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**PHYTOMYXID INFECTION OF THE SEAGRASS *HALOPHILA STIPULACEA*:  
PHYLOGENY, DISTRIBUTION AND NEW INSIGHTS INTO ITS LIFE CYCLE**

**NÁDOROVKA INFIKUJÍCÍ MOŘSKOU TRÁVU *HALOPHILA STIPULACEA*:  
FYLOGENEZE, DISTRIBUCE A NOVÉ POZNATKY O ŽIVOTNÍM CYKLU**

Master's thesis

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**Statement:**

I hereby state that I have completed this thesis by myself and that I have properly cited all literature and other information sources I have used. Neither this thesis nor its parts have been submitted to achieve any other academic title(s).

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## PREFACE

The Earth is a blue planet. Approximately 71% of its surface are covered with ocean waters – an extraordinary environment filled with an inconceivably diverse life, a force influencing our everyday lives by driving the planet’s climate and possibly the womb of the life (as we know it) itself. Yet the vast space below the sea surface represents one of the most unexplored entities in today’s world. Despite the human curious and ingenious nature and the ongoing technological progress, the ocean still remains hardly accessible and somehow distant to a terrestrial being.

*“... Once the sea casts its spell though, it holds one in its net of wonder forever.”*  
(Jacques-Yves Cousteau). And that’s exactly what happened and how this thesis came to life.



**Jacques-Yves Cousteau (1910–1997);** naval officer, explorer, innovator, researcher, filmmaker and much more...

## **ABSTRACT**

Marine phytomyxids (Cercozoa: Phytomyxea) represent a group of obligate biotrophic eukaryotes known for infecting several algae and oomycetes and counted among three taxa generally reported to behave as seagrass-pathogens. Due to their low-key nature, they are observed only sporadically and very limited information about their diversity, life cycles or distribution is available. In order to extend the knowledge of this enigmatic group of marine protists, an extensive research on a phytomyxid found in the invasive seagrass *Halophila stipulacea* was carried out. *In situ* observations, light and scanning electron microscopy and molecular methods were used to approach the species' ecology, geographical range and phylogenetic placement within the class Phytomyxea. The organism was confirmed to be present in the Red, the Mediterranean and the Caribbean Sea, suggesting a potential case of a host-parasite comigration on a global scale. Phylogenetically it represents the first and so far the only described member of the "TAGIRI-5 environmental clade" – possibly a whole new order within the Phytomyxea, outside the already established Plasmodiophorida and Phagomyxida. The infection rates and times of occurrence are specified for all the finding-sites, indicating an interesting seasonal pattern in the Mediterranean Sea. Furthermore, new insights into the phytomyxid's life cycle and dispersal mechanisms are discussed.

## **Key words**

Phytomyxea, invasive seagrass, seagrass parasite, comigration, Tetramyxa, *Plasmodiophora*

## **ABSTRAKT**

Mořské nádorovky (Cercozoa: Phytomyxea) představují skupinu obligátních, biotrofních eukaryot parazitujících na řasách, oomycetech a řadící se mezi tři taxony, obecně považované za patogeny mořských trav. Kvůli svému nenápadnému vzhledu bývají v přírodě pozorovány pouze ojediněle a o jejich diverzitě, distribuci či životních cyklech je dodnes dostupných jen velmi málo informací. Za účelem širšího porozumění těmto záhadným mořským protistům byl v rámci této práce proveden rozsáhlý výzkum nádorovky napadající invazní mořskou travu *Halophila stipulacea*. K přiblížení její ekologie, zeměpisného rozšíření a fylogenetického zařazení v rámci třídy Phytomyxea byla použita široká škála metod, zahrnující pozorování *in situ*, světelnou a elektronovou mikroskopii a molekulární analýzy. Přítomnost daného druhu byla potvrzena v Rudém, Středozezemním i Karibském moři, naznačujíc možnou souběžnou migraci parazita s hostitelem v globálním měřítku. Z fylogenetického hlediska představuje tato nádorovka prvního a dosud jediného popsaného zástupce environmentální skupiny „TAGIRI-5“, která, vedle již ustanovených řádů Plasmodiophorida a Phagomyxida, tvoří zřejmě zcela novou větev třídy Phytomyxea. Shromážděná data o promořenosti jednotlivých zkoumaných populací mořských trav a období výskytu infekce vykazují zajímavý sezónní trend platný pro Středozezemní moře. Práce rovněž diskutuje nové poznatky ohledně životního cyklu a disperzních mechanismů tohoto organismu.

## **Klíčová slova**

Phytomyxea, invazní mořská tráva, souběžná migrace, parazit mořských trav, *Tetramyxa*, *Plasmodiophora*

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## GENERAL INTRODUCTION

Biological symbioses (from the Greek “*symbiōsis*”, meaning “living together”) are close and long-lasting associations between two or more different species of organisms (de Bary 1879), believed to be a significant contributor to the complexity and diversity of the nature surrounding us. The importance of these associations becomes evident while exploring the most productive ecosystems in the world, or even just a single eukaryotic cell. Coral reef communities, a home to almost a third of marine fish species (McAllister 1991) and a source of food for millions of people worldwide, depend completely on the mutualistic relationship between the anthozoans and photosynthetic algae (Cowen 1988). More than 80% of all vascular plants associate with specialized soil fungi, transforming their roots into an organ called mycorrhiza, essential for the uptake of nutrients from the surrounding soil (Brundrett 2009). Finally, the eukaryotic domain in its entire variety would have most-likely never existed without the original union of an archaeal-derived host cell and an  $\alpha$ -proteobacterial endosymbiont (Margulis 1970, Yang et al. 1985).

However, symbiotic relationships represent a whole continuum of interactions not always necessarily beneficial for the species participating. Situations where only one of the two profits, leaving the other not affected (i.e. commensalism) or even harmed (i.e. parasitism) are also important drivers of the evolution on this planet. Besides, in many cases, the causes and consequences of such a coexistence might not become clear at all to the observer. The modern ecology is aware of the fundamental role symbioses play in the maintenance of life on Earth and places emphasis on their further comprehension and use for solving wider biological issues (Lipnicki 2015, Suárez 2018).

The main aim of this thesis is to introduce its readers to a noteworthy example of a plant-protist symbiosis occurring in the marine environment, spotted by chance at the coasts of Turkey in the late summer of 2015. There, an alien seagrass *Halophila stipulacea* was observed colonized by an unknown phytomyxid species (Vohník et al. 2017), an intracellular endosymbiont carrying out part of its life cycle in the plant’s body. An ongoing process of examinations in order to deeper understand the connection of these two organisms is presented. And as it happens in the scientific world, with every answer given, new questions arise.

### Seagrasses

Seagrasses are an ecological, polyphyletic group of flowering plants fully adapted to the submerged life in the marine environment. They have evolved from terrestrial origins and reentered the ocean approximately 100–70 million years ago (Les et al. 1997), adopting many evolutionary and physiological innovations to survive in the increased-salinity environment (Touchette 2007, Papenbrock 2012). Altogether there are 12 genera and only about 60–70 species of these truly marine aquatic angiosperms, confined to four families (Hydrocharitaceae,



Zosteraceae, Cymodoceaceae and Posidoniaceae) within the order Alismatales (den Hartog & Kuo 2007).

The term “sea-grasses” is derived from their morphological resemblance to the terrestrial grasses of the family Poaceae. They can be found in the coastal areas of all but the most polar seas (Green & Short 2003), forming extensive meadows of often strap-shaped leaves growing out of underground rhizomes (Fig. 1). Similarly to terrestrial grasses, seagrasses represent an important primary producer in the oceanic environment, supporting a vast number of grazing herbivores, and provide a habitat to a great range of marine keystone species (Heck Jr. & Valentine 2006, Orth et al. 2006). They also function as ecosystem engineers, affecting the physical and chemical conditions of the ambient water column and sediment. Seagrasses’ roots and rhizomes stabilize and oxygenate the seabed around (Borum et al. 2007), whereas the above-ground canopy acts as a filter, trapping the suspended nutrients and particles, modifies the water-movement by creating an obstruction to its flow (Nepf & Koch 1999) and creates a local microclimate by altering the light intensity, O<sub>2</sub> and pH values (Buapet et al. 2013).



**Fig.1** A mixed meadow of two Mediterranean seagrass species – *Cymodocea nodosa* (in the front) and *Posidonia oceanica* (in the background). Photo © Martin Vohnik

Unfortunately, it is clear that seagrass populations have been declining globally since the beginning of their monitoring in the last century (Waycott et al. 2009). Nearly quarter of all the species is considered threatened or near threatened nowadays by the IUCN Red List database (Short et al. 2011, IUCN 2019). The loss is mainly attributed to the increasing anthropogenic pressure on the coastal zones and the human-driven climate change and is expected to have far-reaching consequences in the future (Orth et al. 2006). Since the importance and the ecological services of these ecosystems are now generally acknowledged, many restoration attempts are luckily being initiated to mitigate the harm already done (e.g. Kirsch et al. 2005, Farrer 2010).

## Phytophyxea

Phytophyxea, commonly referred to as “phytophyxids” or historically also “plasmodiophorids” is a monophyletic group of obligate intracellular, biotrophic eukaryotes infecting a wide range of hosts including crops of a great economic significance (i.e. grapevine, potatoes or cabbage). The group is characterized by complex life cycles (Fig. 2) consisting of some typical features such as cruciform nuclear division, multinucleate plasmodial stage, zoospores with two flagella or formation of environmentally resistant resting spores (Bulman & Braselton 2014). For a long time, the phylogenetic position of these protists stayed uncertain and many scientists believed them to belong among fungi together with slime-molds (today’s Amoebozoa: Mycetozoa) (Sparrow 1960, Waterhouse 1972). Modern molecular studies however placed the group within the phylum Cercozoa and classified its representants into two distinct orders – Plasmodiophorida and Phagomyxida (Cavalier Smith 1996, Bulman et al. 2001).

There are 12 genera currently recognized in the Phytophyxea, inhabiting various hosts and environments (Bulman & Neuhauser 2017), yet only few species have been actually studied and described in depth. A surprising number of black boxes still remains in the knowledge concerning this group, including the life cycles, distribution, or correct taxonomy and nomenclature of a substantial number of the representants. A great potential for further research of the eukaryotic diversity, host-parasite interactions or general microbial ecology therefore seems to be hidden in this field.

