

Rapid Communication**First record of a *Didemnum* carpet ascidian from the southwestern Atlantic Ocean**

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OPEN ACCESS**Abstract**

In the last decades, species of Didemnidae (Tunicata: Aplousobranchia) have attracted attention for overcoming marine barriers, invading coastal areas and causing negative impact to local biodiversity and human facilities. Six years ago, large colonies of a didemnid species were observed growing on artificial substrate in a port in the southeastern Brazilian coast. Samples were collected and morphological and genetic analysis were done. In morphology, the species is similar to *D. vexillum* in colour, tunic surface consistency and internal organisational system. The morphology of the zooids is similar to *D. perlucidum* but differs in the number of stigmata, coils of sperm duct, spicules size and larval aspects. Phylogenetic analysis based on the COI gene indicated the Brazilian samples form a monophyletic group distinct from other species, including *D. perlucidum*, *D. vexillum* and *D. pseudovexillum*. *Didemnum* sp. demonstrated high reproductive activity during the winter season and can grow over native and non-native species, showing high invasiveness potential. *Didemnum* sp. was not recorded anywhere else until now and based on our findings it should be categorised as a non-native species. We highlight the need of a monitoring program and the evaluation of plans for eradication of the species.

Key words: bioinvasion, Ascidiacea, Didemnidae, cryptogenic species, non-native species

Introduction

Ascidians species (Tunicata, Ascidiacea) are considered a nuisance and have attracted attention due to the high number of records among non-native species (NNS), especially considering biofouling communities. Once well-established, ascidians might affect human activities and can be considered marine pests, challenging stakeholders, governmental agencies and also experts (Coutts and Forrest 2007; Lambert 2009; Pérez-Portela et al. 2013; Dias et al. 2016). Several ascidians were likely introduced by shipping through biofouling attached on hull vessels worldwide (Lambert 2007; Bailey et al. 2020). The wide distribution of some marine biofouling species may be a reflection of the increase in shipping traffic and, particularly for ascidians, it has been recently clarified (Pineda et al. 2011;

Silva et al. 2018). There is a massive biodiversity of unrecognised cryptogenic marine species revealing erroneous taxonomical identification and the discovery of previously unknown species (Pérez-Portela et al. 2013; Turon et al. 2020).

The ascidians *Didemnum vexillum* Kott, 2002 and *Didemnum perlucidum* Monniot, 1983 are the two most widespread invasive didemnid species. *Didemnum vexillum* was originally described as native from New Zealand (Kott 2002) but molecular results suggested that it originated from Japan (Stefaniak et al. 2012). Currently, *D. vexillum* is confirmed as invasive in the temperate waters of Europe, North America, and New Zealand (Lambert 2009; Stefaniak et al. 2009). A rapid and massive growth of *D. vexillum* (so called the carpet ascidian) was observed in all infested areas, mostly on artificial substrates at marinas, ports and aquaculture facilities, and the species can be considered an ecosystem engineer because it is capable to adversely modify the habitats, covering large areas and overgrowing several benthic species (Lambert 2009). A very divergent molecular lineage of “*D. vexillum*” found in Roscoff, France was recently described as a new species, *Didemnum pseudovexillum* Turon & Viard, 2020. Although externally indistinguishable from *D. vexillum*, the spicules and larvae of *D. pseudovexillum* are distinct and the molecular data allow the separation of both species (Turon et al. 2020). *Didemnum pseudovexillum* is also recorded from the NW Mediterranean region and possibly represents another introduced species (Turon et al. 2020). *Didemnum perlucidum* was first described from Guadeloupe, in the Caribbean Sea (Monniot 1983). Nowadays the species is recognized as globally widespread (Smale and Childs 2012; Simpson et al. 2016; Dias et al. 2016). Recently the species was found in the Mediterranean Sea (Novak and Shenkar 2020). Concerning the South Atlantic, *D. perlucidum* is recorded from the tropical Western Atlantic, specifically from the southeastern Brazilian coast (Rocha and Monniot 1995). Kremer et al. (2010) observed that some colonies of *D. perlucidum* are able to occupy artificial substrates efficiently. Lins and Rocha (2020) indicated that *D. perlucidum* is associated with negative impacts on shellfish farms in Brazil. At this time *D. perlucidum* has not yet expanded its range along the Brazilian coast (Kremer et al. 2010; Granthom-Costa et al. 2016).

