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Rare phytomyxid infection on the alien seagrass Halophila stipulacea in the southeast Aegean Sea

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Abstract

Phytomyxids (Phytomyxea) are obligate endosymbionts of many organisms such as algae, diatoms, oomycetes and higher plants including seagrasses. Despite their supposed significant roles in the marine ecosystem, our knowledge of their marine diversity and distribution as well as their life cycles is rather limited. Here we describe the anatomy and morphology of several developmental stages of a phytomyxid symbiosis recently discovered on the petioles of the alien seagrass *Halophila stipulacea* at a locality in the southeast Aegean Sea. Its earliest stage appeared as whitish spots already on the youngest leaves at the apex of the newly formed rhizomes. The infected host cells grew in volume being filled with plasmodia which resulted in the formation of characteristic macroscopic galls. The plasmodia eventually cleaved into spores and in the final stage, the dark brown to black galls contained mature resting spores often arranged in duplexes and possessing thick verrucous outer walls. Based on its anatomical and morphological features the phytomyxid was identified as *Plasmodiophora* cf. *halophilae*. Little is so far known about the origin and eco-physiological functioning of this rare symbiosis. However, seemingly similar infection patterns were reported in 1913 on *Halophila ovalis* from Java and in 1995 on *H. stipulacea* from Sicily. Since *H. stipulacea* is of Indo-Pacific origin and no phytomyxid infection was observed on the co-occurring autochthonous seagrasses *Cymodocea nodosa* or *Posidonia oceanica*, we hypothesize that this phytomyxid might have co-migrated into the Mediterranean through the Suez Canal.

Keywords: Alien seagrasses, galls, Lessepsian migrants, marine invasions, Mediterranean Sea, Phytomyxea, *Plasmodiophora halophilae, Tetramyxa*.

Introduction

Phytomyxids (SAR: Rhizaria: Cercozoa: Endomyxa: Phytomyxea), sometimes historically called “plasmodiophorids”, are a monophyletic group of obligate intracellular biotrophs/parasites of many organisms such as oomycetes/water molds, diatoms, brown algae and higher plants (Braselton, 1995; Maier *et al.*, 2000). They possess complex life cycles, occur in terrestrial, freshwater and marine ecosystems and their distribution is likely limited by the availability of suitable hosts (den Hartog, 1965; Karling, 1968; Neuhauser *et al.*, 2014). Phytomyxid taxonomic position was long obscure – they were firstly recognized as a separate family (Plasmodiophoraceae) within Myxomycetes by Zopf (1884), but they have also been considered as either fungi or protists/protoctists (Braselton, 1995; Bulman & Braselton, 2014). Currently, phytomyxids are assigned to the class Phytomyxea within the rhizarian/cercozoan subphylum Endomyxa and the class is divided into two orders, the Plasmodiophorida and the Phagomyxida (Cavalier-Smith & Chao, 2003; Bass *et al.*, 2009).

Terrestrial phytomyxids often act as virus vectors and comprise some important and well-studied parasites of crop plants, such as *Plasmodiophora brassicae* Woronin, *Polymyxa graminis* Ledingham and *Polymyxa betae* Keskin (Karling, 1968; Braselton, 1995). However, significantly less is known about the diversity, distribution and life cycles of aquatic and marine phytomyxids, despite their supposed ecological roles in aquatic food webs and marine ecosystems (Neuhauser *et al.*, 2011a; Neuhauser *et al.*, 2011b). This gap in knowledge is probably due to the difficulties in recognizing and identifying the species not producing galls (e.g., parasites of diatoms) (Neuhauser *et al.*, 2012), while less attention is paid to non-crop parasites (Neuhauser *et al.*, 2014) and targeted research in the marine ecosystem is almost lacking.

In the marine environment, phytomyxids infect brown algae, diatoms and seagrasses (Neuhauser *et al.*, 2011b), the latter group being a guild of vascular flowering monocots adapted to the permanent submerged life in the sea. Regarding phytomyxids infecting seagrasses, the following species have been recorded: *Plasmodiophora bicaudata* J. Feldmann on *Zostera* L. (Feldmann, 1940;

den Hartog, 1989), *Plasmodiophora diplantherae* (Ferdinandsen & Winge) Ivimey Cook on *Halodule* Endl. (syn. *Diplanthera* Thouars) (Ferdinandsen & Winge, 1914; den Hartog, 1965), *Tetramyxa parasitica* K. I. Goebel on *Ruppia* L. (Goebel, 1884; Hisinger, 1887) and, notably to this paper, *Plasmodiophora halophilae* Ferdinandsen & Winge on *Halophila* Thouars (Ferdinandsen & Winge, 1913). The processes connected with phytomyxid infection in the seagrasses are not well described and understood and it is unclear how or whether at all phytomyxids influence seagrass fitness (Neuhauser *et al.*, 2012). Records of phytomyxids infecting seagrasses appear to be relatively scarce and their occurrence is driven by hitherto poorly understood factors (den Hartog & Polderman, 1973; den Hartog, 1989).

