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Occurrence of *Didemnum perlucidum* Monniot F., 1983 on artificial substrates along the Mediterranean coast of Israel

Lion NOVAK[1,2] and Noa SHENKAR[1,2]

[1] School of Zoology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel
[2] Steinhardt Museum of Natural History, Israel National Center for Biodiversity Studies Tel-Aviv University, Tel-Aviv, Israel

Corresponding author: shenkarn@tauex.tau.ac.il

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Abstract

Introductions of non-indigenous ascidian species are highly common nowadays due to their ability to establish successfully on artificial substrates. Didemnid species are of particular concern because of their rapid a-sexual growth and high fecundity. The colonial ascidian *Didemnum perlucidum* Monniot F., 1983 was first described from Guadeloupe Island in the Caribbean, although its native range remains unknown. To date, it has been recorded from numerous sites across the Indo-Pacific and Atlantic oceans. Here, having employed both classic taxonomy and genetic tools to verify its identification, we document for the first time its occurrence in the Mediterranean Sea – on two artificial substrates along the Israeli coast. The ability of *D. pelucidum* to establish reproductive populations despite the harsh environmental conditions of this region, with temperature fluctuations between 16-31°C and a salinity of 38-39 ppt, raises concern regarding this species' potential for introductions at numerous sites across the Mediterranean.

Keywords: Ascidians; marine bioinvasions; aquaculture; Mediterranean Sea.

Introduction

Marine organisms have been transported from place to place by humans throughout history, making the phenomenon of marine bioinvasions a crucial environmental concern (Austen *et al.*, 2018). The Mediterranean Sea, especially the eastern basin, has been frequently reported as being highly susceptible to the introduction of non-indigenous marine species (Zenetos *et al.*, 2012). The eastern basin is a center for marine activities, both commercial and leisure, and features many harbors and marinas. Its unique location also makes it a bridge for species to enter from the Red Sea, a phenomenon termed "Lessepsian invasion" (Por, 1978). Consequently, monitoring programs to detect and follow the dispersal pathways of marine species are increasingly becoming an international effort, such as the EASIN – the European Alien Species Information Network (https://easin.jrc.ec.europa.eu/), and WRiMS – the World Register of Introduced Marine Species (Ahyong *et al.*, 2019), which incorporates both scientists and citizen-science, and receives reports from encounters with such species in the field (Giovos *et al.*, 2019).

Ascidians are well-known for their high introduction potential into a variety of niches, taking advantage of man-made structures, as fouling communities, and surviving in highly polluted areas (Lambert, 2007; López-Legentil *et al.*, 2015; Zhan *et al.*, 2015). One such example is the solitary ascidian *Styela plicata,* with a global distribution and unknown origins, and which is now also found in marinas along the Israeli coasts of the Mediterranean (Novak *et al.*, 2017). Among ascidians, several didemnid species have been introduced into new areas globally, and are notorious for their success in inhabiting new niches and establishing a non-indigenous community, often exploiting artificial constructions and marine vessels (Bullard *et al.*, 2007). *Didemnum vexillum* has been described as an invasive species in many places around the world, with a distribution ranging from temperate regions along both coasts of North America, the UK and Ireland, New Zealand, and northern Europe (Lambert, 2009; Stefaniak *et al.*, 2009; Cottier-Cook *et al.*, 2019), as well as in warmer waters in the northern Mediterranean (Ordóñez *et al.*, 2015). This species, forming carpet-like patches which can spread both horizontally and vertically, is currently defined as a marine pest and a high-risk species in Australia and other countries, posing a threat to native species in the natural environment, as well as overgrowing ropes and nets used in aquaculture (Simpson *et al.*, 2017; Lins *et al.*, 2018; Cottier-Cook *et al.*, 2019).