For the last six years, robust colonies of an unidentified didemnid species have been observed growing on the walls, pillars and cables of the port of Arraial do Cabo, Rio de Janeiro, southeastern Brazil. The colonies have been observed forming pendulous attached to cables, overgrowing on sessile native and non-native species (Figure 1). For the same region, Granthom-Costa et al. (2016) recorded eight *Didemnum* species including *D. galacteum* Lotufo & Dias, 2007, *D. granulatum* Tokioka, 1954, *D. ligulum* Monniot, 1983, *D. perlucidum*, *D. rodriguesi* Rocha & Monniot, 1993,

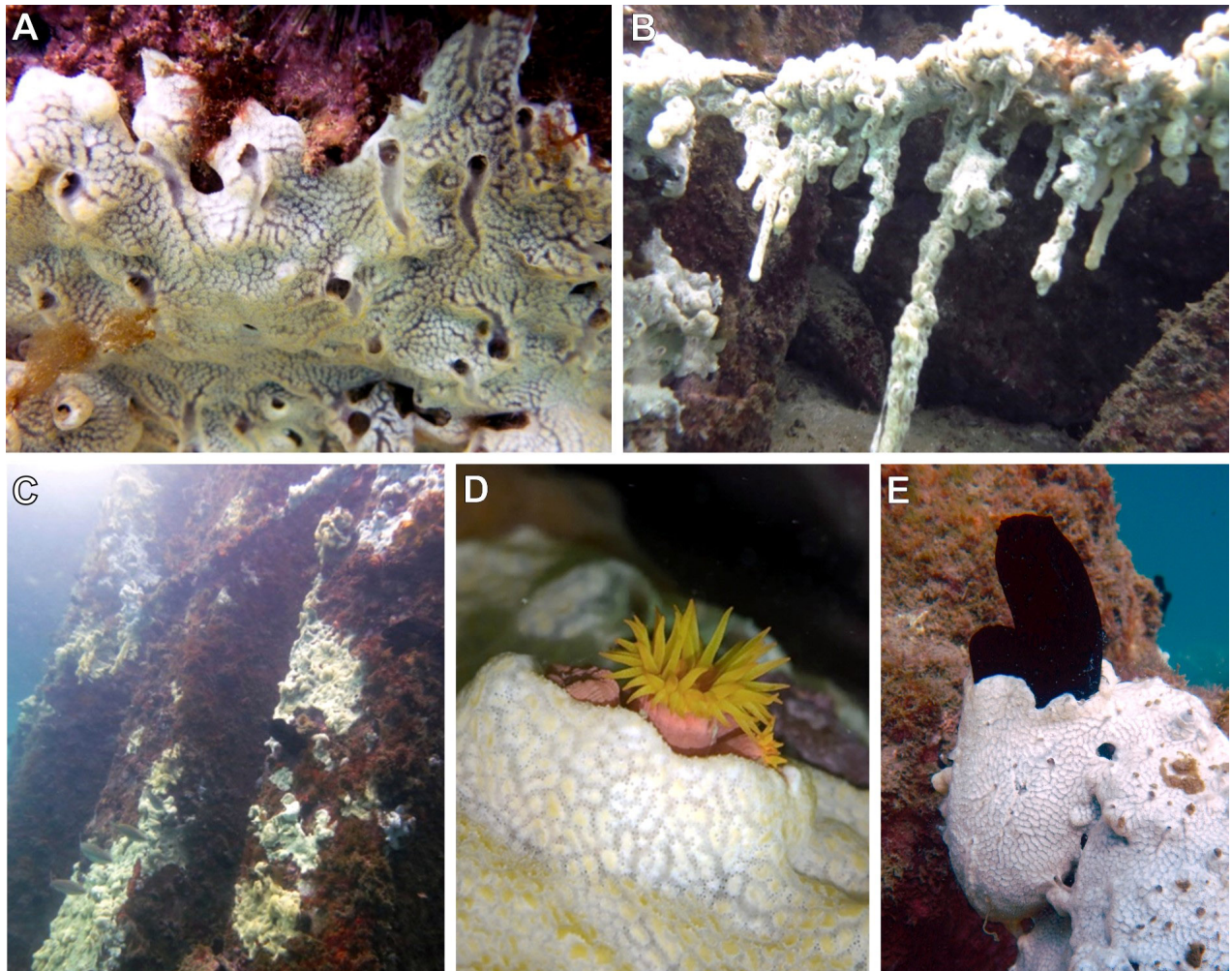


Figure 1. *In situ* observations of *Didemnum* sp. from Arraial do Cabo Bay, southwestern Atlantic. A. Detail of the colony. B. Pendulous growth on iron cables. C. Spreading on artificial substrate. D. Overgrowing on the exotic sun coral *Tubastraea tagusensis* and E. On the solitary ascidian *Phallusia nigra*. (Photos A–C by H. Fabian Messano, D by Alexandre D. Kassuga and E by C. E. L. Ferreira).

D. speciosum Herdman, 1886 and *D. vanderhorsti* Van Name, 1924, based on material collected in 2011. At that time, no colonies of carpet ascidian were observed. Considering the historical invasiveness records of carpet ascidians worldwide and associated concerns, this study aims to 1) report the record of a *Didemnum* carpet species from the southwestern Atlantic Ocean and 2) provide an initial compilation of information about the species including morphological, reproductive, and molecular data.

Materials and methods

Study area

This study was conducted at Arraial do Cabo region (42°00'W–22°44'S), Rio de Janeiro State, Brazil. The area is influenced by coastal upwelling (Valentin and Coutinho 1990) and the irregular geomorphology results in the two areas of distinct environmental characteristics. The embayment consists of a bottle-shaped bay, sheltered from the dominant wind regime, shallow (less than 20 metres) transparent waters and temperatures > 20 °C

