, with fewer (ca. 12 visible, 7 in optical section) and more pointed rays (Fig. 4D), in agreement with previous descriptions (Lambert 2009; Ordóñez et al. 2015).

The thoraces (Fig. 3C) are strongly contracted and measure ca. 0.5 mm. They have six small pointed lobes in the oral siphon, a wide atrial aperture exposing most of the branchial sac and no atrial languet. There are four stigmata rows, the exact number of stigmata could not be counted due to the strong contraction. The thoracic organs break away easily, but when present they lie in the lower part of the thorax and have an ear-like appearance (Fig. 3C). There is a muscular appendix of variable length, but generally shorter than the thorax itself, perhaps due to its contractibility. It originates in the anterior part of the oesophageal neck.

The abdomens reach ca. 0.6 mm; they contain a simple digestive system with an oval stomach. Many zooids have testis, consisting of a single follicle with a coiled sperm duct describing 6-7 turns (Fig. 3D). Some abdomens have also incubating oocytes, generally a single large one, sometimes a second smaller oocyte (Fig. 3E). In some cases, both testis and a small oocyte are present.

There are embryos and larvae in most of the colonies examined from both April 2015 and June 2018. They are free in the basal layer of tunic. The larvae (Fig. 3F-H) measure ca. 0.5 mm. They have 3 adhesive papillae and a variable number of finger-like ectodermal ampullae. Four pairs are present in young larvae and, as they mature, more ectodermal ampullae are added. Careful examination is necessary to assess their number and disposition, but we never observed 6 pairs of ampullae. In contrast, there are always 6 pairs of them in mature *Didemnum vexillum* larvae (Lambert 2009, Ordóñez et al. 2015). Some arrangements found in our specimens are: 4 pairs plus a dorsal unpaired ampulla, 4 pairs plus a single dorsal and a single ventral ampulla, 5 pairs, 5 pairs plus a single dorsal ampulla.

Genetic analyses

Of the sequenced specimens, colonies 1-6 (sampled in 2015) and colonies 7-8 (sampled in 2018), all morphologically assigned here to *Didemnum pseudovexillum* sp. nov., shared the same haplotype, while colonies 9 and 10 (2018), which were identified as *Didemnum vexillum*, had a different haplotype each. The three sequences have been uploaded to GenBank (accession numbers, colonies 1-8: MN952978, colony 9: MN952979, colony 10, MN952980)

The initial *Didemnum* dataset obtained from BOLD comprised 254 records, of which 214 had Barcode Index Numbers (BINs) assigned. They represented 36 nominal species and 51 BINs. This original alignment is available as Online Resource 21. Using the Barcode Gap Analysis tool of BOLD we found an intraspecific distance of 5.84±0.32% (mean±SE) and a distance to the nearest species of 13.29±0.34%. The BIN Discordance Analysis tool of BOLD detected three discordant BINs with multiple species-level designations. Another 20 BINs were taxonomically concordant, while 28 BINs comprised only singletons.

After trimming to 597 bp and collapsing identical haplotypes, we obtained an alignment of 161 sequences, to which we added the sequences obtained in the present study. Congruent with the results of the BIN Discordance Analysis, a preliminary NJ tree (not shown) detected again some sequence misplacement (i.e., sequences assigned to the same species name but appearing in diverse clusters). We then prepared a refined dataset selecting a maximum of 5 sequences belonging to a given species or clade, deleting sequences without species names (except those topologically close to our sequences) and those that were highly divergent and/or had suspicious BLAST results. Note that, for

Didemnum vexillum, we included sequences of the two main clades recognized in Stefaniak et al. (2012) that we named as in that work (Clades A and B). This reduced dataset allowed us to refine the alignment, eliminating gaps introduced by the divergent sequences, to a final length of 582 bp. The final dataset, available as Online Resource 2, comprised 66 sequences, to which a sequence of *Diplosoma listerianum* (GenBank accession number KF791870) was added as outgroup.

The final dataset comprised 20 *Didemnum* species and 29 BINs. We re-ran the BOLD Barcode Gap Analysis, and obtained lower values of intraspecific distance (3.26±0.24%, mean±SE) and distance to the nearest species (12.93±0.19%) than with the initial dataset. With the final dataset there was no discordant BINs (assessed with the Discordance Analysis tool), with 11 concordant BINs and 18 singleton BINs.

The modelTest function of *phangorn* revealed that the best-fit model of nucleotide selection for our *Didemnum* dataset was the General Time Reversible model with a gamma distributed rate variation among sites and a proportion of invariable sites (GTR+G+I). This model was input in the ML tree construction algorithm of Mega and the corresponding phylogenetic tree obtained is depicted in Fig. 5 (G parameter=0.795, I parameter=17.61%). The sequences obtained from specimens sampled in Roscoff either grouped with *Didemnum vexillum* Clade A (colonies 9 and 10), confirming morphological identification, or formed a clade (the single haplotype shared by colonies 1-8) with sequences of two unidentified *Didemnum* species from Catalan harbours, labelled as *Didemnum* sp1 and *Didemnum* sp2 in the work by López-Legentil et al. (2015). The distance between the Roscoff sequences and *Didemnum* sp2 was 2%, and with *Didemnum* sp1 it was 4.9%. This clade of three sequences had a bootstrap support of 99%. The sister clade (albeit poorly supported, <50%) in the tree comprised two sequences identified as *Didemnum cineraceum* (Sluiter, 1898) from Brazil (Oliveira et al. 2017) and one sequence from Australia identified as *Didemnum* cf. *albopunctatum* Sluiter, 1909 (Erwin et al. 2014). The Roscoff sequences had between 12.9 and 16.4% divergence with the sequences of this sister clade.