The Mediterranean Sea is home to four autochthonous seagrass species (*Cymodocea nodosa* (Ucria) Asch., *Posidonia oceanica* (L.) Delile, *Zostera marina* L. and *Zostera noltii* Hornem.) and one allochthonous species (*H. stipulacea* (Forssk.) Asch.; Liliopsida: Alismatales: Hydrocharitaceae) – the latter introduced soon after the opening of the Suez Canal (the so-called Lessepsian route – Ruggiero & Procaccini, 2004). To our knowledge, except of two unpublished findings on *Z. noltii* in the Adriatic Sea (see den Hartog, 1989), no phytomyxid infection has been recorded on the autochthonous seagrasses in the Mediterranean Sea and there is only one published record on the alien *H. stipulacea*: Marziano *et al.* (1995) discovered the typical phytomyxid galls in the petioles of this seagrass off the eastern coast of Sicily and attributed this infection to *Tetramyxa parasitica*. As noted by these authors, a similar infection pattern had been reported on *H. ovalis* (R. Br.) Hook. f. collected in Java, but was attributed to *P. halophilae* (Ferdinandsen & Winge, 1913).

In August 2015, during sampling of *P. oceanica* roots along the Mediterranean coast of Turkey, we discovered a single population of *H. stipulacea* whose individuals bore galls typical for phytomyxid infection in various developmental stages, yet slightly differing in morphology from those reported by Ferdinandsen & Winge (1913) and Marziano *et al.* (1995). Given the poor record of phytomixid infections on seagrasses, we decided to investigate this symbiosis using SEM and TEM in addition to stereomicroscopy and light microscopy, aiming to attract attention and stimulate further investigations on these parasites.

Materials and Methods

Sampling

Collections took place at a site on the southern coast of the peninsula bordering the bay of Marmaris, Turkey (36.79082° N, 28.30403° E) in August 2015, in accordance with a sampling permit #6546593-605-1361 issued by the Turkish authorities, using SCUBA. At the site, *H. stipulacea* grows patchily intermingled with *C. nodosa* (Fig. 1A) in a substrate composed mostly of coarse sand, at a depth of ca. 7–8 meters, while *P. oceanica* occupies greater depths (down to ca. 20 – 25 meters) and grows in a muddy substrate. All three seagrass species were visually checked for phytomyxid infection. Pieces of *H. stipulacea* shoots with infected leaves as well as those bearing fruits and male flower buds were photographed in situ with a Canon G10 camera in a WP-DC28 underwater case, inserted into plastic beakers filled with seawater and transported to the laboratory where the seawater in ca. one half of the beakers was substituted with 30% ethanol. The beakers were then stored in a fridge (6–8°C) until the samples were used for the following investigations. Dried specimens of *H. stipulacea* infected with the phytomyxid were deposited in the Herbarium of the Institute of Botany in Průhonice, Czech Republic (PRA) under the accession number PRA 12834. Specimens stored in 70% ethanol were deposited under the accession number PRA 12835.

Microscopy

Sample preparation and microscopic procedures followed those described in Vohník *et al.* (2015) and Vohník *et al.* (2016). In brief, macro-photographs of the phytomyxid infection were taken using an Olympus SZX12 stereomicroscope equipped with an Olympus DP70 camera. Preparation of paraffin thin sections from the infected petioles was done according to Pazourková (1986). The sections were eventually mounted into permanent slides and screened at high magnifications (400–1000×) with an Olympus BX60 microscope equipped with DIC. The DAPI-stained semi-thin hand sections were observed using an Olympus BX53 microscope equipped with a Jenoptik ProgRes MFCool fluorescence camera. Scanning electron microscopy (SEM) of semi-thin hand sections was performed using a FEI Quanta 200 scanning electron microscope in the ESEM mode at low temperatures (−6°C to −3°C). The protocol for transmission electron microscopy (TEM) was the same as in Vohník *et al.* (2012), the Spurr Resin-embedded and uranyl acetate and lead citrate-contrasted samples were observed using a JEOL 1011 microscope equipped with a digital TEM camera (Veleta, Olympus). Photographs were modified for clarity as needed and assembled into Figures using Paint.net ver. 4.0.13 (dotPDN LLC, Rick Brewster and contributors).

Comparison with previous studies, putative taxonomy of the phytomyxid

In order to assign a putative taxonomic name to the phytomyxid investigated in this study, average diameter of its mature resting spores was calculated from 300 random spore measurements (6 random permanent slides á 50 spores/measurements) which were performed using QuickPHOTO MICRO 2.3 software (Promicra Ltd., Czech Republic) at 1000× magnification. Spore