(Candella 2009). At the inner portion of this bay there is a small port complex, Port of Forno, that supports domestic commercial traffic. The outside of the embayment is deeper and colder ($< 18\text{ }^{\circ}\text{C}$) as a result of direct exposure to a seasonal upwelling. This region is a Marine Protected Area (MPA) since 1997, categorised as Extractive Protected Area in order “to ensure sustainable exploitation and conservation of renewable natural resources for traditional fishing” and where stakeholders coexist, including traditional fishery, touristic activities, recreational boating and port activities (Melo et al. 2009).

Field sampling and morphological study

Colonies of the unidentified *Didemnum* were sampled by scuba diving down to 10 m at Port of Forno ($22^{\circ}58'20''\text{S}$; $42^{\circ}00'51''\text{W}$) initially in June 2016. Colonies were photographed *in situ* using an underwater Canon G15 camera. Colonies were anaesthetised in menthol crystals in seawater for two hours. After that, four colonies were fixed in 96% ethanol for molecular analysis. The rest of them was fixed in 4% formaldehyde and further in 70% alcohol for morphological studies, according to Monniot and Monniot (1972) protocol. Traditional characteristics described by Kott (2001), such as external aspect, colour, growth form of the colonies, zooid morphology, and larvae morphology were observed under a stereomicroscope Zeiss. Tunic tissue with spicules were prepared for analysis being washed in ethanol, dried and selected for observation on a stub. After that, the spicules were observed under a Zeiss Evo 40 scanning electron microscope (SEM). Voucher specimens were deposited at the Scientific Collection of the IEAPM (voucher of morphological collection IEAPM 1237 and tissue collection CT 038).