The species delineation analysis, made with mPTP, identified 19 putative species, mostly coherent with taxonomic identifications (20 nominal species in the tree), but with a few exceptions (Fig. 5). Interestingly, the clade comprising colonies 1-8, *Didemnum* sp2, and *Didemnum* sp1 was identified as one of these putative species. The ABGD method identified 29 distinct entities (i.e. putative species), with again some incongruences with taxonomic identification (Fig. 5). In agreement with the mPTP results, the colonies 1-8, *Didemnum* sp2 and *Didemnum* sp 1 were identified as a single putative species. Finally, the GMYC method identified 30 groups, which were the same as in the ABGD analysis, with the only exception that *Didemnum* sp1 was placed as a separate entity from the one formed by colonies 1-8 and *Didemnum* sp2 (Fig 5).

DISCUSSION

The morphological analyses confirmed that in the Bloscon marina in Roscoff (English Channel, France), *Didemnum vexillum* coexists with a new species, *Didemnum pseudovexillum* sp. nov. Both species are abundant and can be intermingled in the same micro-habitat (here the same walls in the marina studied). There is virtually no external difference between them. On close examination it seems that *Didemnum pseudovexillum* sp. nov. tends to have more oral siphon openings in the darker canal areas, and there is a more marked whitish tinge in the oral siphons due to spicule accumulation. However, in this species, as stated by Lambert (2009) for *Didemnum vexillum* as well, spicule density varies between colonies and even between various parts of the same colony. Clearly, these external characters are too unreliable to be used in the field. On the other hand, the spicules are clearly different and proved a useful diagnostic character. Larvae are also different, as *Didemnum vexillum* larvae have consistently 6 pairs of ectodermal finger-like antero-lateral ampullae, while *Didemnum pseudovexillum* sp. nov. has between 4 and 5 pairs. The number of coils in the sperm duct is also lower (6-7) than in *Didemnum vexillum* (8-11, Lambert 2009; Ordóñez et al. 2015). Finally, a recent study (Casso et al. 2020) showed that the microbiome communities of *Didemnum vexillum* and *Didemnum pseudovexillum* sp. nov. (referred to as *Didemnum* sp. in that work) were also markedly different. In Casso et al. (2020), the microbiome of *Didemnum vexillum* in its native and introduced range was examined, and samples of *Didemnum pseudovexillum* were used for comparison, showing that even congeneric species living in the same kind of environment had species-specific microbiomes.

The phylogenetic tree revealed a clade highly supported by bootstrap analysis (99%) comprising the *Didemnum pseudovexillum* sp. nov. sequences obtained in Roscoff and two sequences previously reported by López-Legentil et al. (2015) from Catalan harbours (NW Mediterranean, Fig. 1). In that work, they were named *Didemnum* sp1 (collected in L'Escala, 42º07.00' N; 3º 08.60' E) and *Didemnum* sp2 (sampled in Port de la Selva, 42º20.20' N; 3º11.90' E). Unfortunately, the specimens from this study are no longer available, but one of us (XT) kept pictures of them and notes. The images revealed colonies small but with the same colouration as the ones from Roscoff. For *Didemnum* sp2 we kept morphological notes and, although the colony was not reproductive, spicules and zooid morphology were in complete agreement with the description of *Didemnum pseudovexillum* sp. nov. Unfortunately, there were no observations available on *Didemnum* sp1. The three methods of species delineation gave overall coherent results, but ABGD and GMYC tended to split the clades into species more than the mPTP method (29-30 vs 19 inferred species). It should be noted that the mPTP analysis yielded results that matched closely the nominal species assignment (20 species), albeit with some exceptions. Concerning our samples, the clade comprising *Didemnum pseudovexillum* sp. nov., *Didemnum* sp2 and *Didemnum* sp1 was recognized

as a putative species by mPTP and ABGD, but *Didemnum* sp1 was placed as a distinct entity by GMYC. The *Didemnum* sp2 sequence was highly similar (98%) to the haplotype observed for the eight colonies sampled in Roscoff (98%), while *Didemnum* sp1 had 4.9% divergence. This slightly higher divergence is likely to explain the discrepancy between the results of the species delineation methods. However, the divergence between *Didemnum pseudovexillum* sp. nov. and *Didemnum* sp 1 is well below the range of interspecies differences in the genus (Stefaniak et al. 2009, and present results). In addition, the tendency of GYMC to over-split has been pointed out in other studies (e.g., Pentinsaari et al. 2017). So, albeit further studies are necessary, we consider colonies 1-8, *Didemnum* sp1 and *Didemnum* sp2 to belong to the same species. Whatever the final placement of *Didemnum* sp 1, *Didemnum pseudovexillum* sp. nov. is present both in Atlantic and Mediterranean harbours. This conclusion implies that, despite genetic COI uniformity in Roscoff, there may be a notable intraspecies genetic variability for that gene. Furthermore, during the genomic study of *Didemnum vexillum* performed by Casso et al. (2019) in the population of Roscoff (not included in that work when it was realized that it was a different species), we found 1,716 polymorphic loci with a mean of 2.72 alleles/locus (authors' unpublished results), a value in the range of the variability found in the *Ddidemnum vexillum* populations analysed (2.71-3.32 alleles/locus, Casso et al. 2019). Thus, the level of genetic variability of *Didemnum pseudovexillum* sp. nov. seems to be as high as that of similar introduced species. Further specific studies are necessary to assess the exact degree of genetic variation in populations of the new species.