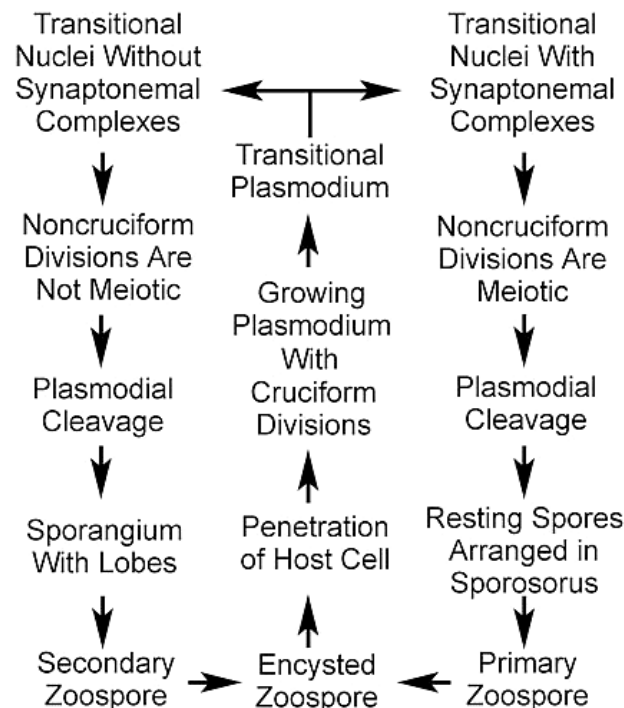


Fig. 2 Generalized life cycle of the group Phytophyxea (from Bulman & Braselton 2014)

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## CHAPTER 1

### ENIGMATIC PHYTOMYXID PARASITE OF THE ALIEN SEAGRASS *HALOPHILA STIPULACEA*: NEW INSIGHTS INTO ITS ECOLOGY, PHYLOGENY AND DISTRIBUTION IN THE MEDITERRANEAN SEA

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*MICROBIAL ECOLOGY, IN REVIEW*



*Halophila stipulacea* (from Hemprich & Ehrenberg 1900)

# **Enigmatic phytomyxid parasite of the alien seagrass *Halophila stipulacea*: new insights into its ecology, phylogeny and distribution in the Mediterranean Sea**

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## **Abstract**

Marine phytomyxids represent often overlooked obligate biotrophic parasites colonizing diatoms, brown algae and seagrasses. An illustrative example of their enigmatic nature is the phytomyxid infecting the seagrass *Halophila stipulacea*, a well-known Lessepsian migrant from the Indo-Pacific to the Mediterranean Sea. In the Mediterranean, the occurrence of this organism was first described in 1995 in the Strait of Messina (southern Italy) and the second time in 2017 in the Aegean coast of Turkey. Here we investigated, using scuba diving, stereomicroscopy, light and scanning electron microscopy and molecular methods, whether this symbiosis is still present in southern Italy, its distribution in this region and its relation to the previous reports. From the total of 16 localities investigated, the symbiosis has only been found at one site. A seasonal pattern was observed with exceptionally high abundance (>40% of the leaf petioles colonized) in September 2017, absence of the symbiosis in May/June 2018 and then again high infection rates (~30%) in September 2018. In terms of anatomy and morphology as well as resting spore dimensions and arrangement, the symbiosis seems to be identical to the preceding observations in the Mediterranean. According to the phylogenetic analyses of the 18S rRNA gene the phytomyxid represents the first characterized member of the environmental clade “TAGIRI-5”. Our results provide new clues about on-site ecology (incl. possible dispersal mechanisms) of the phytomyxid, hint that it is rare but established in the Mediterranean and encourage further research into its distribution, ecophysiology and taxonomy.

## Keywords

plasmodiophorids, invasive seagrass, seagrass symbionts, seagrass disease, *Plasmodiophora*, *Tetramyxa*

## Introduction

Seagrass symbionts represent a very heterogeneous group of epi- and endophytic organisms differing in size from macro- to microscopic species. They occur in all possible niches including the phyllosphere, the rhizoplane and the inside of a seagrass body (either inter- or intracellularly) and perform various functions along the parasitism–mutualism continuum (Ugarelli et al. 2017). In a simplified way, seagrass microbial symbionts can be typically divided into bacteria (Jensen et al. 2007, Garcias-Bonet et al. 2012, Cúcio et al. 2016), fungi (Mata & Cebrián 2013, Venkatachalam et al. 2015, Vohník et al. 2016, Vohník et al. 2017) and protists that are, perhaps except for *Labyrinthula zosterae* (Labyrinthulomycetes), the causative agent of eelgrass wasting disease (Muehlstein et al. 1991, Ralph & Short 2002) considerably understudied.

Among many other taxonomic/ecological groups, seagrass protist symbionts include phytomyxids (SAR: Cercozoa: Phytomyxea), sometimes traditionally referred to as “plasmodiophorids”, an obscure and often neglected clade of biotrophic intracellular symbionts, which, within the marine ecosystem, also colonize brown algae and diatoms (Neuhauser et al. 2012). In contrast to their terrestrial counterparts with often grave impacts on crop plants (Neuhauser et al. 2014), very little is known about marine species, despite their supposed significant ecological roles in marine ecosystems (Neuhauser et al. 2011). Nevertheless, similarly to terrestrial species, they are also treated as parasites, although processes connected with their infection are only poorly understood and for some species it is unclear how or if at all they influence seagrass fitness (Neuhauser et al. 2012). Finally, the reported incidence of seagrass-parasitizing phytomyxids is in general low, rather fragmentary and with driving factors mostly unknown (den Hartog 1965, den Hartog 1989, Bulman & Braselton 2014).

There are four autochthonous seagrass species accommodated in three genera (*Cymodocea*, *Posidonia* and *Zostera*) in the Mediterranean Sea and only a few reports on their colonization by phytomyxids are available. Interestingly, these are limited to *Plasmodiophora bicaudata* infecting *Zostera noltii* at a single locality in southern France (Feldmann 1954, Feldmann 1956) and another one in southwestern Croatia (den Hartog 1989) - the most recent report being from 1971 (den Hartog 1989). The Mediterranean Sea is also home to the alien seagrass *Halophila stipulacea*, one of the first Lessepsian migrants to cross the Suez Canal on its way from the Indo-Pacific (Lipkin 1975), and to our knowledge there are only two published reports on its colonization with phytomyxids in the Mediterranean: one is from the Strait of Messina (Sicily, southern Italy) (Marziano et al. 1995) and one from the Aegean coast



of Turkey (Vohník et al. 2017). This scarcity contrasts with the already large and still expanding distribution area of *H. stipulacea* in the Mediterranean (Gambi et al. 2009, Gambi et al. 2018).

While *P. bicaudata* has been studied and described quite in detail (Feldmann 1940, Feldmann 1956) and is considered to be a specific parasite of the seagrass genus *Zostera* (den Hartog 1989), the identity of the phytomyxid infecting *H. stipulacea* in the Mediterranean Sea is far less clear. Arguably the first phytomyxid reported to infect *Halophila* was described more than 100 years ago as *Plasmodiophora halophilae* on *Halophila ovalis* from today's Nusa Kambangan, Java, Indonesia (Ferdinandson & Winge 1913) but has never been reported again. As a result, some authors consider it a doubtful species (Cook 1933, Dick 2001). The symbiont of *H. stipulacea* in the Mediterranean, firstly reported by Marziano et al. (1995), seems to be related to the one of *H. ovalis* from Java. Indeed, the authors at first “considered the observed parasite to be *P. halophilae*” (p. 165 in Marziano et al. 1995); however, after “further observations on Italian material” (p. 165 in Marziano et al. 1995) they decided to reassign the phytomyxid to *Tetramyxa parasitica*. This decision was based mostly on the observation of spores arranged in tetrads (11% of the observed spore arrangements). However, similarly to the report from the Aegean coast of Turkey (Vohník et al. 2017), most of the spores were actually arranged in dyads (60%) followed by single spores (20%) (Marziano et al. 1995). *Tetramyxa parasitica* was originally described by Goebel (1884) from the salt-tolerant aquatic plant *Ruppia rostellata* in northern Germany. In Europe, it has been time by time found in the Baltic Sea and the North Sea on *Ruppia* and another salt-tolerant aquatic genus *Zannichellia* (Kornaś 1953, den Hartog 1963) but never on seagrasses in the comparably saltier Mediterranean Sea. Most recently, in 2017, a symbiosis morphologically similar to that described by Marziano et al. (1995) was reported on the same host at one site in the Aegean Sea in Turkey and the respective phytomyxid was assigned as *Plasmodiophora cf. halophilae* (Vohník et al. 2017). Indeed, since *H. stipulacea* is originally from the Indo-Pacific region and phytomyxids are obligate biotrophs, it is plausible that both the host and its parasite co-migrated along the Lessepsian route together, suggesting a close taxonomic relationship of the Mediterranean phytomyxid with *P. halophilae* (Braselton 2019).

To investigate the relationship of the phytomyxid symbiosis recently found in Turkey (Vohník et al. 2017) with that previously reported in Sicily (Marziano et al. 1995) as well as to better understand on-site ecology of the respective phytomyxid(s), four sampling campaigns to southern Italy were organized in search for the characteristic galls on *H. stipulacea*. Upon re-discovering the symbiosis in Sicily, we also focused on screening of its development dynamics, colonization levels, possible dispersal modes and its phylogenetic placement based on the analyses of the 18S rRNA gene.

## Materials and methods

### Herbarium material examinations

To decide about the relationship between *P. halophilae* on *H. ovalis* from Java as reported by Ferdinandsen & Winge (1913) and “*T. parasitica*” on *H. stipulacea* from Sicily as reported by Marziano et al. (1913), a series of attempts has been made to obtain herbarium material from the Botanical Museum in Copenhagen, Denmark and the Herbarium of the University of Messina, respectively. Unfortunately, it was impossible to get these specimens on loan as they were unavailable and most probably had been lost (also see Vohník et al. 2017). On the other hand, permanent slides with thin transverse sections of the *H. stipulacea* galls investigated by Marziano et al. (1995) were obtained from the Herbarium in Messina and examined, using light microscopy. Additionally, a few micrographs of phytomyxid spores originating from *H. stipulacea* galls collected by chance at a site in northeast Sicily in around 2015 [Tono (Casabianca) – see Table 1] by one of us (G. M. G.) were also examined.

### Sampling

Four sampling campaigns were carried out during 3 years (December 2015, September 2017, May/June 2018 and September 2018) in southern Italy at 16 different localities (Fig. 1). Eight of them were chosen based on literature searches, i.e. papers reporting *Halophila stipulacea* occurrence (Marziano et al. 1995, Famà et al. 1999, Di Martino et al. 2006, Gambi et al. 2009) and personal communications with local scuba dive operators/scientists, the remaining eight were chosen randomly (Table 1).

Seagrass samples were collected using scuba diving, the collection depth and average water temperature were measured with a diving computer (Freedom, Divesoft, Czech Republic; Vytec or Vyper Novo, Suunto, Finland), seawater salinity was measured during the 2018 campaigns using a portable optical refractometer for seawater RSA1-ATC ([www.refraktometr.cz](http://www.refraktometr.cz), Czech Republic) and in situ photo-documentation was done with a Canon G10 camera in a WP-DC28 underwater case. Collected samples were stored in plastic tubes filled with seawater which was upon surfacing substituted with 30% ethanol in seawater in ca. half of the samples. Samples were then transported to the laboratory for further examination; representative specimens were washed with tap water and transferred to a 70% ethanol solution in deionized water or dried at room temperature and then deposited in the Herbarium of the Institute of Botany, Czech Academy of Sciences, Průhonice (PRA).