A closely-related species, *Didemnum perlucidum* Monniot F., 1983, has also been added to the "Western Australian Prevention List for Introduced Marine Pests", having been first spotted there on artificial substrates in 2010 (Smale & Childs, 2012; Simpson *et al.*, 2016). It is now found also in natural habitats, dominating seagrass beds across wide stretches from tropical to temperate waters in western Australia and overgrowing mussels and oysters in aquaculture farms (Muñoz *et al.*, 2015; Simpson *et al.*, 2016). *D. perlucidum* is considered a cryptogenetic species, and has been spotted in different locations around the world, probably originating from the Indo-Pacific region. Originally considered a tropical species, it is now spreading to temperate regions, having reached a latitude as far north as Japan, where it is co-occurring with *D. vexillum* (Muñoz *et al.*, 2015; Dias *et al.*, 2016). During 2019 we received reports from the aquaculture maintenance team at Mikhmoret, Mediterranean coast of Israel, of an unknown colonial species overgrowing the fish-cage nets. Here, having employed molecular and morphological taxonomy to verify its identification, we document for the first time, the occurrence of *D. perlucidum* on artificial substrates in the Mediterranean Sea.

Materials and Methods

Sample collection and preservation

Didemnum tissue samples were collected by SCUBA diving at two sites along the Mediterranean coast of Israel. The first site – the fish cages – is an aquaculture artificial structure floating in the water column (32.4126° N, 34.8348° E). The structure stretches from sea level to a depth of 18 m, with the sea bottom at a depth of 36 m. *Didemnum* colonies were found covering the solitary ascidians and other invertebrates growing on the structure (Fig. 1a,b). The second site – named AR01 – is an artificial structure located at a depth of 15 m (32. 48981° N, 34.876507° E). Samples from both sites were kept in seawater and a coolbox until arrival at the lab.

For fixation and morphological identification, tissues were narcotized with menthol crystals and then fixed in 4% formaldehyde in filtered seawater buffered with sodium borate, following G. Lambert's guide for relaxing and preserving ascidians for morphological analysis. Formula/liter: 850 ml seawater, 50 ml distilled water, 100 ml of 37% formaldehyde, 1 gr sodium borate. The material is deposited at the Steinhardt Museum of Nat-

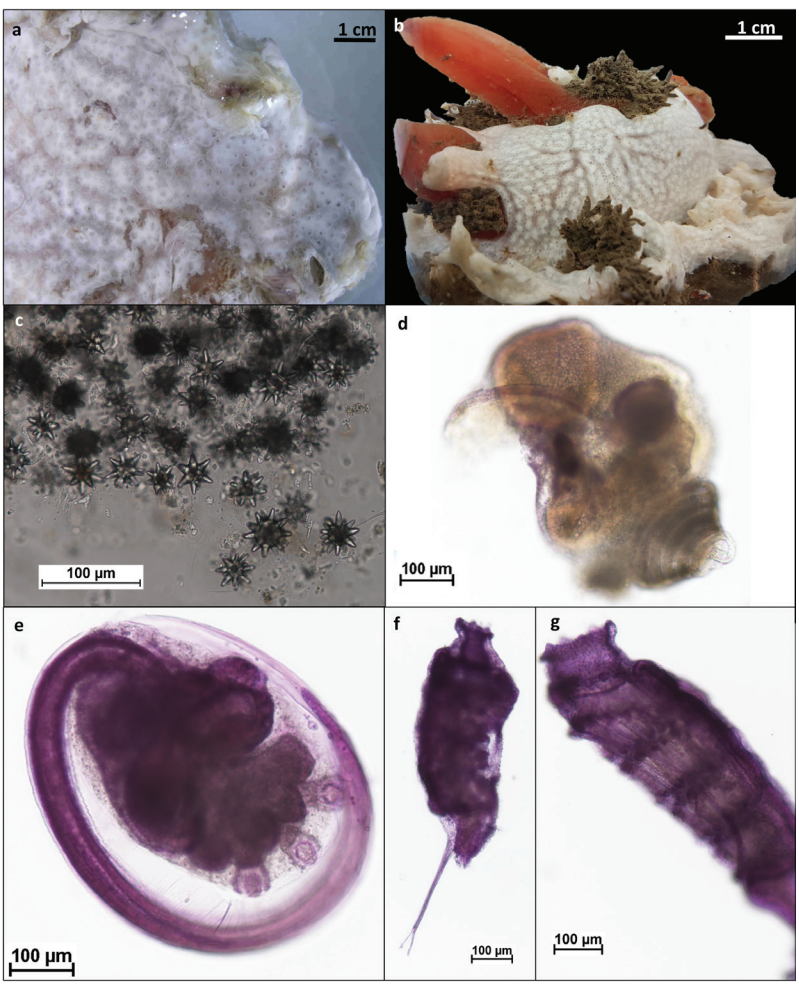

Fig. 1: *Didemnum perlucidum* characteristics. (a) Colony overgrowing a bivalve, and overgrowing the solitary ascidian *Herdmania momus* (b); conical spicules (c); abdominal part of the zooid with testis surrounded by 6 coils of sperm duct (d); larva with four pairs of ampullae and three long adhesive papillae (e); zooid branchial part with a wide six- lobed oral siphon, and four rows of elongated stigmata (f,g). Photos: N. Shenkar, L. Novak.