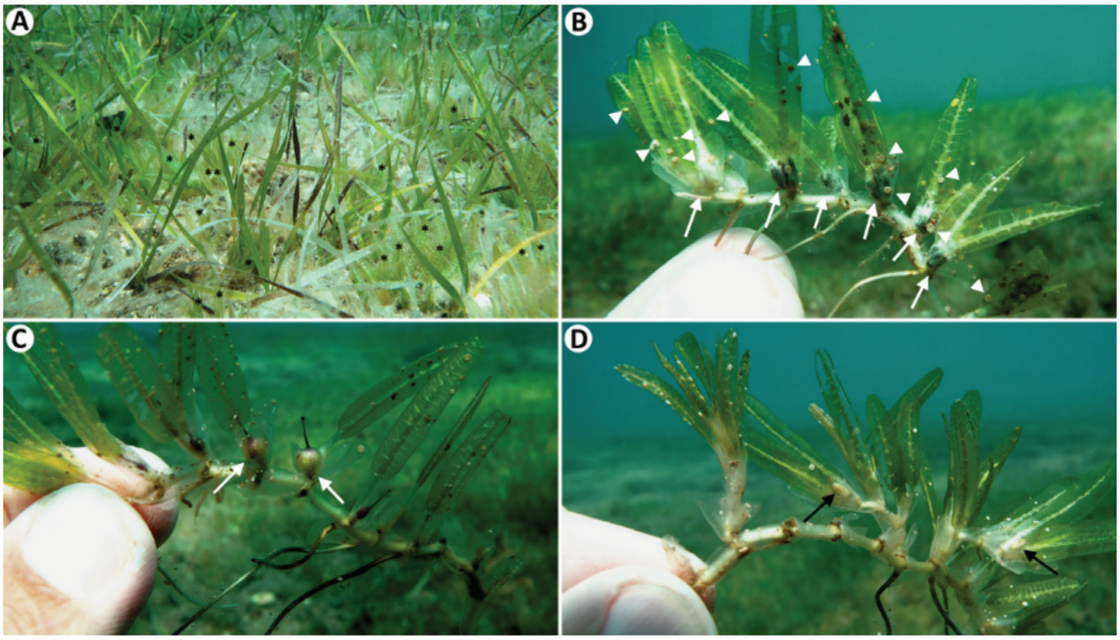

Fig. 1: *Halophila stipulacea* and its habitat in the SE Aegean Sea, its phytomyxid infection and fruits and male flower buds. Fig. 1A: *H. stipulacea* (asterisks) growing in a coarse sandy substrate intermingled with *Cymodocea nodosa*. Fig. 1B: Whitish and blackish developmental stages of the phytomyxid infection (arrows) in situ. Note clusters of Foraminifera dwelling on the surface of the *H. stipulacea* leaves (arrowheads). The phytomyxid infection was originally mistaken for seagrass fruits (Fig. 1C, arrows) and male flower buds (Fig. 1D, arrows).

aggregation evaluation was performed for the same 6 random slides at the same magnification. Additionally, the dimensions of the plasmodia and cleaved plasmodia occurring in the first two developmental stages (whitish and yellowish, respectively; see Results) as well as the dimensions of the host cells filled with the resting spores in the third developmental stage (blackish; see Results) were measured using the same software as above. All measurements as well as the resting spore aggregation were compared with the data presented in Ferdinandsen & Winge (1913) and Marziano *et al*. (1995).

Results

Morphology

The phytomyxid infection (Fig. 1B) was found only on *H. stipulacea*, not on the co-occurring seagrasses *C. nodosa* and *P. oceanica,* and was originally mistaken for its fruits (Fig. 1C) and developing male flower buds (Fig. 1D) which also occurred close to the bases of the seagrass leaves. However, upon close examination using a dissecting microscope, both the fruits (Fig. 2A) and the male flower buds (Fig. 2B) significantly differed in their morphology from the phytomyxid infection which was, unlike the fruits and buds, always restricted to the leaf petioles (Fig. 2C). The youngest stage of the infection occurred already at the tips of the seagrass rhizomes, at the bases of the very youngest leaves (Fig. 2D). Sometimes, the infection did not lead to an enlargement of the infected petioles, i.e., to formation of the typical galls; instead, the petioles retained their thin shape despite being infected by the phytomyxid (Fig. 2E). Rarely, the phytomyxid infection seemed to be connected with necrosis of the infected leaves, especially around the central vascular tissue (Fig. 2F). Three developmental stages of the reported phytomyxid infection could be distinguished on the screened seagrass specimens, differing in their color (whitish, yellowish, blackish; Figs 2C and 2G) and anatomy (see below). All these stages were often present on single rhizomes in a few centimeters of their length (Fig. 2G).

Anatomy

As seen during the stereomicroscopic observations, the youngest developmental stage was present already in the petioles of the youngest leaves, sometimes even before their blades started to develop (Figs 3A and 3B). The infected cells were apparently enlarged and their whole volume was filled with the developing phytomyxid. The first whitish stadium was characterized by the host cells being filled with young sporogenic plasmodia (Fig. 3C). These plasmodia grew in size (alongside with the host cells) and started to be concentrated close to the host cell walls (Figs 3D to 3H). Later stages were characterized by the occurrence of what we interpreted as cleaved plasmodia with the phytomyxid matter arranged into relatively flat polygonal or elliptic formations (Figs 3F to 3L) containing many nuclei visible using fluorescent microscopy and DAPI staining (Figs 3K and 3L).