For measurement of the intensity of the phytomyxid colonization (found only at one locality), three microsites with the presence of the symbiosis were randomly chosen in 2017 and 2018 and at each of them, one plastic box for a diving mask (approx. volume 1.5 l) was filled with the seagrass biomass (leaves + rhizomes + roots). Upon surfacing, all collected leaves were



**Fig. 1** Localities investigated in this study. **a** Study sites were located in the transition zone between the Western and the Eastern Basin of the Mediterranean Sea (black rectangle). **b** Detailed locations of the investigated sites in the Strait of Messina and its surroundings (dots). Scale bar = 100 km. Original maps were downloaded from the USGS National Map Viewer (public domain at <http://viewer.nationalmap.gov/viewer/>) for **Fig. 1a** and the Maps at the CIA (public domain at <https://www.cia.gov/library/publications/the-world-factbook/index.html>) for **Fig. 1b**. The resulting **Fig. 1** is similar but not identical to the original images and is therefore for illustrative purposes only

checked for the presence of the symbiosis and according to gall coloration assigned into the three following categories: i) the early developmental stage containing sporogenic plasmodia (referred to as the “whitish” stage in Vohník et al. (2017)), ii) later developmental stages containing cleaved plasmodia and fully developed resting spores (referred to as the “yellowish” and “blackish” developmental stages in Vohník et al. (2017), respectively) and iii) no colonization.

### **Stereomicroscopy, light and scanning electron microscopy**

Firstly, randomly selected rhizomes with leaves as well as detached leaves from the 2018 collection (see Results) were screened with an Olympus SZX12 stereomicroscope. Secondly, paraffin thin sections from both infected and non-infected petioles were prepared as detailed in Vohník et al. (2015). These sections were eventually mounted into permanent slides and screened at high magnifications (400 – 1000×) with an Olympus BX60 upright microscope equipped with differential interference contrast. Micrographs were taken with an Olympus DP70 camera using QuickPHOTO MICRO 2.3 software (Promicra Ltd., Czech Republic), the Deep Focus mode was employed when needed (Promicra). Thirdly, 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 to –3 °C). Micrographs were modified for clarity (adjustment of brightness and contrast) as needed.

## Measurements

The measured parameters as well as measurement approaches were the same as in Vohník et al. (2017), i.e. we focused on gall dimensions (length and width), infected cell dimensions, plasmodium diameter and spore diameter. Additionally, we measured dimensions (length and width) of non-infected petioles. Galls and non-infected petioles were measured using the SZX12 stereomicroscope while the rest of the parameters were measured using the upright BX60 microscope (see above) and the QuickPHOTO MICRO 2.3 software. All measurements were done by a single person (V. K.) and compared with those published in previous studies.

## DNA extraction, amplification and sequencing

Sterile tweezers and a scalpel were used to cut as much of the uninfected plant tissue off the gall as possible. The rest of the gall was surface sterilized in 10% SAVO (common household bleach, Unilever ČR Ltd., Czech Republic; 100% SAVO contains 47 g/kg, i.e. 4.7% sodium hypochlorite = NaClO) for 1 min and rinsed twice in sterile deionized water. The DNA was then extracted from the remaining tissue using DNeasy Plant Mini Kit (QIAGEN Inc., Venlo, Netherlands) following the manufacturer's instructions.

To amplify the phytomyxid's 18S rRNA gene sequence, a nested PCR with the following protocol was carried out. The reaction mixture (25 µl) consisted of 12.5 µl of Plain Combi PP Master Mix (Top-Bio s.r.o., Praha, Czech Republic), 1.5 µl of each primer (final concentration 0.6 µM), 0.8 µl of 20 mg/ml Bovine Serum Albumin, 2 µl of the extracted DNA and 6.7 µl of deionized H<sub>2</sub>O. For the first round PCR (95 °C for 5 min initial denaturation, followed by 35 cycles of 95 °C for 35 s, annealing at 50 °C for 35 s, extension at 72 °C for 10 min, and 72 °C for 10 min final elongation) universal eukaryotic primers MedlinA (CTGGTTGATCCTGCCAG) (Medlin et al. 1988) and EK-1498R (CACCTACGGAAACCTTGTTA) (Marande et al. 2009) were used. For the second round PCR (95 °C for 5 min initial denaturation, followed by 35 cycles of 95 °C for 35 s, annealing at 54 °C for 35 s, extension at 72 °C for 10 min, and 72 °C for 10 min final elongation) set of primers specific for phytomyxids: Plas1f (TCAGTGAATCTGCGGATGGC) (Neuhauser et al. 2014) and PHMX-1570R (GCKARTTGCAAGMSGCAAGC; redesigned from the primer Plas1r (Neuhauser et al. 2014) to cover all Phytomyxea species) were used.

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Inc.). The purified products were then Sanger sequenced by MacroGen Europe Laboratory (MacroGen Inc., The Netherlands), using the 577F (GCCAGCAGCCGCGGT), 577R (ACCGCGGCTGCTGGC), 1055F (GGTGGTGCATGGCCG) and 1055R (CGGCCATGCACCACC) sequencing primers (Elwood et al. 1985). The newly determined

sequence of the 18S rRNA gene of the phytomyxid symbiont of *Halophila stipulacea* has been deposited in GenBank under the accession number MN128644.

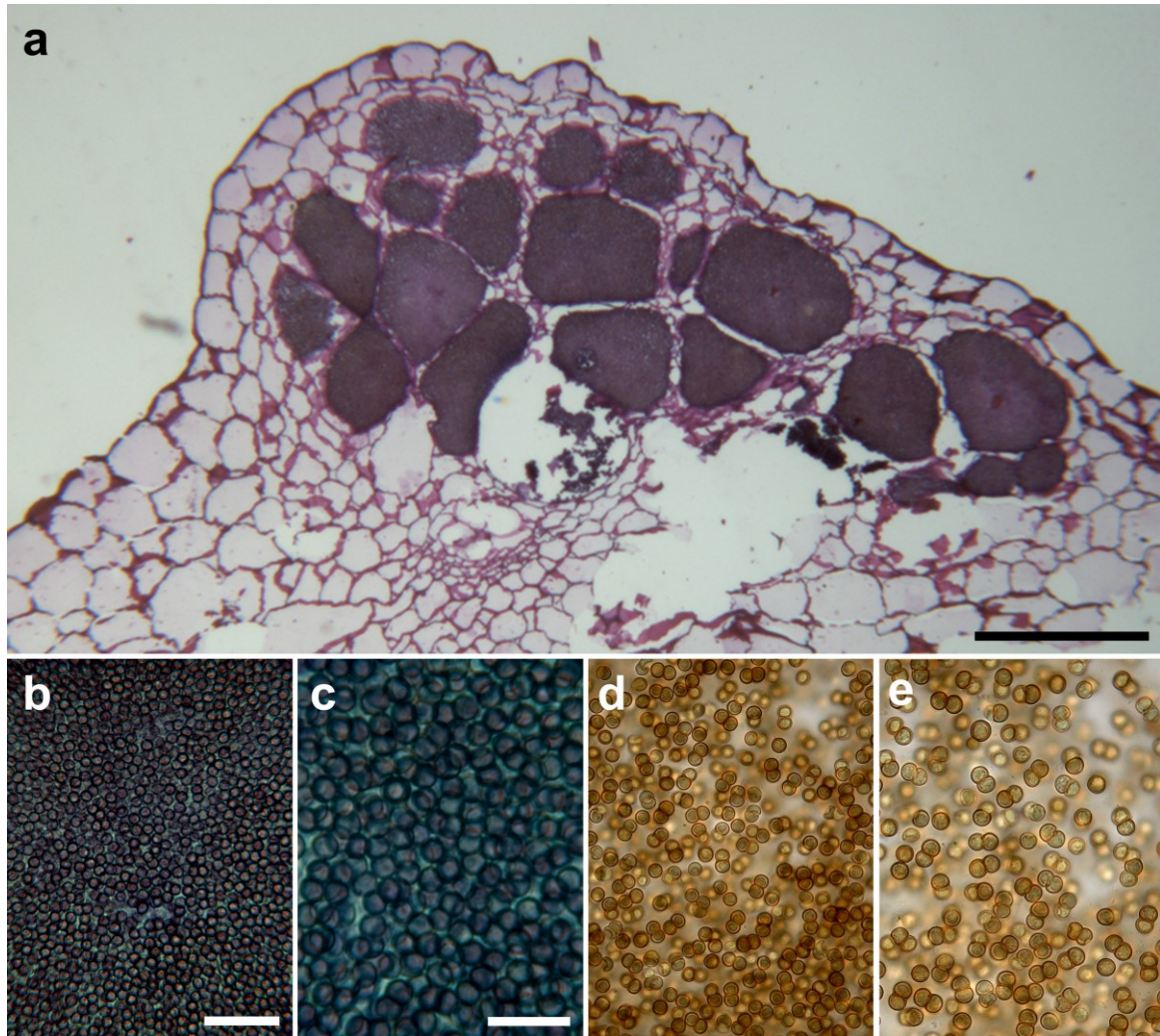
### **Molecular phylogenetic analysis**

A dataset of aligned 18S rRNA gene sequences was created. It contained the newly determined sequence, 16 Phytomyxea sequences retrieved from GenBank, 12 environmental 18S rRNA gene sequences identified by BLAST to be affiliated with Phytomyxea, and 11 sequences of vampyrellids, *Placopus*, “novel clade 9” and *Penardia* and its relatives, used as outgroups. The sequences were aligned using MAFFT (Kato et al. 2002) on the MAFFT 7 server (<https://mafft.cbrc.jp/alignment/server/>) with the G-INS-i algorithm at default settings, followed by manual correction and exclusion of poorly aligned sites in BioEdit 7.0.4.1 (Hall 1999). The final dataset contained 1725 aligned sites and is available upon request. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian Inference (BI) methods. The ML analysis was performed using RAxML 8.0.0 (Stamatakis 2014) under the GTRGAMMAI model, with 10 random taxon additions to generate starting trees. Bootstrap support was inferred from 1000 pseudoreplicate datasets. BI was performed using MrBayes 3.2.2 (Ronquist et al. 2012) under the GTR + I +  $\Gamma$  + covarion model. Two parallel runs of four MCMCs were run for 3000000 generations at which point the mean standard deviation of split frequencies based on the last 75 % of generations was < 0.01. The trees were sampled every 500th generation. The first 25 % of trees were removed as burn-in.

## **Results**

### **Herbarium material examinations**

We found the permanent slides from Marziano et al. (1995) to be closely comparable to the galls collected by Vohník et al. (2017) in Turkey as well as to the material collected in this study, both in terms of the endophytic infection pattern (Fig. 2a) and the spore size (Fig. 2b, c). However, it was impossible to reliably determine the frequency of the different spore arrangements due to the high spore density in the Marziano et al. (1995) slides. On the contrary, the different spore arrangements were clearly recognizable in the micrographs of the recent findings by G. M. Gargiulo (Fig. 2d, e): most of the spores formed dyads or were single, which is in agreement with all published observations of this symbiosis in the Mediterranean (see above).