Molecular study

DNA of four colonies was obtained using the DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. The mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the primers TunF and TunR from Stefaniak et al. (2009). PCR reactions containing a final volume of 25 μL with 5.0 μL of 5x buffer (Promega), 0.5 μL of dNTP (0.2 mM each dNTP), 1.0 μL of each primer (0.4 $\mu\text{M}/\mu\text{L}$), 0.125 μL of Taq polymerase (5U/ μL), up 100 ng template DNA and ultrapure water to 25 μL . PCR cycle conditions were an initial denaturation of 4 min at $95\text{ }^{\circ}\text{C}$, followed by 1 min at $94\text{ }^{\circ}\text{C}$, 1 min at $39\text{ }^{\circ}\text{C}$, and 1.5 min at $72\text{ }^{\circ}\text{C}$ for 40 cycles, and a final extension for 10 min at $72\text{ }^{\circ}\text{C}$. PCR product was purified with the ExoSAP-IT enzyme (USB Scientific). The sequencing was conducted in the Platform of Sequencing-LABCEN/CCB in the UFPE/Brazil. Consensus sequences were checked for contamination using the BLAST-n search in the GenBank website (<http://www.ncbi.nlm.nih.gov>). The four colonies sequenced resulted

in the same COI haplotype deposited in the GenBank under the accession number KX641024. The dataset for the phylogenetic analysis included the unidentified didemnid species plus sequences of other 23 *Didemnum* species obtained from GenBank. In addition, COI sequences of *Corella eumyota* Traustedt, 1882, *Diplosoma spongiforme* (Giard, 1872) and *Clavelina meridionalis* (Herdman, 1891) were included in the analysis (Supplementary material Table S1). *Clavelina meridionalis* was selected as an outgroup. The alignment of the COI was generated in MUSCLE (Edgar 2004) using default settings. Nucleotide substitution saturation was evaluated with Xia's method implemented in DAMBE version 5.2.31 (Xia and Xie 2001). Maximum Likelihood analysis was executed in RAxML version 7.2.6 (Stamatakis 2006) and node support was assessed with non-parametric bootstrapping (BS) with 1000 replicates. Phylogenetic tree was visualised in FigTree v.1.4.0 (Rambaut 2012) and edited for publication in Corel Photo-Paint X7.

Results

Field observation and morphology

Colonies of the unidentified *Didemnum* species were first recorded in June 2016 growing on artificial structures such as cables and concrete pillars (Figure 1). Nowadays (November 2022), the colonies are occupying large areas mostly between two to six metres depth, and remain confined to the breakwater. In the field, the colour is an opaque white to yellowish and there is no association of epibionts. The colonies were observed successfully covering species of different taxonomic groups such as hydrozoans, vermetids, barnacles, native and non-native, such as *Tubastraea* spp. corals and the ascidian *Phallusia nigra* (Figure 1). The colonies are also able to adapt to grow around the substrate and form fingerlike projections as they grow along cables (Figure 1).

Family Didemnidae Giard, 1876

Genus *Didemnum* Savigny, 1816

***Didemnum* sp.**

Material examined: [IEAPM 1237] Brazil, Arraial do Cabo (Cais do Anel, 22°58'15.629"S; 42°1'4.249"W); artificial substrate (concrete); depth. 2–4 m; col. HF Messano, 03/June/2016. [IEAPM 1238] Brazil, Arraial do Cabo (Cais do Anel, 22°58'15.629"S; 42°1'4.249"W); artificial substrate (cables), depth. 2–4 m; col. HF Messano, 21/ July/2016.

Colonies are encrusting, crumbly, slightly wrinkled and large in size (more than 12 cm of diameter). The preserved material in formalin always becomes white with some yellow spots around the cloaca.

The tunic shows a high number of spicules observed on the surface with approximately four millimetres of thickness. Calcareous spicules < 30 µm,