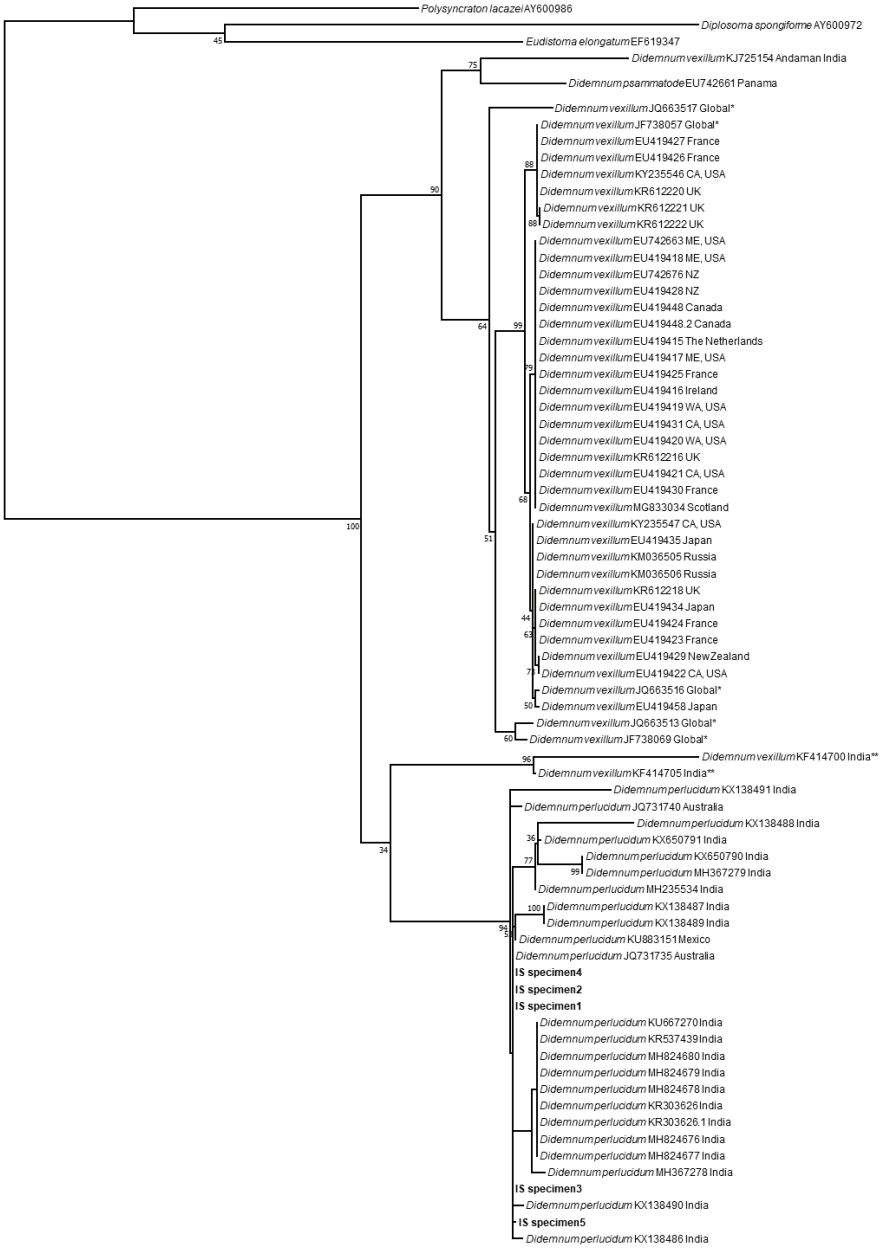

Fig. 2: A Maximun Likelihood phylogenetic tree based on *COI* sequences of *D. vexillum* and *D. perlucidum* from GenBank (NCBI). Bootstrap values are shown at nodes. Listed are location and GenBank accession numbers for each sequence. *Accession numbers obtained from Stefaniak *et al.* (2009), specific location not listed. **Unverified sequences. The Israeli sequences are listed in bold.