**Fig. 2** Photographs of earlier findings of the phytomyxid infection in *Halophila stipulacea* in Sicily. **a** Transverse section of a gall on *H. stipulacea* examined by Marziano et al. [21]. **b, c** Micrographs of mature resting spores from the galls examined by Marziano et al. [21]. **d, e** Micrographs of mature resting spores from galls on *H. stipulacea* collected by G. M. Gargiulo in around 2015 near Tono (Casabianca), Sicily (see **Table 1**). Note that most of the spores are arranged in dyads. Scale bars: **a** = 300  $\mu\text{m}$ ; **b** = 40  $\mu\text{m}$ ; **c** = 20  $\mu\text{m}$ ; **d, e** = unavailable

## Sampling

Out of the four sampling campaigns, only two were successful in terms of phytomyxid infection findings (Table 1). In December 2015 no *H. stipulacea* populations were found at the localities visited. In September 2017, a population of *H. stipulacea* was discovered at one locality in Messina at the depth between 11 and 14 meters with a relatively high phytomyxid colonization rate (Table 2). In May/June 2018 several new localities with *H. stipulacea* were found but there was no sign of the infection at any of them. At the locality in Messina where the infected plants had been found the previous year, many rhizomes were observed with the leaves broken off, but no galls or other signs of the infection were observed. However, in September 2018 the infection was detected at the same site again, although this time the percentage of the colonization was slightly lower (Table 2). Water salinity did not differ between the two samplings in Messina in 2018 and was 40‰.

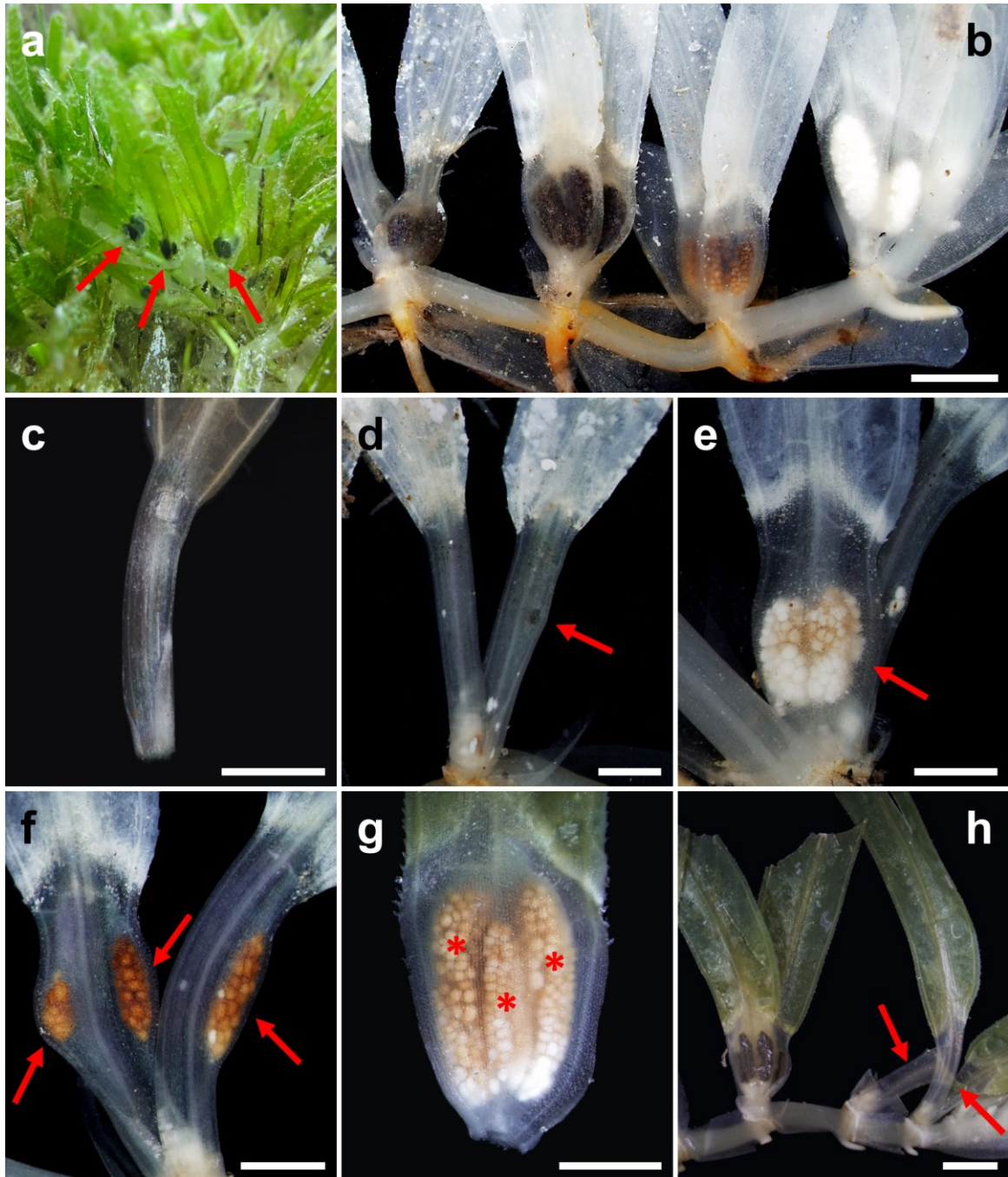
When present at the site, the infection was immediately visible to the naked eye (Fig. 3a) and even its different developmental stages could be easily recognized by their different coloration (Fig. 3b), as previously described in Materials and methods.

### **Stereomicroscopy, light and scanning electron microscopy**

When the petiole was infected with the phytomyxid, its usually long and thin proportions (Fig. 3c) changed. According to the extent of the infection (i.e. the number of the infected host cells), the morphological modification of the petiole altered from hardly detectable (Fig. 3d) to the formation of the characteristic galls, i.e. the petiole was considerably shorter and swollen (Fig. 3e). The galls comprised the petiole tissue with one or typically two clusters of infected parenchyma cells, each located on one side along the central vascular tissues of the petiole (Fig. 3f). In rare occasions of exceptionally heavy infection a third cluster was formed (Fig. 3g).

In agreement with Vohník et al. (2017), all three morphologically distinguishable developmental stages of the infection were often found on the same rhizome with the youngest leaves showing signs of the earliest infection stage (white colored galls containing sporogenic plasmodia) to the mature infection stage (dark colored galls filled with mature resting spores) usually being developed somewhere around the third youngest node (Fig. 3b). However, there were also cases (although very seldom) where the youngest leaves of an already infested rhizome seemed not infected (Fig. 3h).

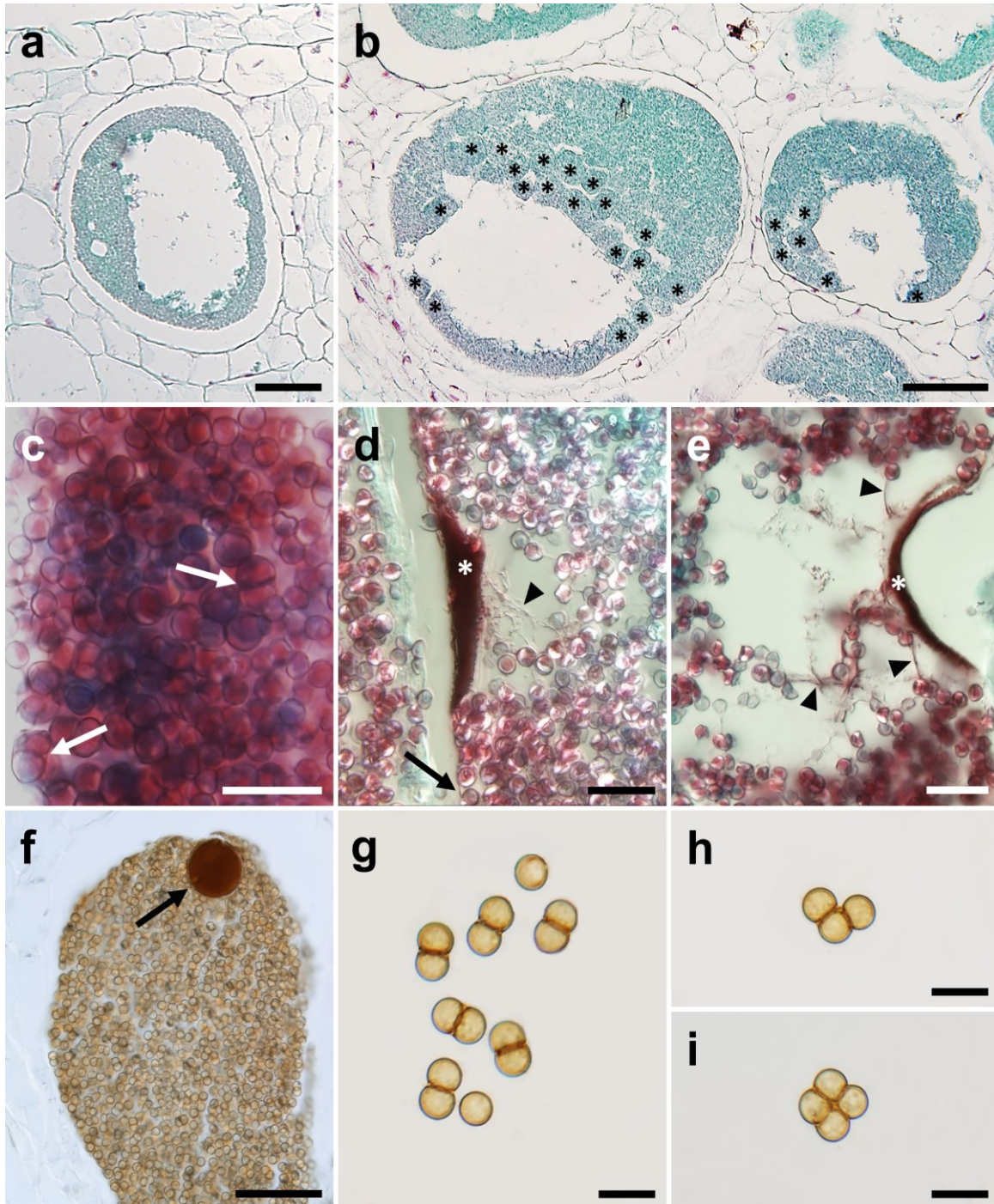




**Fig. 3** Morphological changes in *Halophila stipulacea* petioles due to phytomyxid infection. **a** Plants with visible phytomyxid infection (arrows) observed *in situ*. **b** A rhizome with different developmental stages of the infection, distinguishable by the coloration of the modified petioles/galls. **c** Typical morphology of a healthy *H. stipulacea* petiole. **d** A petiole with a very indistinctive infection (arrow), where the morphological changes are hardly noticeable. **e** A swollen and shortened petiole with a well-developed gall (arrow). **f** Host cells infected with the phytomyxid lie very close to each other and form characteristic clusters (arrows) in the petiole tissue. **g** Three clusters (asterisks) of infected cells formed in a single petiole. **h** A rare observation of phytomyxid-free young leaves (arrows) on a previously infected rhizome. Scale bars: **a, b** = 4 mm; **c-g** = 2 mm; **h** = 5 mm

Using light microscopy, in thin sections of the white colored galls each infected host cell could be easily localized by the presence of a sporogenic plasmodium causing a significant enlargement of the cell (Fig. 4a, b). The cytoplasm of the plasmodium often concentrated on the periphery of the host cell creating a hollow structure (Fig. 4a) and, in many cells, its cleavage