The sister clade of *Didemnum pseudovexillum* sp. nov. comprised two sequences of *Didemnum cineraceum* from Brazil (Oliveira et al. 2017). This species has been reported from both sides of the Atlantic and the Pacific (Monniot 1983; Monniot and Monniot 1994; Monniot 1995; Rocha and Bonnet 2009; Lambert 2019). It has a very different type of larva (twice as large and gemmiparous, Monniot 1983; Neves 2015). The sister clade included also a sequence identified as *Didemnum* cf. *albopunctatum* by Erwin et al. (2014). This Australian specimen had a very different colony aspect and spicules. This sister clade is thus unlikely to be the same species, as also supported by the three methods used in the species delineation analysis.

The native versus non-native status of the new species is unclear, and it should be classed for the time being as cryptogenic (Carlton 1996). It is, however, noteworthy that *Didemnum pseudovexillum* sp. nov. has been found, so far, only on artificial structures, and it displays a disjoint distribution across the Mediterranean Sea and the English Channel, two distinct biogeographic provinces. It is thus tempting to classify the new species as non-native in these places, or at least in one of the two provinces. Numerous NIS, among them many ascidians, are shared by Mediterranean and English Channel harbours, such as *Botrylloides violaceus* Oka, 1927 and *Botrylloides diegensis* Ritter & Forsyth, 1917 (Viard et al. 2019). This pattern might be due to bivalve aquaculture activities, known to host many native and non-native tunicates (Carman et al., 2010), which might act as a relay towards other artificial habitats

such as marinas. Non-native colonial tunicates, including *Didemnum* and *Botrylloides* species, might have been “hitch-hiked” with imports of oysters and mussels between Mediterranean and Atlantic regions of France and Spain. A more complete knowledge of the current geographic distribution and habitat is necessary to assign a definite status to *Didemnum pseudovexillum* sp. nov.

In the presence of a species suspected of being introduced, extreme care should be taken before describing it as a new species to ensure that it has not been described elsewhere. Failure to recognize a species as introduced and the creation of a new name for it leads to the so-called “pseudo-indigenous species” (Carlton 2009), a problem that has already occurred in ascidians. For instance, *Didemnum vexillum* was “re-described” as *Didemnum vestum* Kott in Kott (2004a) in New England. *Styela clava* Herdman, 1881, was similarly “re-described” as *Styela mammiculata* Carlisle, 1954 in the English Channel (Millar 1960). *Clavelina phlegraea* Salfi, 1929 was the name given to Mediterranean specimens of *Clavelina oblonga* Herdman, 1880 (Ordóñez et al. 2016).

To avoid the pseudo-indigenous species problem, we revised all described species of *Didemnum*. There are 237 species recognized in the Ascidiacea World Database (http://www.marinespecies.org/ascidiacea/, Shenkar et al. 2019) as of December 2019. For each species we consulted primary literature (original descriptions whenever possible) and assessed colony aspect and spicules in the first place. In species where these characters were coherent with *Didemnum pseudovexillum* sp. nov. we further checked the literature for zooid and larval descriptions. The results of this perusal showed that the species found in Roscoff had not been previously described. Some species showing similarities are listed below. Of note here is that, with a few exceptions, there are no COI data for these species, and obtaining genetic information would be invaluable to complement the morphological perusal done.

Didemnum perlucidum Monniot, 1983 is another introduced species that forms large investing colonies on artificial substrates, and is widespread in tropical and subtropical waters worldwide (Smale and Childs 2012; Dias et al. 2016; Lambert 2019). However, this species is usually whitish, and the spicules are different, with fewer and more pointed rays, from those of *Didemnum pseudovexillum* sp. nov. (Monniot 1983; Neves 2015). Genetically, *Didemnum perlucidum* is also clearly different from the new species (Fig. 5).

Didemnum lahillei (Hartmeyer, 1909) has honey-coloured colonies with sparse spiculation. It can be abundant in shallow waters in Europe (Lafargue and Wahl 1987). However, the spicules are burr-like and the larvae have 5-6 pairs of ectodermal ampullae (Lafargue and Wahl 1987).

Didemnum psammatodes (Sluiter, 1895) is an invasive species, often reported from harbours, occurring in all warm waters (Kott 2001; Monniot 2016). It can form large colonies, sometimes with tri-dimensional structure, and has brownish colour and sparse spiculation. It is characterized by the abundance of faecal pellets embedded in the colony, which is not observed in *Didemnum pseudovexillum* sp. nov. In addition, the spicules of *Didemnum psammatodes*

include burr-like spicules (Monniot 1983; Kott 2001) not present in *Ddidemnum pseudovexillum* sp. nov. In our phylogenetic tree (Fig. 5), *Didemnum psammatodes* appears closely related to *Didemnum vexillum*, but markedly different from *Didemnum pseudovexillum* sp. nov.