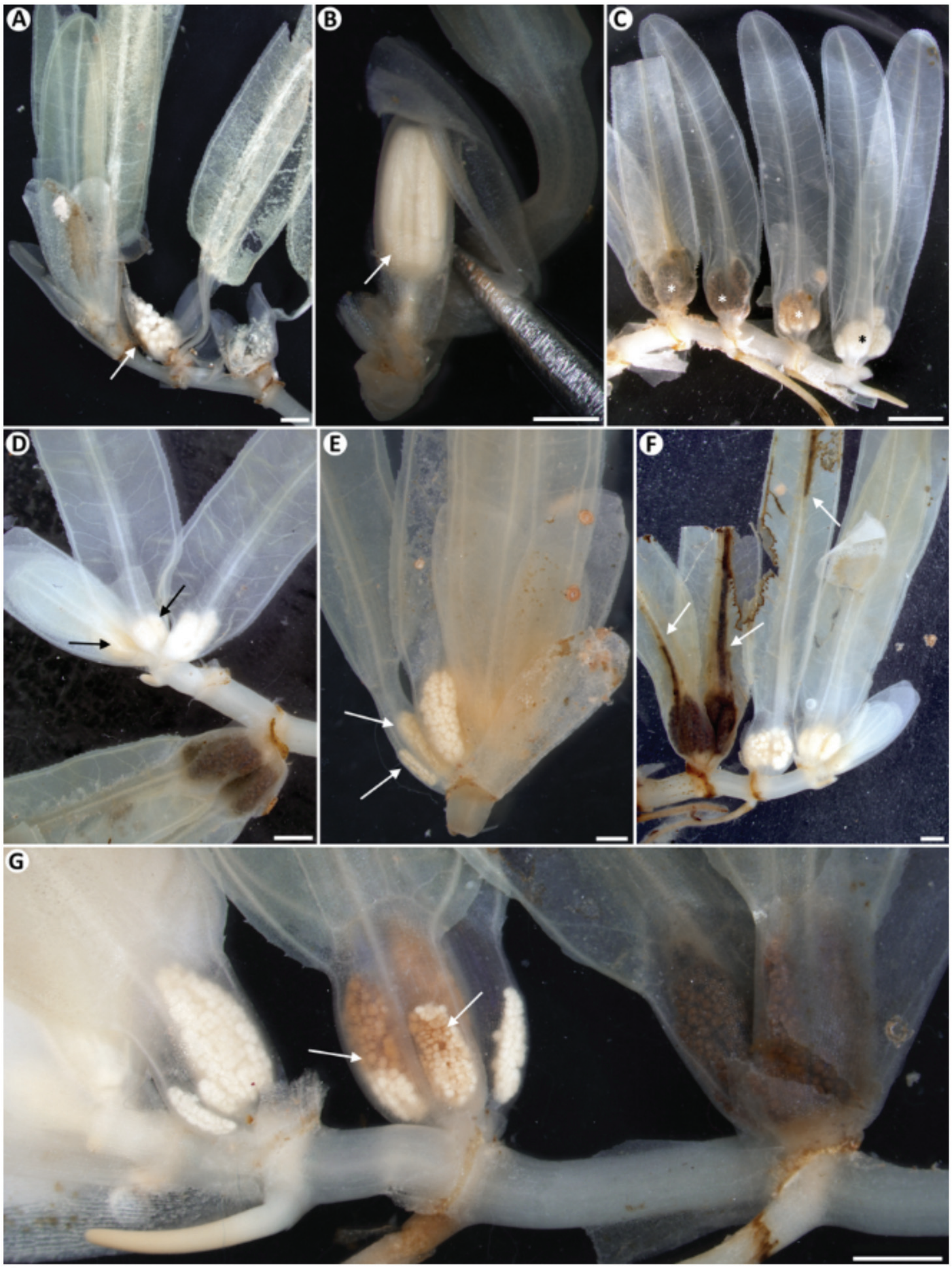

Fig. 2: Morphology of the phytomyxid infection on *Halophila stipulacea.*
Fruit with seeds (Fig. 2A, arrow) and a male flower bud (Fig. 2B, arrow) significantly differing in morphology from the typical phytomyxid infection (Fig. 2C, asterisks). Fig. 2D: The youngest phases of the phytomyxid infection in the youngest leaves (arrows). Fig. 2E: The leaf petioles being recognizable even when infected (arrows). Fig. 2F: Late phytomyxid infection connected with apparently necrotrophic development of the central vascular tissues of the infected leaves (arrows). Fig. 2G: A rhizome displaying several developmental phases of the phytomyxid infection, differing in color from white to ochre to dark brown/black – color transition being apparent even within single galls (arrows). All bars = 2 mm except Fig. 2C where bar = 5 mm. The decolorization of the leaves is due to their storage in ethanol solution.

The cleaved plasmodia enlarged and became elliptical/irregular in the second yellowish stadium (Fig. 4A) and as a consequence, they eventually filled the whole host cell volume (Figs 4B). Later, these plasmodia broke up into smaller spherical young resting spores with yet not fully developed cell walls (second cleavage; Figs 4D-4F). In the final stage, these spores became mature, i.e., with developed cell walls, and filled the whole volume of the infected cells (Figs 5A to 5C), occurring singly (44%) or more frequently in duplexes (56%) (Fig. 5C and 5D; Table 1). The mature resting spores possessed relatively thick verrucous cell walls with many fine protuberances on their outer surface (Figs 5E and 5F).

Combining the morphological and anatomical observations, three different stages of the phytomyxid development can be delimited as follows: a) the whitish