ural History, National Research Center, under voucher number SMNHTAU-AS26087-AS26092. Samples were observed using a Nikon SMZ18 stereomicroscope, and a Nikon Eclipse NI-U light microscope. We used the original descriptions by Monniot (1983) and Rocha & Monniot (1995) for the morphological study, in addition to professional advice provided by Prof. Rocha, Universidade Federal do Paraná, Brazil. Tissue samples for DNA extraction were either stored directly at -80ºC or kept in absolute ethanol.

DNA extraction and data processing

DNA extractions were performed using the Power Plant DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). PCR amplification of the mitochondrial gene *COI* was performed using the Tun_forward (5'- TCGACTA-ATCATAAAGATATTAG -3') and Tun_reverse2 (5'-AACTTGTATTTAAATTACGATC -3') primers developed by Stefaniak *et al.* (2009). Both DNA and PCR products were visualized on 1% agarose gels to ensure quality and presence of amplicons. Sequencing of PCR products was done on a ABI 3500xl Genetic analyzer (Applied Biosystems, ThermoFisher Scientific) in the ZABAM unit of the Faculty of Life Sciences, Tel-Aviv University.

Data analysis

Sequences were aligned and trimmed in BioEdit 7.2.5 (Hall, 1999). Maximun Likelihood analysis was conducted in RAxML v.8.1.2, as implemented in raxmlGUI v.1.5 (Silvestro & Michalak, 2012), with the GTRCAT model of sequence evolution and 100 random-addition replicates. Nodal support was assessed with 1000 bootstrap replicates.

Results and Discussion

Employing both classic taxonomic observations and genetic tools, the specimens from the Israeli Mediterranean coast were identified as *Didemnum perlucidum* Monniot F., 1983. According to Monniot's (1983) original description from Guadeloupe, and that of Monniot & Monniot (1996) from other western Pacific locations, *D. perlucidum* zooids are characterized by a relatively short and wide oral siphon with six sharp lobes and 6-8 elongated stigmata in four rows (Fig. 1f,g). The thorax of the Israeli specimens is relatively long in well-relaxed specimens, measuring 630-650 µm in length and 230-289 µn in width. The distinguishable long and thin retractor muscle can reach a length of 300 µm, and tends to break during examination. The sperm duct displays 6 coils (Fig. 1d,f), matching specimens from Brazil (Rocha pers. comm.), in contrast to the 7-8 coils described for specimens from the western Pacific (Monniot & Monniot 1996). Similarly to the samples from Brazil and other locations, the colony of the Israeli specimens had created a thin mat of 1-2 mm in

Table 1. Salinity and temperature range in previously recorded regions of *Didemnum perlucidum* †coordinates from referenced manuscript, ‡coordinates from Google maps. Average salinity and temperature were extracted from Bio-ORACLE v2.0 (Assis *et al.,* 2017). *Marks cases in which salinity and temperature data were obtained from the original publication.

Region	Salinity (ppt)	Temperature (°C)	Reference
†Israel	38-39	17-29	Current study, Herut *et al.,* 2017.
†Western Australia*	33-36	17-24	Muñoz *et al.,* 2015
‡New Caledonia	35-36	23-28	Monniot & Monniot, 1996
‡Palau	34	28-30	Lambert, 2002
†Guam	34-35	28-30	Paulay *et al.*, 2002, Monniot & Monniot, 2001, Lambert, 2003
†Japan	34-35	17-29	Dias *et al.*, 2016
‡Papua New Guinea	34-35	30-32	Smale & Childs, 2010
‡Indonesia	33-34	30-32	Monniot & Monniot, 2001
‡Maldives	35-36	29-31	Monniot & Monniot, 2001
‡India	32-34	27-30	Jaffar Ali *et al.*, 2015
†Philippines	34	27-31	Monniot & Monniot, 2001
‡Tanzania, Zanzibar	35	26-30	Monniot & Monniot, 1996,1997
†Senegal	35-36	19-29	Monniot & Monniot, 1994
†Madeira islands	36-37	18-24	Canning–Clode *et al.,* 2013
‡Guadeloupe	35-36	26-30	Monniot, 1983
‡Belize	36	26-31	Goodbody, 2000
†Panama (Pacific coast)	31-34	26-29	Carman *et al.,* 2011
‡USA (Atlantic coast)	33-36	21-30	Lambert, 2002
†Mexico (Atlantic coast)	36-37	24-31	Dias *et al.,* 2016
†Mexico (Pacific coast)	34-35	22-31	Dias *et al.,* 2016
†Venezuela	36-38	25-30	Rocha *et al.*, 2010
†Colombia (Atlantic coast)	34-37	26-30	Dias *et al.,* 2016
‡Southern Brazil*	34	19-28	Rocha & Monniot, 1995, Kremer *et al.*, 2010
†Hawaii	35	25-27	Godwin & Lambert, 2000
‡Tahiti	36	27-30	Monniot *et al.*, 1985
†Galapagos	34-35	21-27	Witman & Smith 2003, Lambert, 2019