Didemnum spumosum Kott in Kott, 2004b, reported from Australia, has complex, three-dimensional colonies and similar zooid and spicule morphology. However, the sperm duct has more coils (10) and the larvae are larger than in *Didemnum pseudovexillum* sp. nov. (0.75 mm, Kott 2004b).

Didemnum mesenbrinum Monniot in Monniot et al. 2001, forms large crusts covering all substrata in South Africa. Its colour is whitish or cream and the spicules are not very abundant (Monniot et al. 2001). The spicules are similar to the ones of *Didemnum pseudovexillum* sp. nov., but the atrial aperture of the zooids is different, being narrow or even slit-like (in contracted thoraces) instead of exposing most of the branchial sac as in the new species.

We summarize in Table 1 the main morphological differences between the new species and the three widespread invasive species in the genus (*Didemnum vexillum, Didemnum perlucidum, Didemnum psammatodes*) as well as with the closest species in our genetic tree (*Didemnum cineraceum*).

In conclusion, a new species of *Didemnum* is described which is present in some Atlantic and Mediterranean marinas. It can be dominant in fouling communities on artificial substrates, as it was the case in the marina of Roscoff (Britanny, France), where all the colonies sampled in 2015 and more than half of those collected in 2018 were *Didemnum pseudovexillum* sp. nov. Morphological and genetic data support the establishment of a new species. Its status should be considered cryptogenic until more information can be gathered, but it is likely an introduced species of unknown origin.

This case study adds to previous ones (e.g. *Botrylloides* spp., Viard et al. 2019) calling for caution when using field survey methods (such as RAS, or BioBlitz surveys), based on easy-to-use external morphological characters, to monitor colonial tunicates. This is unfortunate as these taxa are among the most invasive species at a global level. It is important to note that fast field assessment surveys, such as RAS, are a powerful and needed tool, allowing a cost-effective surveillance of large territories with a high temporal frequency (Campbell et al. 2007; Kakkonen et al. 2019). They actually proved effective to monitor the spread of already reported NIS (e.g., Cohen et al. 2005; Bishop et al. 2015) as well as to discover novel NIS (e.g., *Asterocarpa humilis* (Heller, 1878), Bishop et al. 2013). We thus certainly do not suggest that these field assessment methods should be abandoned. However, we do advocate for regular control of species lists obtained with these methods, for instance by means of genetic barcoding methods or by request to taxonomic specialists (if available). This would ensure the correctness of NIS lists, particularly in the context of surveillance programmes, such as the Marine Framework Strategy Directive, as any mistake can be propagated in public databases. In the case of *Didemnum vexillum*, because of its external morphological similarity with

Didemnum pseudovexillum sp. nov., observation of diagnostic molecular, such as COI sequencing, or morphological characters, such as spicules, should be compulsory, as well as keeping voucher specimens fixed in both formalin and ethanol. Our findings also imply the need for checking previous reports of *Didemnum vexillum* because of potential confusion with the new species.

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Ethical Approval: All applicable international, national, and/or institutional guidelines for animal testing, animal care and use of animals were followed by the authors.

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Data Availability Statement: The sequences obtained in this study have been deposited in GenBank with accession numbers MN952978-80. All datasets analysed during this study are included as supplementary information files.

Author Contribution Statement: XT and FV conceived the research. FV contributed samples. MC and MP generated and analysed genetic data, with contribution from FV and XT. XT analysed morphological details and wrote the

first draft of the manuscript. All authors contributed to the manuscript and approved its contents.

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Table Legends

Table 1. Summary of the main morphological characters of *Didemnum pseudovexillum* sp. nov. compared to the three widespread invasive species of the genus (*Didemnum vexillum, Didemnum psammatodes, Didemnum perlucidum*) and with the closest species in the genetic tree (*Didemnum cineraceum*).

Figure Legends

Fig. 1 Map of southwestern Europe with indication of the type locality of *Didemnum pseudovexillum* sp. nov. (Atlantic), and the two localities where its presence has been inferred from previous data (Mediterranean). The map has been drawn with package rworldmap of R (https://cran.r-project.org/web/packages/rworldmap/index.html).

Fig. 2 Images of several colonies from the marina of Bloscon (June 2018). Images a and d correspond to *Didemnum pseudovexillum* sp. nov.; images b and c to *Didemnum vexillum*. Scale bars: 1 cm. Picture credits: L. Lévêque, F. Viard – Station Biologique de Roscoff.

Fig. 3 *Didemnum pseudovexillum* sp. nov. a, image of the colony surface; b, colony section, arrows point to canals; c, ventral view of a thorax, showing a thoracic organ (arrow); d, abdomen with testis; e, abdomen with a large and a small oocyte; f-h, images of three different larvae. Scale bars: a and b, 2 mm; c-h: 250 μm (note common scale bar).

Fig 4 a-c, spicules from three colonies of *Didemnum pseudovexillum* sp. nov.; d, spicules from a colony of *Didemnum vexillum* from the same marina. Scale bars: 20 μm

Fig 5 Maximum Likelihood tree of the *Didemnum* dataset. For each branch, GenBank accession number and sequence id is provided. Numbers in main branches indicate bootstrap support values (when >50%). Clades suggested to correspond to species are indicated by asterisks (mPTP method), by inverted triangles (ABGD method, and by triangles (GMYC method). The three clades of *Didemnum vexillum* (following the same names as in Stefaniak et al. 2012) are indicated.