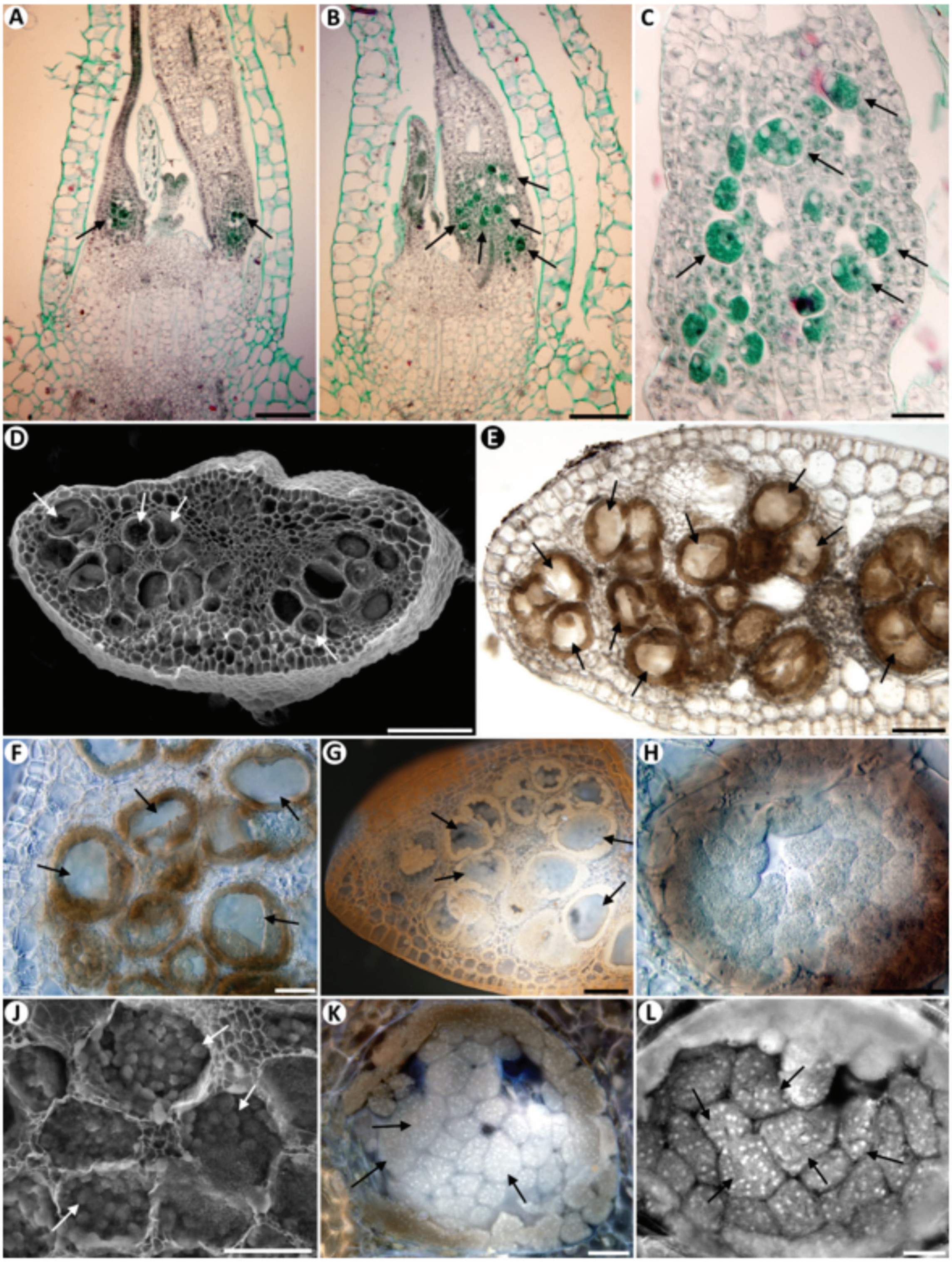

Fig. 3: Anatomy of the phytomyxid infection on *Halophila stipulacea.*
Figs 3A&B: The youngest stage of the phytomyxid infection in the petioles of the youngest leaves (arrows). Bars = 200 µm. Fig. 3C: Infected cells apparently enlarged and filled in with sporogenic plasmodia of the developing phytomyxid (arrows). Bar = 50 µm. Figs 3D–H: Phytomyxid structures filling the infected host cells interpreted as cleaved sporogenic plasmodia (arrows). Cross-sections of leaf petioles. Bars D = 500 µm; E, G = 200 µm; F, H = 50 µm. Figs 3J–L: Cleaved sporogenic plasmodia close to the host cell walls and arranged into relatively flat polygonal or elliptic formations (arrows) with many nuclei visible using fluorescence microscopy and DAPI staining (K, L). Bars J = 200 µm; K = 30 µm; L = 25 µm.