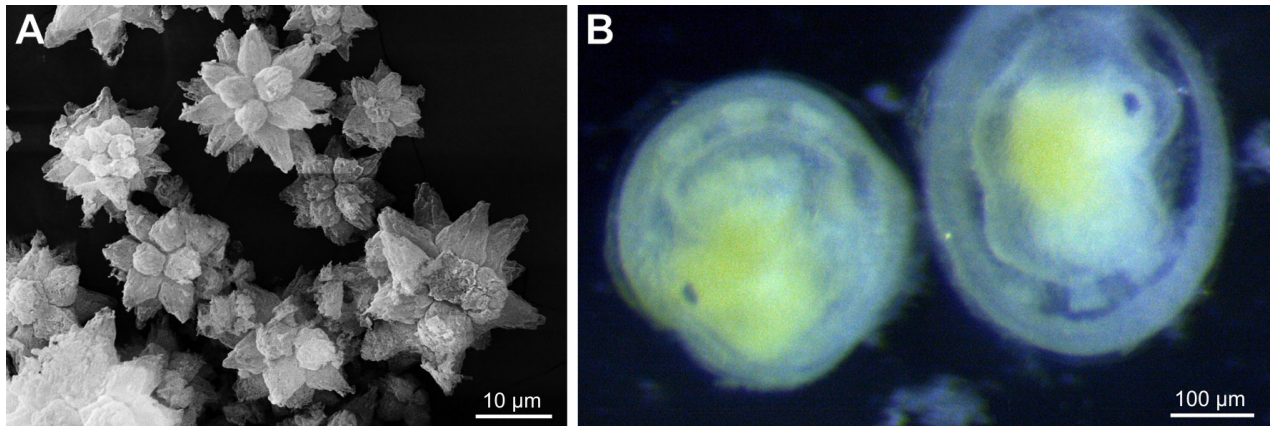


Figure 2. Morphology of *Didemnum* sp. A. SEM micrograph of spicules. B. Larvae.

stellate-shaped with 8–12 conical rays in optical transverse section (Figure 2A). Cloaca is apparent and large forming channels devoid of spicules in direction to the middle of the colony which are reducing in width – near the centre, the width of the channels is smaller. The organisational system is complex and includes between 5–16 individuals.

Each individual is approximately divided in thorax and abdomen 1.2 mm in total, yellowish colour always. Thorax slightly smaller than abdomen with large branchial aperture. Oral syphon with six triangular lobes. Pharynx has four rows of stigmata, the first one with seven stigmata with wide atrial aperture. Short muscular process projection from the esophageal-rectal peduncle. Testis spherical surrounded by five coils of the sperm duct. Larvae are oval shaped with 3 mm in length; yellow even when preserved in formalin. Three short adhesive papillae and four pairs of ectodermal ampoules with 3/4 of the tail involving the trunk. Sensory vesicles, including ocellus and otolith, are in the mid-dorsal region of the larval trunk (Figure 2B). They are incubated in the basal region of the colonies in high density.

Molecular phylogenetic analysis

The molecular phylogenetic analysis included the largest number of *Didemnum* species up to date (23 species, 109 COI sequences), including different haplotypes of the well-known invasive *D. vexillum* and *D. perlucidum*. In our tree (Figure 3), *Didemnum* sp. from southeastern Brazil is monophyletic with maximum support (bs = 100) and sister to *D. perlucidum*.

Didemnum vexillum is divided in two clades, one with samples from Japan (Group A) and the other clade with sequences from Japan, USA, France, UK and Russia (Group B). *Didemnum vexillum* is sister (bs = 99) to *D. spadix* and *D. psammatoles*. *Didemnum granulatum* is not monophyletic and divided in two distinct clades with high (bs = 98) and maximum support (bs = 100). *Didemnum pseudovexillum* is monophyletic (bs = 91) with unclear relationship to other species and not closely related