Supplementary Material

Online Resource 1 Fasta file containing the initial alignment of *Didemnum* sequences downloaded from BOLD systems.

Online Resource 2 Fasta file with the final, refined *Didemnum* alignment used in the phylogenetic analyses.

Table 1

Species	Colour	Spicule density	Spicule size (μm)	Spicule shape[1]	Sperm duct turns	Ampullae (pairs)	Remarks	References
Didemnum pseudovexillum	yellowish/orange	low	20-40	10 rays/blunt	6-7	4-5		This work
Didemnum vexillum	yellowish/orange	low	20-60	7 rays/pointed	8-11	6		This work, Kott (2002), Ordóñez et al. (2015)
Didemnum psammatodes	cream, brown, gray	low	<35	many rays/burr-like[2]	6-8	4	fecal pellets embedded	Monniot (1983), Kott (2001), Neves (2015)
Didemnum perlucidum	white, gray, yellow, brown	low	<40	6-9 rays/pointed	6-8	4		Monniot (1983), Neves (2015)
Didemnum cineraceum	brown, black, deep purple	low	15-30	many rays/burr-like	7-9	6-10	gemmiparous larva	Monniot (1983), Neves (2015)

[1] Number of rays in optical section given

[2] Kott (2001) describes spicules with short conical rays in addition to the burr-like spicules

Figure 1

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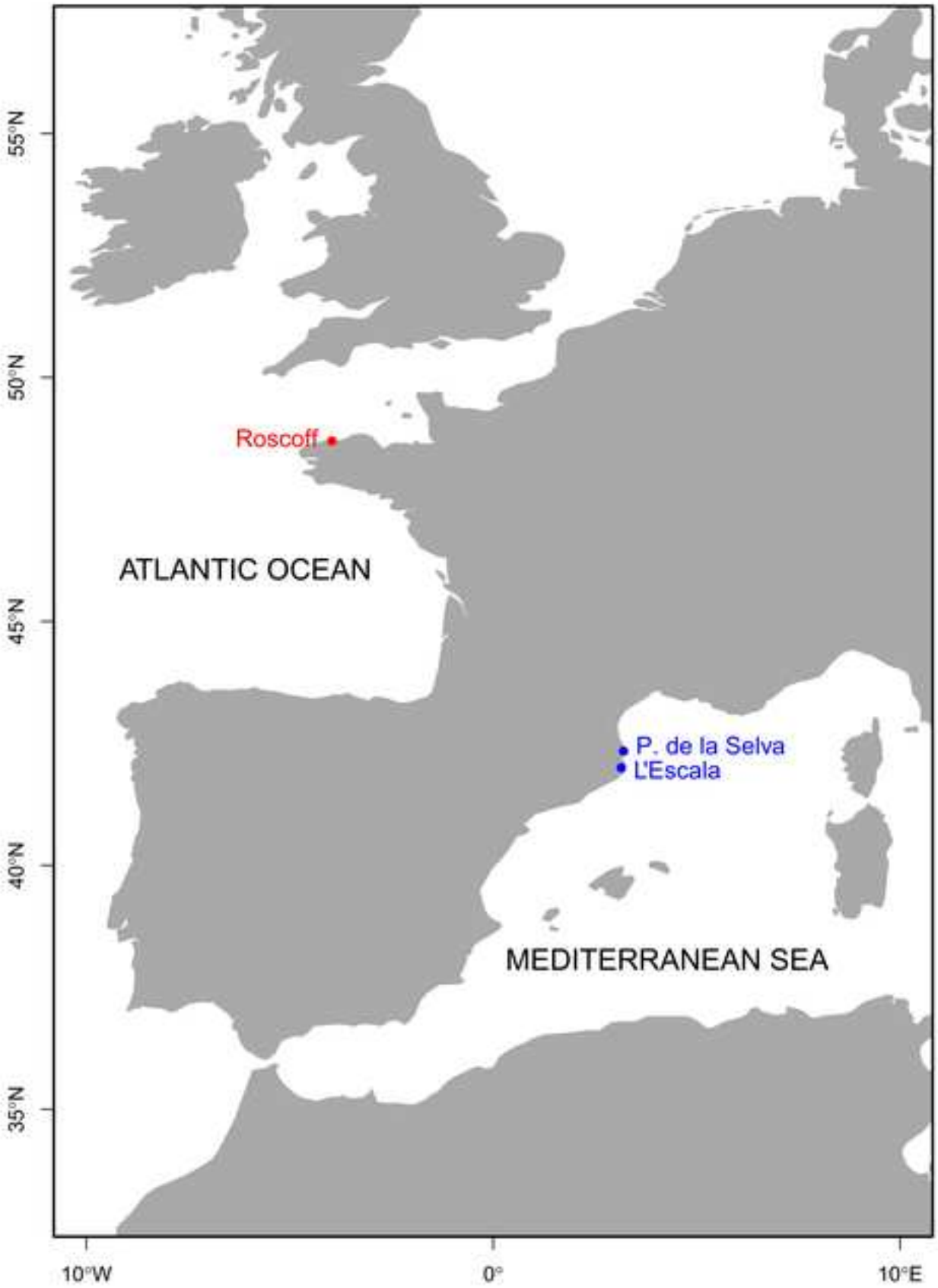