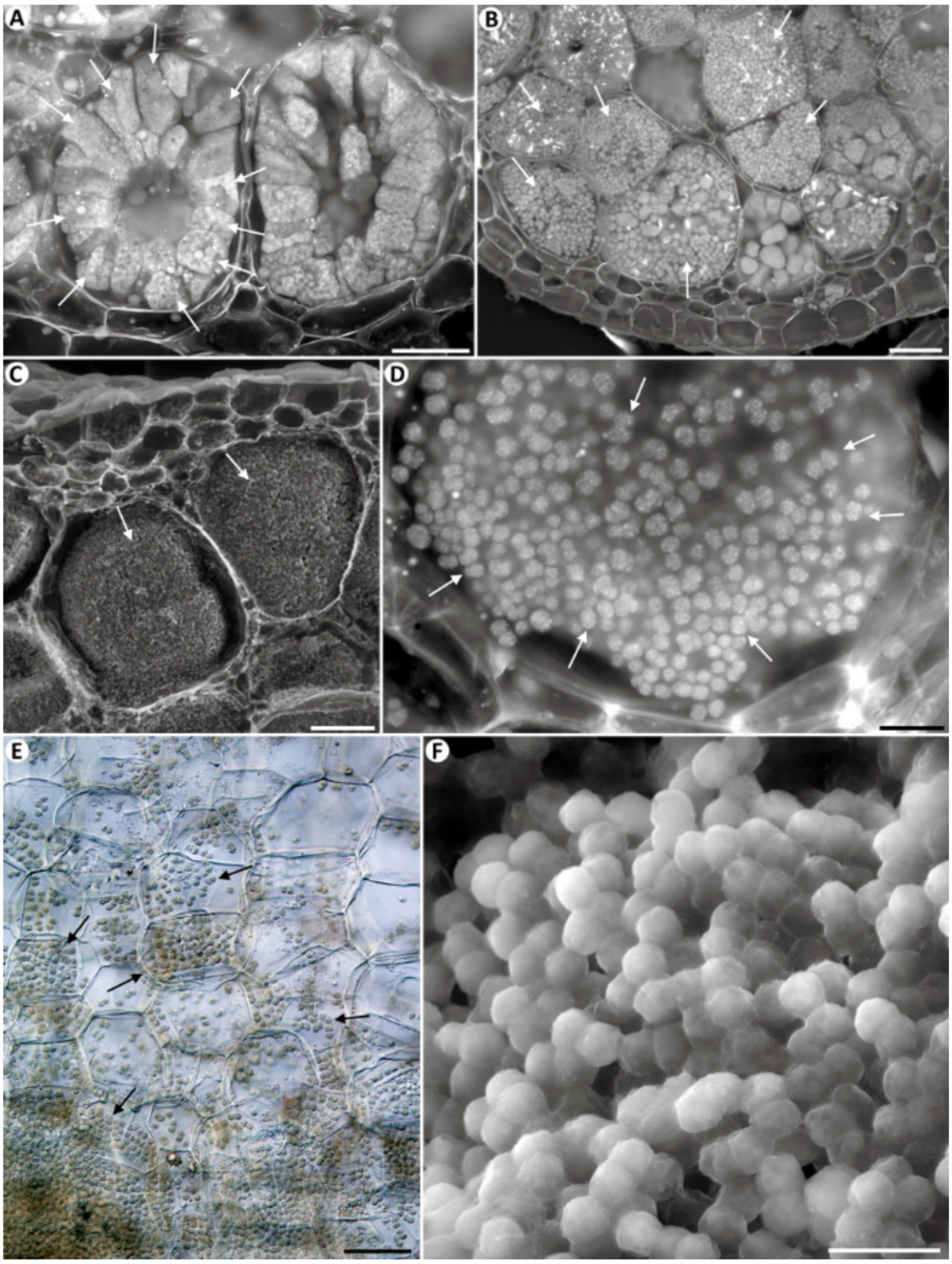

Fig. 4: Development of the phytomyxid symbiont from plasmodia to young spores.
Fig. 4A: Flat polygonal/elliptic formations interpreted here as cleaved sporogenic plasmodia (see above) eventually becoming enlarged and elliptical/irregular in the second yellowish stadium of the phytomyxid infection (arrows). Bar = 100 µm. Fig. 4B: Enlarged elliptical/spherical structures eventually filling the whole volume of the infected cells (arrows). Bar = 200 µm. Figs 4C–E: Enlarged structures eventually breaking up into smaller spherical units interpreted as young resting spores with yet not fully developed walls (arrows). Bars C = 100 µm; D, E = 50 µm. Fig. 4F: Young resting spores (SEM). Bar = 10 µm.

stage, which involves development of early plasmodia, concentration of plasmodia in the periphery, and the first cleavage of plasmodia which are still multinucleate and growing; b) the yellowish stage, when plasmodia get elliptical/irregular, still growing and typically filling the whole lumen of the infected cell, eventually cleaving to form young spores that become mature (spore maturation being the transition from the yellowish to the blackish stage); and c) the blackish stage, when mature resting spores typically fill the whole lumen of the infected cell.

Comparison with previous studies, putative taxonomy of the phytomyxid

Unfortunately, the Java collections of *H. ovalis* (i.e., the holotype of *P. halophilae*) are no longer available in the Botanical Museum in Copenhagen. Nevertheless, the average mature spore diameter calculated here (5.03 µm) is comparable to that reported by Ferdinandsen & Winge (1913) for *P. halophilae* (5 µm) and slightly smaller than that reported by Marziano *et al.* (1995)