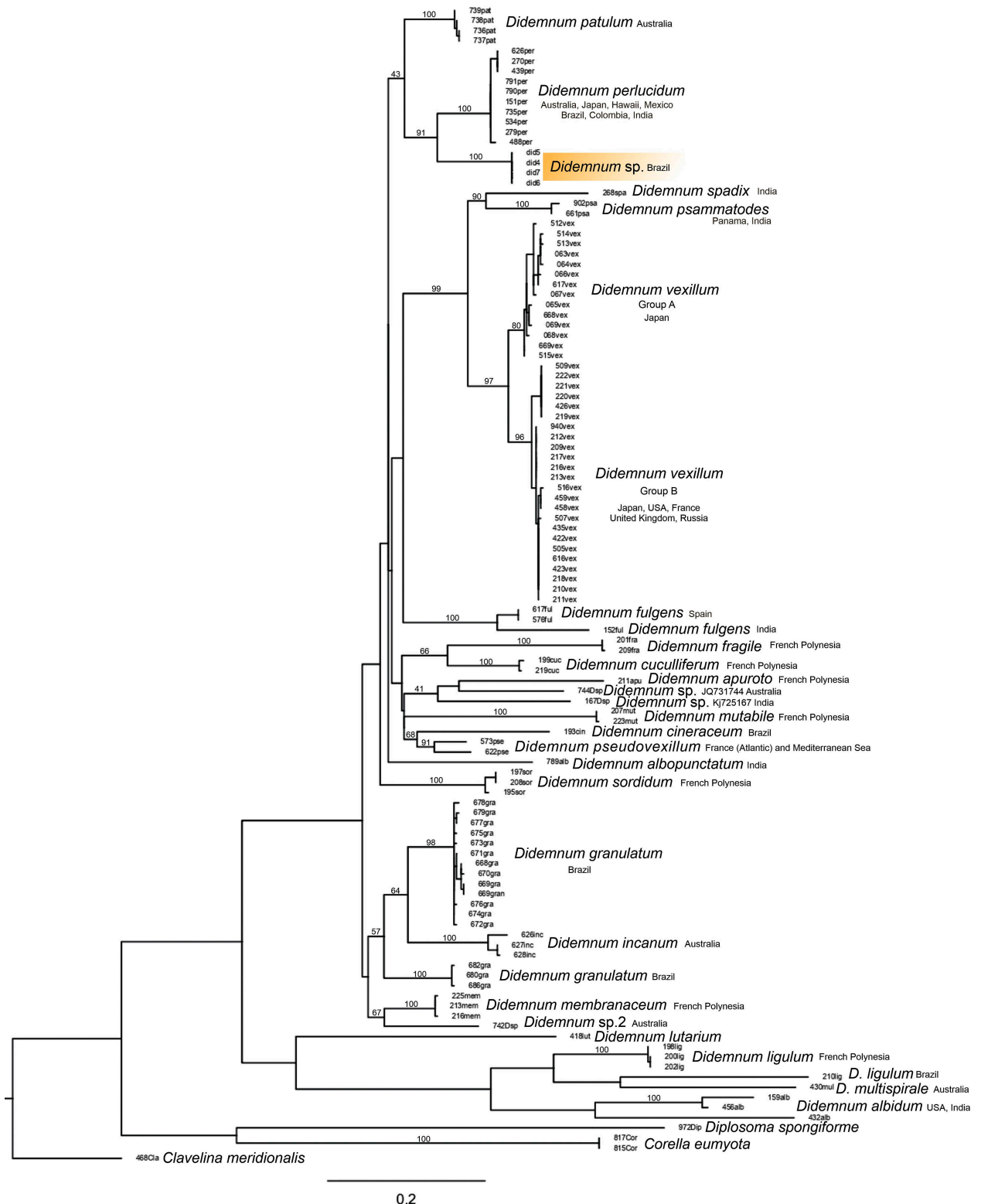


Figure 3. Maximum Likelihood inferred COI gene tree, including 24 *Didemnum* species. Tree rooted using *Clavelina meridionalis* as outgroup. Node support was assessed with non-parametric bootstrapping with 1000 replicates. Bootstrap values indicated above the branches.

to *D. vexillum* and *D. perlucidum*. Most of the other *Didemnum* species included in the analysis are monophyletic e.g. *D. patulum* (bs = 100), *D. mutabile* (bs = 100), *D. sordidum* (bs = 100), *D. incanum* (bs = 100), among others (Figure 3).