Figure 2

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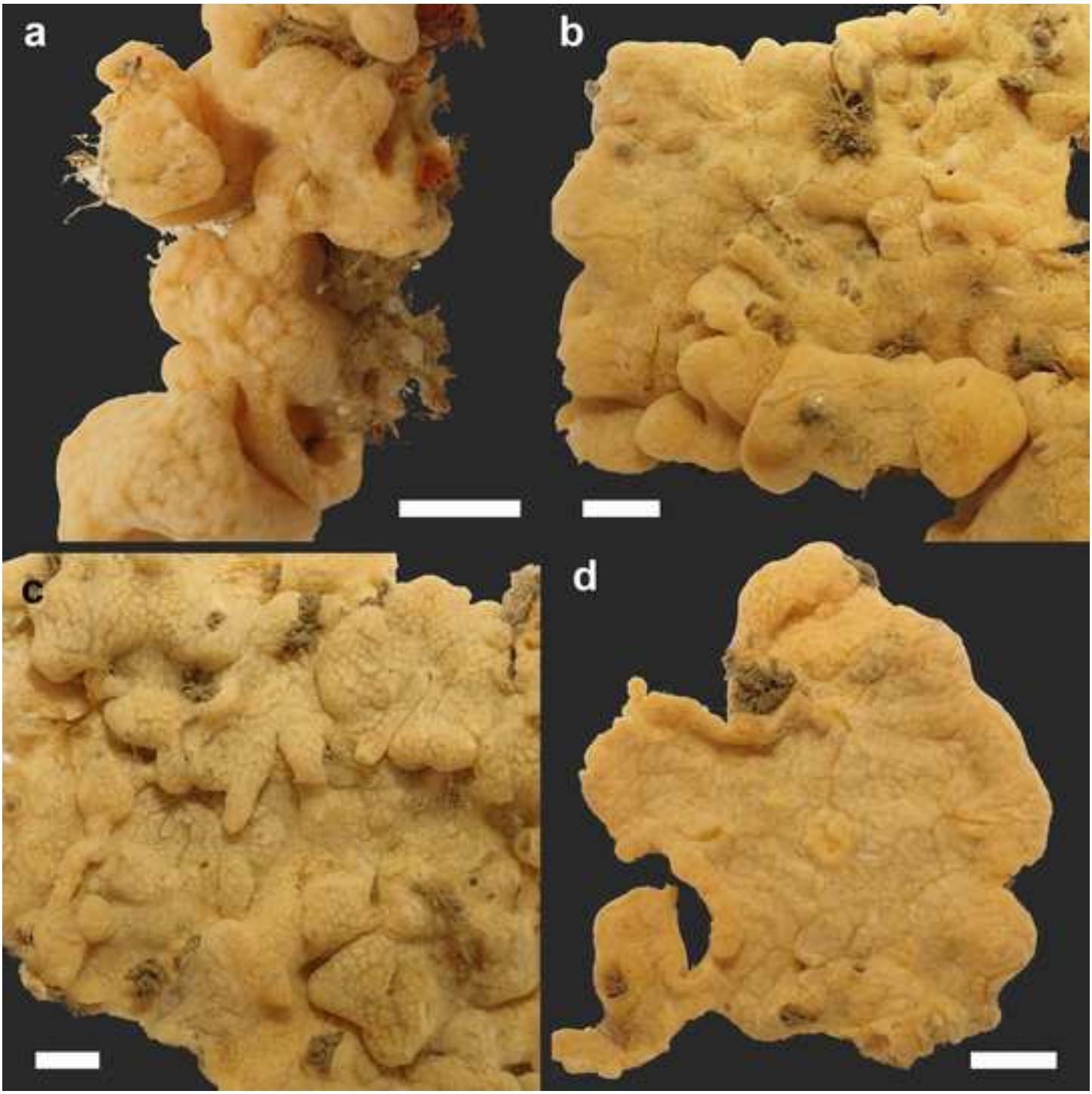