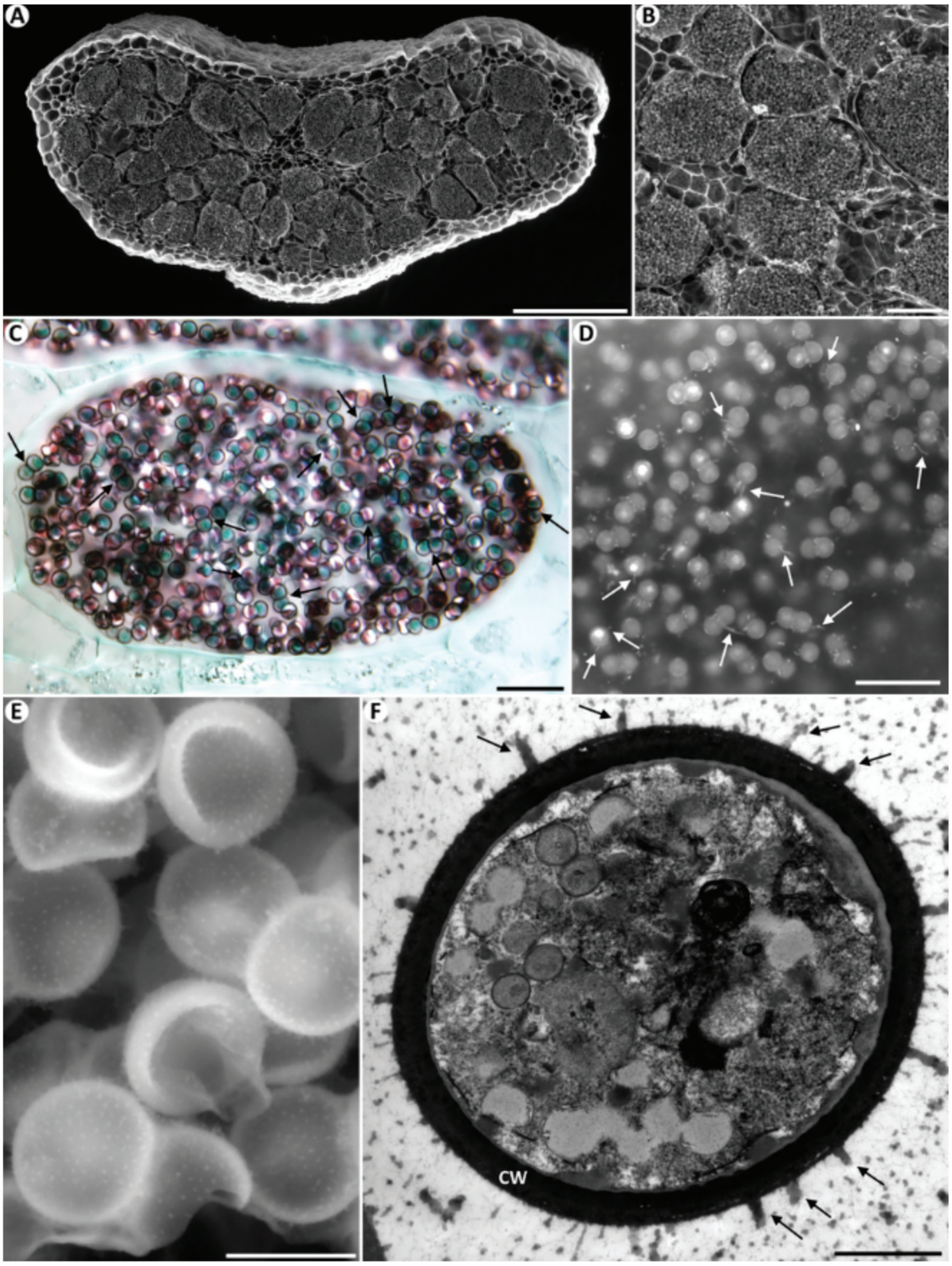

Fig. 5: Morphology and anatomy of the phytomyxid mature resting spores.
Fig. 5A: Resting spores filling most of the volume of an infected *Halophila stipulacea* petiole. Bar = 500 µm. Fig. 5B: Mature spores typically filling the whole lumen of the infected host cells. Bar = 100 µm. Fig. 5C: Mature spores occurring singly but mostly in duplexes (arrows). Artificial coloration due to DIC, bar = 20 µm. Fig. 5D: Mature spores possessing thin protrusions of different lengths (arrows; DAPI). Bar = 25 µm. Fig. 5E: Regular short and thin protrusions on the surface of mature spores (SEM). Bar = 5 µm. Fig. 5F: Ultrastructure of a mature resting spore, with apparently thick cell wall (CW) and many wart-like protuberances on its outer surface (arrows; TEM). Bar = 1 µm.

(5.8 µm). The spores were observed either as single cells (44%) or aggregated in dyads (56%) (Table 1). In contrast to Marziano et al. (1995), no triads or tetrads were observed (Table 1). The average dimensions of the infected cells in the latest stage of the phytomyxid development, i.e., when filled with mature resting spores, were 172.2 x 127. 6 µm, which is less than those reported by Ferdinandsen & Winge (1913) (i.e., ca. 300 µm in diam.). On the other hand, the average dimensions of the cleaved plasmodia measured here (48.3 x 38.2 µm) are comparable to the plasmodium dimensions reported by Ferdinandsen & Winge (1913) for *P. halophilae* (30 – 60 µm in diam.). Given these similarities, and the close taxonomic relationship between *H. ovalis* and *H. stipulacea*, the alien nature of *H. stipulacea* in the Mediterranean Sea as well as the apparent absence of the phytomyxid in neighboring seagrasses at the sampling locality (Marmaris), we assigned the phytomyxid reported here as *Plasmodiophora* cf. *halophilae*.

Table 1. Comparison of published reports on phytomyxid infections on the seagrass *Halophila.*

Reference	Location	Host species	Suggested phytomyxid species	Gall/swelling diameter (mm)	Infected cell dimensions (μm)	Plasmodium diameter (μm)	Spore diameter (μm)	Spore aggregates
Ferdinandsen and Winge (1913)	Noesa Kembangan = Nusa Kambangan (Java, Indonesia)	*Halophila ovalis*	*Plasmodiophora halophilae*	not measured (10 x 8 according to the picture)	isodiametric, diameter ca. 300 (“cellulae hospitis”)	30 – 60 (“myxamebae”)	avg. 5 range unknown (n unknown)	only single depicted
Marziano *et al*. (1995)	Naxos and Riposto (Sicily, Italy)	*Halophila stipulacea*	*Tetramyxa parasitica*	up to about 20 x 5	not measured	not observed	avg. 5.8 range 5.0 – 6.5 (n = 1713)	single (20%) dyads (60%) triads (6%) tetrads (11%)
this study (samples collected in 2015)	Marmaris (Turkey)	*Halophila stipulacea*	*Plasmodiophora* cf. *halophilae*	average length 3.8 (n = 78, min. 3, max. 6) average width 2.6 (min. 2, max. 3)	average length 172.7[1] (n = 148, min. 64, max. 318) average width 127.6[1] (min. 56, max. 209)	average length 48.3[2] (n = 34, min. 32, max. 66) average width 38.2[2] (min. 22, max. 56)	avg. 5.03 range 4.0 – 6.0 (n = 300)	single (44%) dyads (56%)

[1] dimensions of host cells in the final blackish developmental stage recorded here, i.e., when filled with resting spores.