**Fig. 4** Anatomy of different developmental stages of the phytomyxid infection in *Halophila stipulacea*. **a** Sporogenic plasmodium with the cytoplasm gathered close to the host cell periphery; stained with safranin/Fast Green FCF. **b** Cleavage of the sporogenic plasmodium into smaller plasmodia (asterisks); stained with safranin/Fast Green FCF. **c** Early formation of resting spores with many spores of unusually large size (arrows); stained with safranin/Fast Green FCF. **d, e** Dark colored structures of unknown origin (asterisks) located close to the membrane (arrow) surrounding the newly forming resting spores. Filaments attached to both the structure and the spores are visible (arrowheads); stained with safranin/Fast Green FCF. **f** A dark colored compact structure as above (arrow) in a cell filled with mature resting spores. **g** Dyads and single resting spores. **h** An illustrative example of a resting spore triad. **i** An illustrative example of a resting spore tetrad. Scale bars: **a** = 100  $\mu$ m; **b** = 200  $\mu$ m; **c-e** = 20  $\mu$ m; **f** = 50  $\mu$ m; **g-i** = 10  $\mu$ m

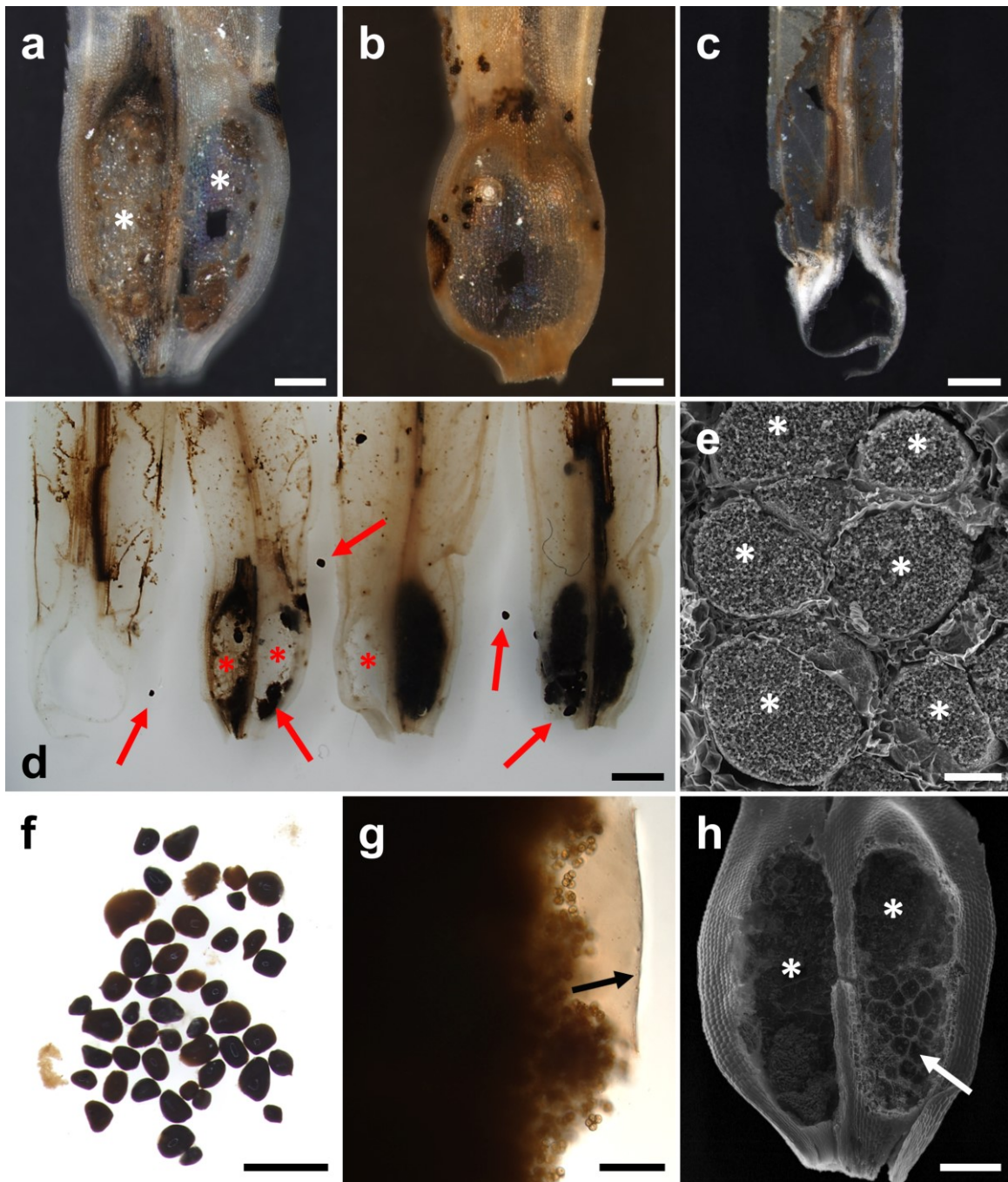
into smaller polygonal to elliptical subunits was apparent (Fig. 4b) (also compare with Vohník et al. 2017). In more mature galls, formation of resting spores could be seen with many spores being noticeably larger than the common size at this point (Fig. 4c). In each host cell, the spores were surrounded by a membrane and dark colored structures of unknown origin were often

observed close to the membrane (Fig. 4d). Sometimes, visible filaments were coming out of these structures, often with attached spores (Fig. 4d, e). In the host cells filled with mature resting spores, these structures seemed more compact and were usually of a rounded shape (Fig. 4f). The resting spores formed mostly dyads (60%) or stayed single (31%) although some triads (6%) and tetrads (3%) were also present (Fig. 4g-i, Table 3). During the determination of the phytomyxid colonization percentage in September 2018, few older detached leaves were noticed in the samples. Their gall tissue had already started to disintegrate, but the original infection pattern was still easily recognizable (Fig. 5a-d). Upon further examination we discovered that the resting spores fell out from the decaying petioles not individually, but as massive aggregations surrounded by a membrane (Fig. 5d-g), leaving empty cavities where the infected enlarged cells had been once located (Fig. 5d and h).

### **Measurements**

According to our measurements, well-developed galls cause shortening of the petiole by approximately two thirds of its average length (i.e. 8.7 mm; n=34, min. 6.0 mm, max. 11.7 mm) and almost doubling of its average width (i.e. 1.6 mm; min. 0.9 mm, max. 2.3 mm) (see Table 3). The gall average dimensions measured in this study (3.2 x 2.6 mm) are very similar to those published in Vohník et al. (2017), although the resting spore diameter (5.5 µm) and dimensions of the infected cells (260.3 x 198.8 µm) were slightly larger here (Table 3). These numbers differ significantly from the dimensions characteristic for *Tetramyxa parasitica* as originally described in Cook (1933) (resting spore diameter 3.5 µm, plasmodium diameter 15–30 µm), further confirming that this phytomyxid is not *T. parasitica* (also see Braselton 2019).

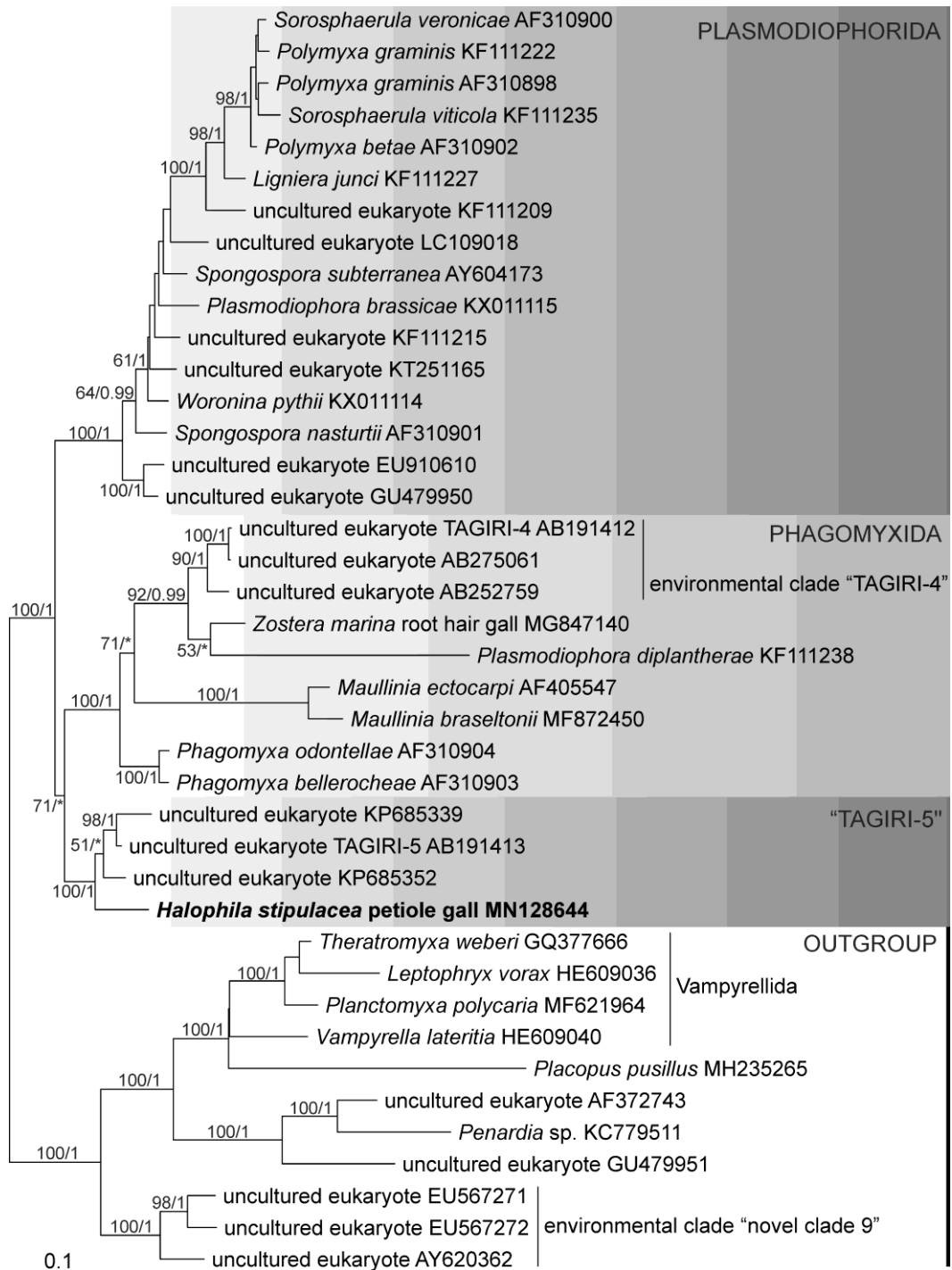




**Fig. 5** Gall disintegration and spore release. **a–c** Old *H. stipulacea* leaves with thick swollen petioles indicating former phytomyxid infection/galls. Asterisks mark empty cavities created by the release of spore aggregations into the environment. **d** Different stages of the gall emptying: spore aggregations (arrows) are visibly falling out of the damaged petioles, leaving empty cavities (asterisks) in the host tissue. **e** A SEM micrograph of mature phytomyxid spore aggregations (asterisks) still situated in the petiole tissue. **f** Individual spore aggregations after their release from a disrupted gall. **g** Each spore aggregation is visibly coated by a membrane (arrow). **h** A SEM micrograph of an empty gall with hollow cavities (asterisks) and visible imprints of formerly present enlarged infected host cells (arrow). Scale bars: **a, b, f, h** = 1 mm; **c, d** = 2 mm; **e** = 100  $\mu$ m; **g** = 50  $\mu$ m

### Molecular phylogenetic analyses

The phylogenetic tree of Phytomyxea inferred from the 18S rRNA gene sequences is shown in Fig. 6. Phytomyxea was recovered as monophyletic with maximum support and split into three robust clades (bootstrap support, BS 100, Bayesian posterior probability, BPP 1),



**Fig. 6** Phylogenetic tree of Phytomyxea, based on the 18S rRNA gene sequences, constructed by the maximum likelihood in RAxML (GTRGAMMAI model). Shaded boxes highlight the three major clades of Phytomyxea. Values at branches represent statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes); support values below 50/.90 are not shown or are represented by an asterisk. Newly determined sequence in bold.