Figure 3

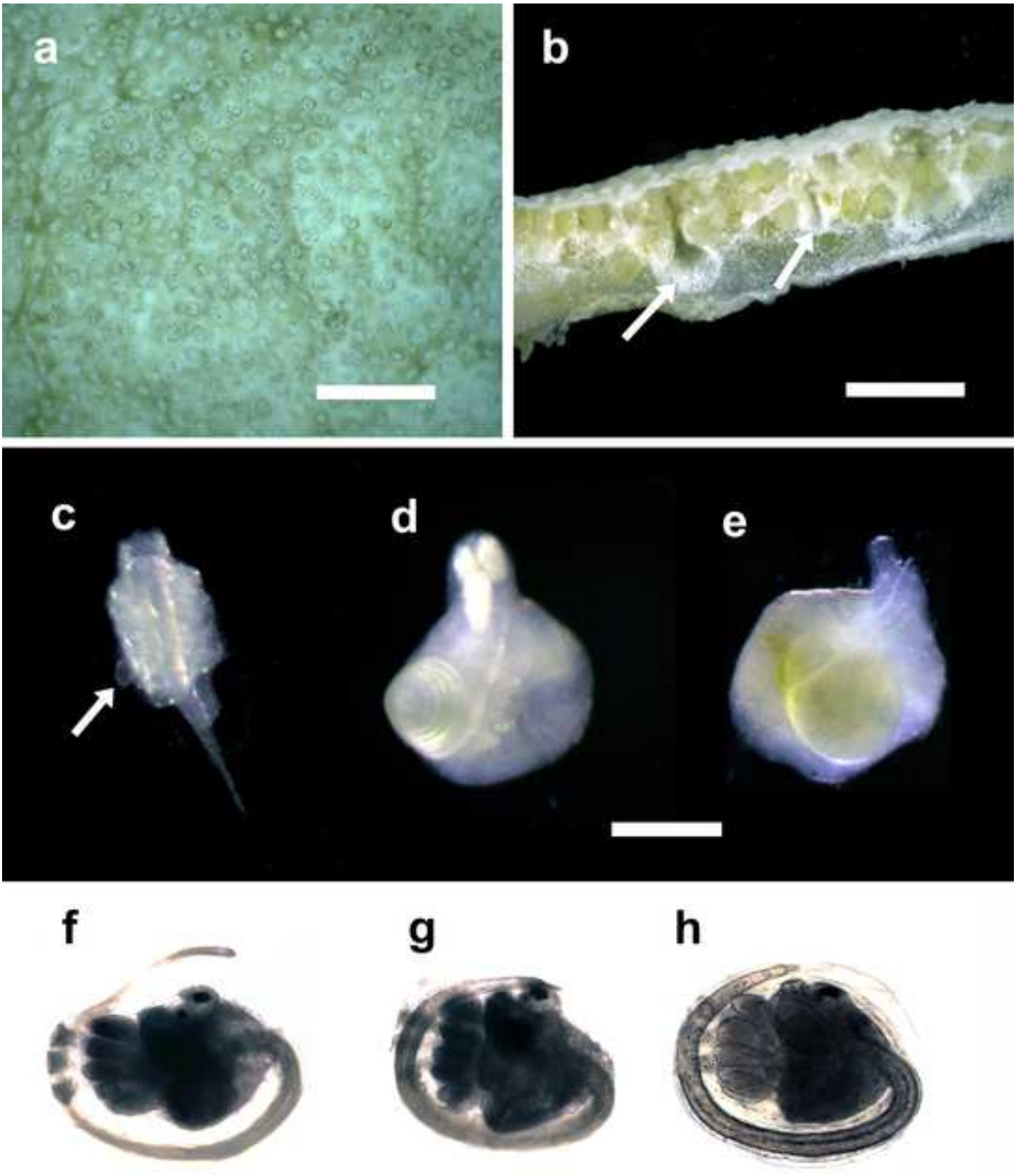

Figure 4

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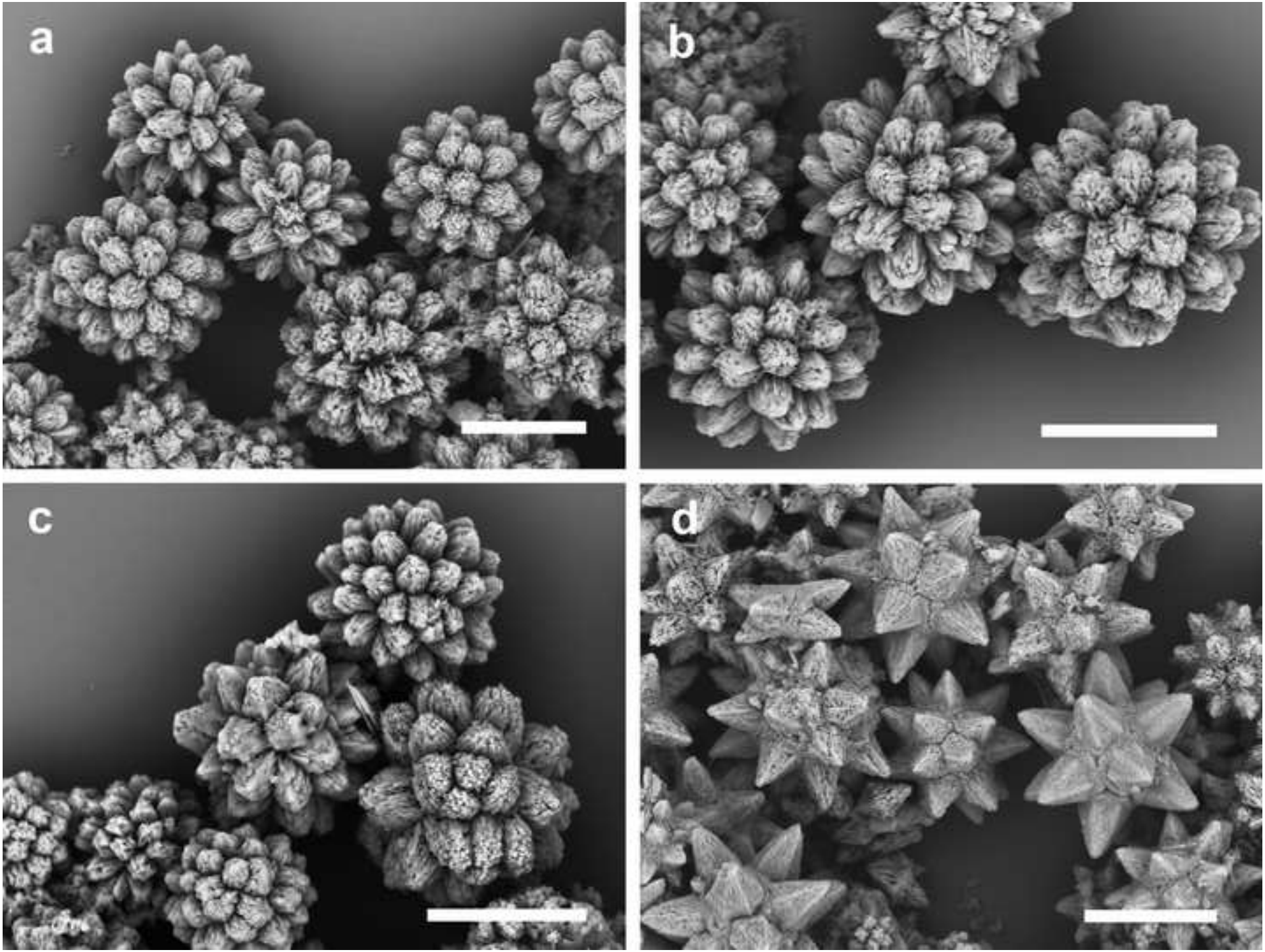