[2] dimensions of young sporogenic plasmodia at the first (whitish) developmental stage recorded here.

Discussion

Galls, the characteristic features of phytomyxid infection, have been reported on seagrasses continuously since the 1880s and from today´s point of view it is admirable that these were from the beginning correctly ascribed to phytomyxids/plasmodiophorids, without the aid of molecular or advanced taxonomic tools (Goebel, 1884; Hisinger, 1887; Ferdinandsen & Winge, 1913; Ferdinandsen & Winge, 1914). However, with a few exceptions (Ferdinandsen & Winge, 1914; Karling, 1944), the graphic documentation of the reported infections was usually modest and often restricted to simple drawings of the whole infected plants, occasionally coupled with drawings of the infected cells with plasmodia/spores (e.g., Goebel, 1884; Hisinger, 1887; Ferdinandsen & Winge, 1913; den Hartog, 1965; den Hartog & Polderman, 1973). In addition, many authors noted that their finds were more or less accidental and that phytomyxid infection on seagrasses was conspicuously infrequent (den Hartog, 1989; Bulman & Braselton, 2014). For example, Marziano *et al.* (1995) noted that the infection they found along the east coast of Sicily on *H. stipulacea* was present in less than 1% of the screened leaves, and their observation was the only published report on a phytomyxid infecting seagrasses in the Mediterranean Sea.

Our discovery, too, was accidental and occurred only in one relatively small *H. stipulacea* population (the phytomyxid infection reported here was absent at five other localities in Egypt, three in Greece, one in Malta and two in Turkey visited after the discovery; M.V., unpublished observations). Moreover, when samples were collected underwater, the phytomyxid galls were originally mistaken for *H. stipulacea* fruits and/or male flower buds and thrown away. As the low frequency of phytomyxid findings on seagrasses is in conflict with the theory that their cosmopolitan distribution is limited only by the occurrence of suitable hosts (e.g., den Hartog, 1965; Neuhauser *et al.*, 2014), we suggest that one possible explanation could be that the phytomyxid galls have simply been overlooked or mistaken for other parts of the seagrass body, especially when observed underwater. At a closer look, however, the differences are apparent (cf. Figs 1 and 2).

The symbiosis reported here is morphologically similar to the infection described by Marziano *et al.* (1995) on Sicilian *H. stipulacea* and attributed to *Tetramyxa parasitica.* Our observations also partially resemble the infection described by Ferdinandsen & Winge (1913) on *H. ovalis* collected close to today´s Nusa Kambangan (Java, Indonesia) and attributed to *P. halophilae*. Additionally, the arrangement and overall appearance of the spores reported here resemble those described by Ferdinandsen & Winge (1914) for *P. diplantherae* (syn. *Ostenfeldiella diplantherae* Ferdinandsen & Winge) on *Halodule wrightii* Asch. (syn. *Diplanthera wrightii* (Asch.) Asch.) from the Danish West Indies (today´s US Virgin Islands). Molecular identification of our material will expectedly reveal its phylogenetic placement within the currently recognized lineages of Phytomyxea (cf. Neuhauser *et al.*, 2014), but its relationship to *T. parasitica* is to be hampered by the lack of any available sequences of this species in the NCBI-GenBank database (May 2017). However, the original specimens of the infected Sicilian *H. stipulacea* (Marziano *et al.* 1995) should be deposited in the herbarium at the University of Messina, Italy and their investigation could confirm or reject the hypothesis that our material is conspecific (we have been unable to obtain a loan of the Marziano's et al. specimens). *Plasmodiophora halophilae* was collected only once (Ferdinandsen & Winge, 1913) and neither a molecular sequence nor the original specimens are available (see Results).

Halophila stipulacea was firstly spotted in the Mediterranean Sea in Rhodes in 1894 (Fritsch, 1895), which makes it one of the arguably oldest Lessepsian migrants (Lipkin, 1975). Subsequently, it has spread through the eastern Mediterranean to the western basin, reaching south-west Italy (Biliotti & Abdelahad, 1990; Cancemi *et al.*, 1994; Procaccini *et al.*, 1999; Gambi *et al.*, 2009) and north-eastern Tunisia (Sghaier *et al.*, 2011). However, prior to this study it has been reported only once in association with a phytomyxid in the Mediterranean (Marziano *et al.*, 1995). Considering that phytomyxids are obligate biotrophs and a similar phytomyxid infection has been recorded on autochthonous *H. ovalis* in Indonesia (Ferdinandsen & Winge, 1913), it is parsimonious to assume that the phytomyxid reported here, *Plasmodiophora* cf. *halophilae*, was introduced to the Mediterranean together with its host. Alternatively, given the predisposition of phytomyxids for even cross-kingdom host shifts (Neuhauser *et al.*, 2014), our phytomyxid could have lived in the Mediterranean on a non-seagrass host and switched to *H. stipulacea* when it became available as a suitable alternative host.

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