Plasmodiophorida, Phagomyxida, and the environmental clade “TAGIRI-5” (see Murúa et al. 2017); the latter two appeared closely related, though the relationship was not highly supported (BS 71, BPP <0.9). The newly determined sequence of the phytomyxid colonizing *Halophila stipulacea* clearly belonged to the clade “TAGIRI-5”, representing its first characterized member.

## Discussion

The rediscovery of the phytomyxid symbiosis after 20+ years in the Strait of Messina suggests that the respective phytomyxid is either well established at least in some of the local *H. stipulacea* populations or that it has been repeatedly introduced into this area, either as infected seagrass fragments or in the form of resting spores/spore aggregations. Indeed, the Strait of Messina is a relatively narrow but important seaway connecting the Eastern Mediterranean Basin with the Tyrrhenian Sea so the possible repeated introductions could have happened through commercial and leisure traffic, similarly to the situation with *H. stipulacea* in the Caribbean (Wilette et al. 2014), perhaps being facilitated by the notorious Strait currents periodically changing their directions and intensity (<http://www.correntidellostretto.it/>). However, the observed absence of the characteristic galls at most localities with *H. stipulacea* investigated in this study suggests that the phytomyxid is either rare (as has been suggested for *Plasmodiophora bicaudata* (den Hartog 1989)) or overlooked (similar to the case of *Plasmodiophora* cf. *halophilae* at the Turkish locality in the Aegean Sea (Vohník et al. 2017)).

The fact that the symbiosis was very common in Messina (>40 % of the leaf petioles colonized) in September 2017, then totally absent in May/June 2018 and then again common (~30 %) in September 2018 importantly adds to our so far very limited knowledge on ecology of this peculiar symbiosis as it seems that the phytomyxid survives a significant part of its life cycle outside the host, probably in the form of environmentally resistant resting spores/spore aggregations. Additionally, this apparent seasonality may explain the very low number of reports on its occurrence and the observed differences in colonization rates. In the same area in Sicily, Marziano et al. (1995) reported less than 1% of the petioles colonized (with no details on the sampling date) whereas in this study, we found more than 40% of the petioles colonized in September 2017. Thus, our findings emphasize the need for periodic screening of the investigated localities and could serve as a warning against premature conclusions on the distribution and population dynamics of marine phytomyxid species.

According to the phylogenetic analyses based on the 18S rRNA gene sequence, the organism studied here is closely related to the phagomyxids, being a member of the clade “TAGIRI-5”, which also comprises environmental clone sequences from the East China Sea (Jiang et al. 2016) and the coast of Japan (Takishita et al. 2005). The “TAGIRI-5” clade certainly represents at least a new genus of Phytomyxea and the organism studied by us represents its first characterized member. For its accurate taxonomic treatment, a morphological and phylogenetic comparison with *Plasmodiophora halophilae* is necessary. However, we were unable to obtain the type specimen of *P. halophilae* on loan from the Botanical Museum in Copenhagen, Denmark (i.e. the place where it was originally deposited by H. Jensen, see Ferdinandsen & Winge 1913, Anonymous 2014) and, unfortunately, it seems to be irretrievably lost. Moreover, to our best

knowledge, no phytomyxid infection has been reported on *Halophila ovalis* in Java, Indonesia since the paper by Ferdinandsen and Winge (1913) so at present, such a comparison is impossible. The *H. stipulacea* phytomyxid should be also compared with *Tetramyxa parasitica*, despite that this phytomyxid seems to be specific for salt-tolerant aquatic plants such as *Ruppia* and *Zannichellia* (Goebel 1884, Kornaś 1953, den Hartog 1963) and certain *Potamogeton* species (Karling 1968, Tur et al. 1984) Indeed, except the paper by Marziano et al. (1995), to our best knowledge it has never been reported on *Halophila* or any Mediterranean seagrass. These taxonomic issues will be eventually solved by molecular approaches but at the moment, *T. parasitica* sequences are not available in the NCBI-GenBank database (accessed June 2019). This obviously holds true also for *P. halophilae*.

Based on the anatomy and morphology of the galls as well as appearance and arrangement of the spores it is almost certain that the phytomyxid reported in Marziano et al. (1995), Vohník et al. (2017) and here represents the same species, thus extending its known distribution range from the northeastern part of the Eastern Mediterranean Basin to the borderline with the Western Basin. Since *H. stipulacea* is a well-known Lessepsian migrant and phytomyxids are obligate symbionts/parasites, there are two perhaps equally parsimonious scenarios available to explain the origin of this phytomyxid symbiosis in the Mediterranean Sea: 1) the symbiosis arrived to the Mediterranean as a whole, i.e. both the host and its intracellular symbiont travelled through the Suez Canal together or 2) the seagrass and the phytomyxid travelled separately, i.e. as phytomyxid-free seagrass fragments and environmentally resistant resting spores/spore aggregations, either in parallel or at various times, and formed the characteristic symbiosis only after the first contacts in the Mediterranean Sea. Alternatively, and perhaps less parsimoniously, considering the predisposition of phytomyxids for even cross-kingdom shifts (Neuhauser et al. 2014), today's *H. stipulacea* phytomyxid could have lived in the Mediterranean in symbioses with alternative hosts and switched to *H. stipulacea* once it arrived through the Suez Canal and colonized suitable habitats. In any case, given the lack of the symbiosis at the site in Messina in May/June 2018 and its return in September 2018, it seems plausible that the phytomyxid has found favorable conditions for completing its life cycle even close to the current northwesternmost limit of the distribution of *H. stipulacea* in the Mediterranean (Gambi et al. 2009, Gambi et al. 2018), from the release of the resting spores to the formation of the zoospores which colonize host tissues, eventually triggering the formation of the characteristic petiole galls full of the resting spores (see Neuhauser et al. 2011). It would therefore be interesting to investigate the phytomyxid spore bank not only at the site in Messina but all along the coast of the Strait of Messina, perhaps using approaches similar to those common in terrestrial phytomyxid research (Buczacki & Ockendon 1978, Takahashi & Yamaguchi 1987).

In May/June 2018, no galls were observed at the locality in Messina but many *H. stipulacea* rhizomes were observed without older leaves. Additionally, many already dead

leaves mostly with galls at various stages of disintegration and releasing resting spores/spore aggregations were found at the same place in September 2018. It is unclear whether the observed leaf shedding was facilitated or even triggered by the phytomyxid infection at the point when the resting spores were mature and ready to disperse into the surroundings. Nevertheless, spore dispersal aided by the dead leaves would certainly be of a significant advantage both in terms of dispersal range and spore protection. The latter may be further enhanced by formation of the spore aggregations enveloped in a protective membrane as observed in this study (Fig. 5e-g). Some phytomyxids may, interestingly, cause increased uprooting of the host rhizomes (den Hartog 1989, Walker & Campbell 2009, Elliot et al. 2019), which can also facilitate the dispersal of resting spores. Such a phenomenon has however not been observed in the case of *H. stipulacea* – *Plasmodiophora* cf. *halophilae* symbiosis.

## Conclusions

Our study shows that the phytomyxid symbiosis on *H. stipulacea* reported in the Mediterranean Sea firstly in 1995 and secondly in 2017 may be actually more common than previously thought and that targeted research throughout the whole year is necessary to fully understand its distribution as well as the infection process of the respective phytomyxid. DNA-based investigations (including *P. halophilae* and *T. parasitica*) are needed to elucidate the identity of the phytomyxid and its taxonomic position among the already described marine species. Investigations into the phytomyxid spore bank, including areas in the northwest Mediterranean Sea where *H. stipulacea* is currently absent, may further expand our understanding of its population dynamics, dispersal modes and future infection potential.

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## Tables

**Table 1** List of the localities investigated in this study during four sampling campaigns (2015-2018)

Locality <sup>a</sup>	GPS coordinates	December 2015 sampling		September 2017 sampling		May/June 2018 sampling		September 2018 sampling		Herbarium vouchers (PRA acc. numbers <sup>b</sup> )
		<i>H. stipulacea</i>	colonization	<i>H. stipulacea</i>	colonization	<i>H. stipulacea</i>	colonization	<i>H. stipulacea</i>	colonization	
<u>Bufalaro (Augusta)</u>	N37.20618°, E15.18371°	not visited (nv)		nv		yes	no	nv		14516-18
<i>Capo di Milazzo</i>	N38.24284°, E15.25299°	nv		no	-	nv		v		-
<i>Capo Meli (Plemmirio)</i>	N37.00508°, E15.31377°	no	-	no	-	nv		nv		-
Capo Peloro I (Messina)	N38.26446°, E15.65088°	nv		no	-	nv		nv		-
<u>Capo Peloro II (Messina)</u>	N38.27249°, E15.65215°	nv		no	-	yes	no	yes	no	14515
Capo Santa Croce	N37.24187°, E15.25622°	nv		nv		no	-	nv		-
<b><i>Messina</i></b>	N38.22915°, E15.57089°	nv		yes	yes	yes	no	yes	yes	13417-19
Milazzo	N38.23126°, E15.24984°	nv		nv		no	-	nv		-
<i>Ognina</i>	N36.97924°, E15.25901°	no	-	nv		nv		nv		-
<u>Palinuro</u>	N40.03024°, E15.27642°	nv		yes	no	nv		nv		-
Priolo Gargallo	N37.15451°, E15.21804°	nv		nv		no	-	nv		-
<i>Riposto</i>	N37.73436°, E15.20512°	no	-	nv		nv		nv		-
Scilla	N38.25445°, E15.72604°	nv		nv		no	-	nv		-
St. Mark Beach (Recanati)	N37.80108°, E15.24877°	no	-	nv		nv		nv		-
<u>Tindari</u>	N38.14344°, E15.04839°	nv		yes	no	nv		nv		-
<u>Tono (Casabianca)</u>	N38.29102°, E15.56636°	nv		yes	no	nv		nv		-

<sup>a</sup>Random localities are in normal font, localities with expected *H. stipulacea* presence (based on literature or personal communications) are in italics, localities where *H. stipulacea* was actually found are underlined and the only locality with phytomyxid colonization is in bold.

<sup>b</sup>PRA = Herbarium of the Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic.