width overgrowing other organisms. The mat displayed a marbled white-gray pattern due to the distribution of the stellate spicules across the colony, which resulted in a very bright white color in areas of high density and a grayish color along the cloacal canals, in which there were fewer spicules (Fig. 1a,b). The conical spicules measured 15-25 µm from the center (Fig. 1c). As noted by Rocha & Monniot (1995), the Israeli specimens also bore numerous rounded larvae measuring about 500 µm, with four pairs of ampullae and three long adhesive papillae.

Barcoding of five specimens from the Israeli coast resulted in five sequences of 588bp in length of the *COI* mitochondrial gene. The phylogenetic tree we constructed, together with other didemnid sequences, strongly supports our morphological identification, as they group with other known *D. perlucidum* sequences from NCBI GenBank database, including those identified in Australia (Fig. 2). This clade is distinct from *D. vexillum* and other didemnid species. Belonging to the same *D. perlucidum* clade, but with weak correlation, two *D. vexillum* from India were also present (KF414700,705). However, we suspect their accurate taxonomic identification is indeed *D. vexillum*. Specimen 5 from Israel was found to differ from the other four sequences from Israel in two bp (less than 0.3 % difference), and therefore is located on a separate branch. Specimen 5 was the only sample taken from the artificial reef site – AR01, while the others were from the fish cages. Although site AR01 is located near the fish cages, it is not an open-water site like the fish cage facilities; but, rather, an artificial reef lying on the seafloor at a depth of 15 m. Further molecular analysis may contribute to our understanding of whether the Israeli populations of *D. perlucidum* are the result of a single or numerous introductions.

D. perlucidum is now a common fouler of artificial substrates around the world, including the Pacific, Atlantic, and Indian oceans (Table 1). Detailed studies of the salinity and temperature ranges at the sites where it has been previously recorded have revealed its exceptional ability to establish populations in water temperatures as low as 17°C and as high as 32°C, with a salinity range of 31 ppt to 39 ppt. Samples were often found with larvae, and several studies have indicated that this species is highly reproductive, probably breeding throughout the year (Lambert, 2002; Muñoz & McDonald, 2014), with an ability to produce an average of 42.7 larvae per cm^2 (Kremer *et al.*, 2010). As indicated by both Muñoz & McDonald (2014), and Kremer *et al.* (2010), larval production is highest during the warmer months (24°C in western Australia, 28°C in Brazil). However, 30% of the colonies in Brazil were also reproductive during winter, when water temperature drops to 19°C. Therefore, the colder water temperatures across the Mediterranean Sea may not be a sufficient physical barrier to *D. perlucidum,* and we anticipate its future rapid spread there, mainly on artificial substrates. The eradication of colonial ascidians has proven successful only for targeted substrates, such as aquaculture facilities in Canada and artificial substrates in a New Zealand harbor that are routinely cleaned and maintained at high cost (Switzer *et al.,* 2011; Roldheiser *et al.*, 2012). Due to the rapid growth and high a-sexual reproduction of *Didemnum* fragments (Carman *et al.*, 2014; Muñoz *et al.*, 2015), together with the hospitable temperature and salinity range in the Mediterranean Sea, it would seem that no effective action can be taken to eliminate *D. perlucidum* populations there. Nonetheless, further studies monitoring its expansion across the Mediterranean, its potential impact, and possibilities for control, are strongly recommended.

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