Discussion

Species identification

Didemnum is the most speciose genus among the Didemnidae family with 241 valid species, most of them from tropical regions (Monniot et al. 1991; Kott 2001; Shenkar et al. 2022). The genus has a complicated taxonomic history because there are few morphological characters, so the larvae have become an important tool for species identification. Lambert (2009) highlighted the misunderstanding regarding the identification of *Didemnum* species worldwide, being a challenge to make the final decision about a final species identification. *Didemnum* sp. from Arraial do Cabo shares similarity in external morphology with *D. vexillum*, such as colour, tunic surface consistency, organisational system, and growth strategies (Lambert 2009). On the other hand, the morphology of the zooids is similar to *D. perlucidum* but there are differences in the number of stigmata (six in the first and second rows), numbers of coils of sperm duct (eight coils), maximum spicules size (40 µm), number of conical rays in optical transverse section (five rays) and larval aspects (Monniot 1983; Monniot and Monniot 1996; Rocha et al. 2015). Our phylogenetic tree based on the COI barcoding gene clearly indicates that the newly detected carpet ascidian from southeastern Brazil is not *D. vexillum* nor *D. pseudovexillum* or even *D. perlucidum*. It is also distinct from the all other 23 species included in the analysis. However, due to absence of clear diagnostic morphological characters and considering the total diversity of the genus we provisionally do not describe it as a new species.

Species spreading and potential impacts

Didemnum sp. is observed growing on artificial substrate down to eight metres in the breakwater of Port of Forno. The highest density has been observed between two to six metres depth. The initial spreading and the intense colonisation indicated that this a suitable local condition for the reproduction of the founder population. Dispersion in the area and surroundings is possible and can initiate at any time. The dispersion could be partially favoured by the local circulation pattern, in addition to hundreds of small boats of fisheries, recreational and tourism activities that might act as vectors of dispersal of *Didemnum* sp. The environmental consequences might be disastrous (Zabin et al. 2014) since the port is located inside a marine protected area that hosts high marine benthic biodiversity (Batista et al. 2020).

Didemnum sp. was reproductively active all year long and during the local winter season (between June to September, *personal observation*) the analysed colonies were full of larvae. The high larval production suggests that the species is well-adapted and uses both sexual and asexual reproductive

strategies for spread and growth (Kremer et al. 2010). Despite the fact that specific identification is not confirmed for *Didemnum* sp., the species fills the 10 criteria of Chapman and Carlton (1991) for NNS designation that are: 1) appearance in local regions where not found previously; 2) initial expansion of local range subsequent to the introduction; 3) association with human mechanism(s) of dispersal; 4) association with or dependence on other introduced species; 5) prevalence or restriction to new or artificial environments; 6) relatively restricted distribution on a continent compared to distributions of native species; 7) isolated populations on different continents or in isolated oceans; 8) insufficient active dispersal capabilities to account for the observed distribution of the species; 9) insufficient passive mechanisms of dispersal (drifting or phoresy) and 10) it has closest morphologic and/or genetic affinities to species groups occurring elsewhere in the world. Thus, based on our findings *Didemnum* sp. should be categorised as a non-native species. It is important to note that it has no record of occurrence anywhere else in the world until now.

We highlight our concern for the need of a monitoring program and the evaluation of plans for eradication of the species. These actions can reduce the risk of a new invasion event as the case of *D. vexillum* spread in New Zealand (Lambert 2009). A comprehensive review about the invasion of *D. vexillum* in New Zealand pointed to failures in biosecurity management due to conflicting views of experts about the status of the species. As pointed out by Coutts and Forrest (2007) when *D. vexillum* was considered a threat it was too late for effective action for control and all attempts at eradication failed.

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Authors' contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Luciana Vieira Granthom Costa, Vinicius Padula, Francisca Andrea Silva Oliveira and Héctor Fabian Messano. The first draft of the manuscript was written by Luciana Vieira Granthom Costa with the contributions of Luciana Vicente Resende de Messano, Francisca Andrea Silva Oliveira and Vinicius Padula supervised by Ricardo Coutinho. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics and permits

We are grateful to Instituto Chico Mendes para Biodiversidade e Conservação (ICMBio) for sampling permission to the first author (SISBIO number 35930).

Availability of data and material

All zoological material used in genetic and morphological studies are deposited in the Scientific Collection of Instituto de Estudos do Mar Almirante Paulo Moreira – IEAPM.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Ascidian species included in the molecular phylogenetic analysis based on the COI gene.

This material is available as part of online article from:

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