**Table 2** Phytomyxid infection levels in the seagrass *Halophila stipulacea* collected in this study in Messina (Sicily, southern Italy). Each year, three samples of *H. stipulacea* biomass were collected at the site with the presence of the phytomyxid infection (see Table 1) for an estimation of its intensity. For further details see Materials and Methods

Collection date	Colonization type	Sample #1	Sample #2	Sample #3	Averages per collection
September 2017	white galls	107 petioles (14.70 %)	61 (13.23 %)	60 (10.91 %)	12.95 %
	yellow to black galls	261 (35.85 %)	136 (29.50 %)	100 (18.18 %)	27.84 %
	no colonization	360 (49.45 %)	264 (57.27 %)	390 (70.91 %)	59.21 %
September 2018	white galls	115 (12.91 %)	29 (4.16 %)	30 (3.13 %)	6.74 %
	yellow to black galls	345 (38.72 %)	129 (18.48 %)	96 (10.01 %)	22.40 %
	no colonization	431 (48.37 %)	540 (77.36 %)	833 (86.86 %)	70.86 %

**Table 3** Comparison of reports on phytomyxid infections in the seagrass genus *Halophila*

Reference	Location	Host species	Suggested phytomyxid species	Gall/swelling diameter (mm)	Infected cell dimensions (µm)	Plasmodium diameter (µm)	Spore diameter (µm)	Spore aggregations
Ferdinandsen & Winge (1913)	Nusa Kambangan (Java, Indonesia)	<i>Halophila ovalis</i>	<i>Plasmodiophora halophilae</i>	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)	<i>Halophila stipulacea</i>	<i>Tetramyxa parasitica</i>	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%)
Vohník et al. (2017)	Marmaris (Turkey)	<i>Halophila stipulacea</i>	<i>Plasmodiophora</i> cf. <i>halophilae</i>	length 3 – 6 (avg. = 3.8, n = 78) width 2 – 3 (avg. = 2.6)	length 64 – 318 <sup>a</sup> (avg. = 172.7, n = 148) width 56 – 209 <sup>a</sup> (avg. = 127.6)	length 32 – 66 <sup>b</sup> (avg. = 48.3, n = 34) width 22 – 56 <sup>b</sup> (avg. = 38.2)	avg. 5.03 range 4.0 – 6.0 (n = 300)	single (44%) dyads (56%)
this study/the samples collected in 2017	Messina (Sicily, Italy)	<i>Halophila stipulacea</i>	putative <i>Plasmodiophora</i> cf. <i>halophilae</i>	length 0.9 – 5.4 (avg. = 3.2, n = 74) width 1.2 – 4.4 (avg. = 2.6)	length 169 – 384 <sup>a</sup> (avg. = 260.3, n = 66) width 124 – 327 <sup>a</sup> (avg. = 198.8)	length 117 – 332 <sup>b</sup> (avg. = 222.8, n = 36) width 99 – 273 <sup>b</sup> (avg. = 184.8)	avg. 5.5 range 5.0-7.0 (n = 300)	single (31%) dyads (60%) triads (6%) tetrads (3%)

<sup>a</sup>Dimensions of host cells in the final blackish developmental stage, i.e. when filled with resting spores.

<sup>b</sup>Dimensions of young sporogenic plasmodia at the first (whitish) developmental stage

## CHAPTER 2

### CONQUEST OF PARADISE II: CO-MIGRATION OF AN ALIEN SEAGRASS AND ITS PHYTOMYXID PARASITE FROM THE INDO-PACIFIC THROUGH THE MEDITERRANEAN TO THE CARIBBEAN SEA



*Halophila stipulacea* (from Hemprich & Ehrenberg 1900)

## Introduction

*Halophila stipulacea* (Hydrocharitaceae: Alismatales) is a tropical/subtropical seagrass native to the Indo-Pacific region (Ostenfeld 1914, den Hartog 1970) that represents one of the most recent invaders in the seagrass world (Willette et al. 2014, Smulders et al. 2017). Starting as a Lessepsian migrant which crossed the Suez Canal via shipping and colonized sea-beds in the Eastern Mediterranean ports (den Hartog 1972, Lipkin 1975), it was eventually dragged all the way to the Caribbean Sea (most likely with recreational yachts) where it has continued to spread rapidly since its first observation in Grenada in 2002 (Ruiz & Ballantine 2004). In the tropical Caribbean waters, it is considered a highly invasive species capable of forming dense mats and displacing local seagrass populations within few weeks (Willette & Ambrose 2012). In the considerably colder Mediterranean Sea, it rather behaves as an opportunist and, despite its alleged ability to outcompete the native angiosperm *Cymodocea nodosa* (Sghaier et al. 2014), recent research indicates that it does not negatively affect the carbon stock of the indigenous Mediterranean seagrasses (Apostolaki et al. 2019). Thanks to its immigration status, *H. stipulacea* has quickly become one of the most studied marine phanerogams in the world. Many works were published to this date concerning its distribution, physiology, microbiome or its effect on associated marine biota. Interestingly, *H. stipulacea* was also reported several times to host a phytomyxid parasite forming specific malformations (i.e. galls) in the plant's petioles (Marziano et al. 1995, Vohník et al. 2017, Chapter 1).

Along with two other groups of protists, Oomycota and Labyrinthulomycetes (Sullivan et al. 2018), and potentially also viruses (Park et al. 2018, Van Bogaert et al. 2019), phytomyxids (Cercozoa: Phytomyxea) are commonly counted as important seagrass pathogens, although this being mainly because of the phytopathological nature of their terrestrial representatives parasitizing on crop plants (e.g. *Plasmodiophora brassicae*, *Spongospora subterranea*, or *Sorosphaerula viticola*). Due to the historical absence of the genus *Halophila* in the Mediterranean Sea and the occurrence of a morphologically similar phytomyxid (*Plasmodiophora halophilae*) in the petioles of *H. ovalis* collected by Hjalmar Jensen in Java (Ferdinandson & Winge 1913), a hypothesis was brought up, that *H. stipulacea* and its parasite had co-migrated through the Suez Canal and established in the Mediterranean jointly (Vohník et al. 2017, Chapter 1). The theory development however seemed to reach a dead end, when the type and only material of *P. halophilae* turned out to be irretrievably lost (Chapter 1).

In the ongoing process of digging through the relevant literature on the marine Phytomyxea, a surprising information was stumbled upon eventually, that the first observation of a phytomyxid infecting *H. stipulacea* was not in fact presented by Marziano et al. (1995) from Sicily as commonly thought and reported by the major contemporary reviews dealing with Phytomyxea (Bulman & Braselton 2014, Bulman & Neuhauser 2017). In 1974, long before

Marziano et al. (1995), two Israeli scientists published a detailed description of this symbiosis from the Gulf of Aqaba (Red Sea) and assigned the organism to a new species – *Tetramyxa marina* (Lipkin & Avidor 1974). Their manuscript, intuitively supporting the later hypothesis of the seagrass-parasite co-migration through the Suez Canal, has been surprisingly overlooked by everyone except Tur et al. (1984) reporting on a phytomyxid found in Argentina a review on straminipilous fungi by Dick (2001).

In the last few decades, tens of co-introduced parasites were noted in the oceans, many spilling over the immunologically naive native populations leading to their significant reduction (Torchin et al. 2002, Goedknecht et al. 2016). Moreover, an increasing number of marine organisms' mass mortality events caused by a microbial agent is being recorded (Muehlstein et al. 1991, Carella et al. 2014, Clemente et al. 2014, Vázquez-Luis et al. 2017 and many others). A strong urge has therefore risen to inspect the potentially pathogenic taxa, understand their ecology and try to predict their dispersal and impact on the affected populations. It was suggested many times that temperature changes, pollution/habitat degradation and human-induced species introductions can substantially facilitate the emergence of diseases and cause irretrievable damage to the balance of the marine environment (Harvell et al. 1999, Williams 2007, Marcogliese 2008).

Considering the ecological value of seagrass meadows – seagrass beds count among one of the most productive and complex oceanic ecosystems, providing habitat, nursery-ground and major food source for countless species of the marine biota (McRoy & McMillan 1977, Duffy 2006, Duarte et al. 2010); and the invasive potential of *H. stipulacea*, it seemed essential to attempt to broaden the knowledge of its phytomyxid parasite. In order to do so, the Lipkin & Avidor (1974)'s lead was followed and *Tetramyxa marina* was examined. Also, new important findings concerning the symbiosis' global distribution were delivered in cooperation with three foreign researchers.

## **Materials and methods**

### ***Tetramyxa marina* type material observation**

The type material of *Tetramyxa marina* was deposited by Lipkin & Avidor (1974) in the herbarium collection of the Phycological Laboratory, Dept. of Botany, Tel Aviv University under a code "PHYT. 2261". It comprised samples preserved in 4% formalin, and thin paraffin sections mounted in permanent slides. All the stored material was thoroughly examined and documented, using light microscopy and a Canon G10 camera. The material preserved in formalin was unfortunately in a poor state (Fig. 1) due to a damaged container lid and was not suitable for any further investigations.





Fig. 1 *Tetramyxa marina* type material “PHYT. 2261”; samples originally stored in 4% formalin. Photo © Martin Vohnik

### Distribution mapping

The infected specimens newly presented here were collected in 2018 and 2019 at nine different localities in the Red, the Mediterranean and the Caribbean Sea-s (for the sampling sites details, see Table 1). The localities at Taba border-crossing (Israel), Eilat (Israel) and Maagana (Egypt), all located in the Gulf of Aquaba, were discovered as a result of thorough search and observation of the seagrass meadows near the original collection sites described by Lipkin and Avidor (1974). The rest of the localities were usually discovered randomly by researchers carrying out parts of their other research (see Acknowledgements). The infected plants often grew on a coarse sand substrate in the depths ranging from 2–24 meters. The depth and average water temperature at the sampling sites were measured with a diving computer (Freedom, Divesoft, Czech Republic; Vytec, Vyper Novo or D4i, Suunto, Finland). Samples were collected using scuba diving or snorkeling and stored in 30–50% ethanol or 3% formalin, or dried and mounted on sheets for herbarium storage. They are available in the Herbarium of the Institute of Botany, Czech Academy of Sciences, Průhonice (PRA) and the Herbarium of the Department of Plant Sciences, Tel Aviv University (TAU) (for the specimen codes, see Table 1). A part of the samples kept in ethanol was later transferred into 70% ethanol solution in deionized water and deposited in PRA. Another part of the samples was kept in a refrigerator (5–7° C) until used for the following examinations.