Figure 5

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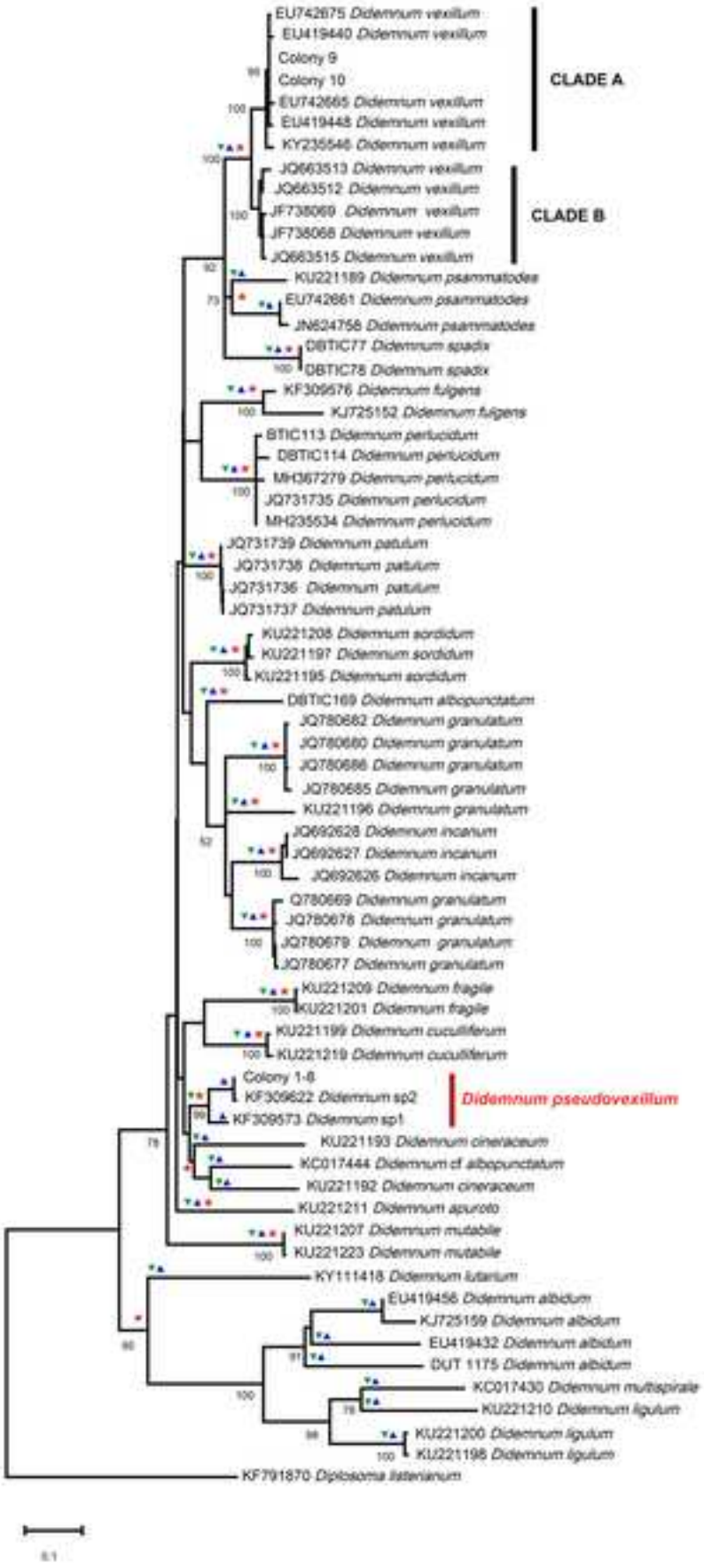

Online resource 1

Online resource 2

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Supplementary Material

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