**Table 1** Summary of reported observations of the seagrass *Halophila stipulacea* infected with a phytomyxid

source	locality	GPS	date of observation	depth	temperature	infection rate	substrate	herbarium code
Lipkin & Avidor (1974)	Israel: Eilat	N/A	Jul 1961, Jun 1963	0.5–7 m	N/A	< 1%	coralligenous sand	not deposited
Lipkin & Avidor (1974)	Egypt: Taba	N/A	Mar 1969	0.5–7 m	N/A	< 1%	coralligenous sand	not deposited
Lipkin & Avidor (1974)	Egypt: Shorat El Manqata	N/A	Dec 1969	0.5–7 m	N/A	< 1%	coralligenous sand	PHYT 2261 (damaged)
Lipkin & Avidor (1974)	Egypt: Ras Burqa	N/A	Aug 1973	0.5–7 m	N/A	< 1%	coralligenous sand	not deposited
Marziano et al. (1995)	Italy: Sicily, Naxos/Riposto	N/A	N/A	N/A	N/A	< 1%	N/A	lost
Vohník et al. (2017)	Turkey: Marmaris	N 36.79082° E 28.30403°	Aug 2015	7–8 m	28° C	N/A, but common	coarse sand	dried: PRA-12834 in 70% EtOH: PRA-12835
Kolátková et al. ( <i>in review</i> )	Italy: Sicily, Messina	N 38.22915° E 15.57089°	Sep 2017, Sep 2018	11–14 m	17° C	30–40%	sand	dried: PRA-13417-8 in 70% EtOH: PRA-13419
this study	Israel: Taba border-crossing*	N 29.49764° E 34.91328°	Jan 2018	8–24 m	22° C	up to 4%	sand	dried: PRA-13846 in 70% EtOH: PRA-13847
this study	Israel: Eilat, North-western beach	N 29.53986° E 34.94780°	March 2018	9 m	22° C	up to 4%	sand	dried: TAU-3098
this study	Israel: Eilat, North beach	N 29.53466° E 34.97285°	May 2018	0.5–1 m	26° C	up to 4%	sand	dried: TAU-3321
this study	Israel: Eilat, North beach	N 29.54503° E 34.97199°	May 2018	0.5–1 m	26° C	up to 4%	sand	dried: TAU-3330
this study	Israel: Eilat, North beach	N 29.54542° E 34.97020°	May 2018	0.5–1 m	26° C	up to 4%	sand	dried: TAU-3350
this study	Bonaire: Lac Bay*	N 12.10065° W 68.24009°	Nov 2015, Sep 2018, Apr 2019	2–3 m	30.2° C	up to 3%	coarse sand	dried: PRA-15024 in 70% EtOH: PRA-15117
this study	Martinique: Fort de France, La Française beach*	N 14.59994° W 61.06805°	Jul 2018	2 m	28° C	< 1%	muddy sand	too few galls
this study	Greece: Samos*	N 37.69774° E 26.97058°	Oct & Nov 2018	5–7 m	21.5° C	up to 3%	sand	to be deposited
this study	Egypt: Maagana*	N 29.10598° E 34.67545°	Feb 2019	6 m	23° C	< 1%	sand	too few galls

\* samples from these localities were used for DNA isolation and amplification of the 18S rRNA gene

At the localities in Israel and Bonaire, the infection rate was calculated herbarium (Table 1) following the protocol described in Chapter 1. At the Samos locality (Greece) 10 benthic chambers (surface area of a single chamber = 0.071 m<sup>2</sup>) were installed in the *Halophila stipulacea* meadows. All the leaves from the chambers were then collected, dried, and later screened for the phytomyxid infection; the percentage of infected leaves was determined. At the rest of the localities the infection was too scarce for any reasonable quantitative calculation.

When present, populations of *H. ovalis* were inspected as well during the sampling in the Gulf of Aquaba. Unfortunately, no signs of any phytomyxid infection were discovered in this species.

### **Stereomicroscopy, light microscopy**

The collected material was observed closely with an Olympus SZX12 stereomicroscope. Paraffin thin sections from the infected plant tissues were produced following (Pazourková, 1986) and mounted into permanent slides. The permanent slides were then examined at high magnifications (400–1000 x) with an Olympus BX60 upright microscope. Photos were taken with an Olympus DP70 camera using QuickPHOTO MICRO 2.3 software (Promicra Ltd., Czech Republic). Photographs were modified for clarity (adjustment of sharpness, brightness and contrast) as needed.

### **DNA extraction, amplification and sequencing**

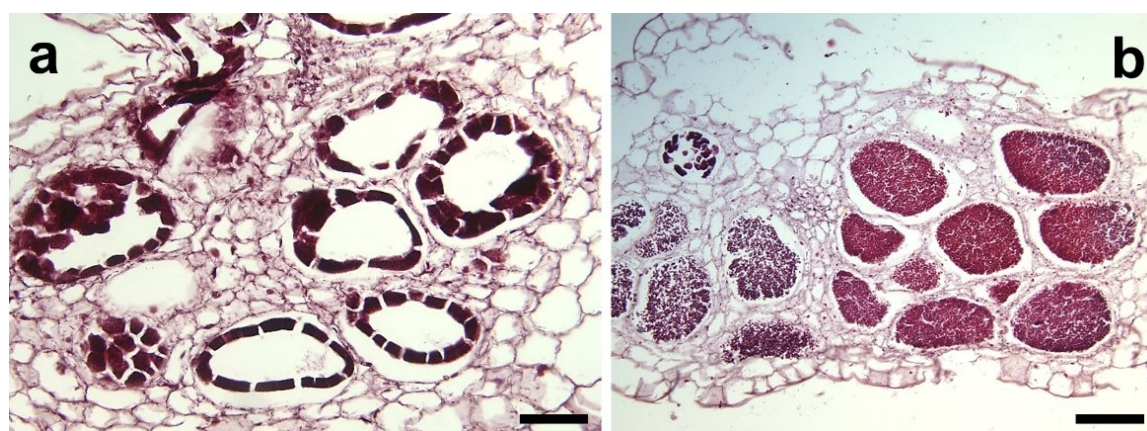
DNA was isolated and amplified from the samples of *H. stipulacea* from five of the newly presented localities (Table 1). DNA isolation and amplification procedures followed those described in Chapter 1, with a minor alternation in the purification kit used.

Briefly, the DNA was isolated from the infected plant tissue using the DNeasy Plant Mini extraction kit (QIAGEN Inc., Venlo, Netherlands) according to the manufacturer's instructions. A nested PCR was carried out to obtain the 18S rRNA gene sequence, using the universal eukaryotic primers MedlinA (Medlin et al. 1988) and EK-1498R (Marande et al. 2009) in the first PCR round and Phytomyxea-specific primers Plas1f (Neuhauser et al. 2014) and PHMX-1570R (Chapter 1) in the second PCR round. For the detailed PCR protocol and primer sequences please see Chapter 1. The amplicons were visualized by gel electrophoresis (1.5% agarose gel) and purified using QIAquick PCR Purification Kit (QIAGEN Inc.) or Zymoclean Gel DNA Recovery Kit (Zymo Research Corp., California, the USA). The purified products were sequenced by Macrogen Europe Laboratory (Macrogen Inc., The Netherlands) using the 577F, 577R, 1055F and 1055R sequencing primers (Elwood et al. 1985).

## Results

### *Tetramyxa marina* type material observation

Due to the poor condition of the samples originally preserved in 4% formalin, it was impossible to verify the relation of *Tetramyxa marina* to the phytomyxid reported in *H. stipulacea* from the Mediterranean Sea based on its morphological features. All the available plant material was discolored and dried up (Fig. 1), and no galls were discovered among the crumbling leaves. However, the macrographs published by Lipkin & Avidor (1974) do not leave much room for doubts. Additionally, when the permanent slides were screened at high magnification, they showed characteristics clearly comparable to the phytomyxid described in Vohník et al. (2017) and Chapter 1 (Fig. 2a, b).

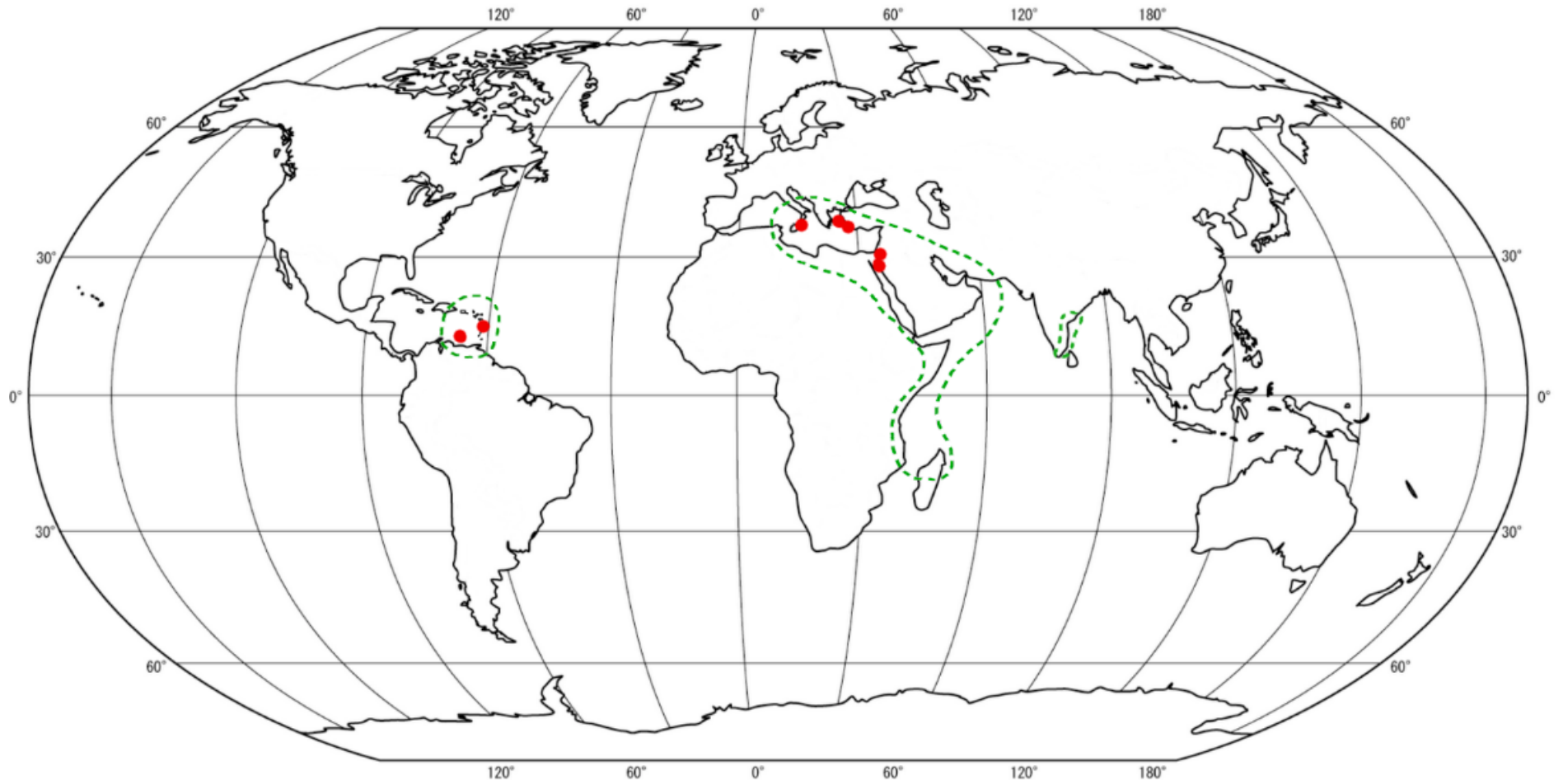


**Fig. 2** Anatomy of *Halophila stipulacea* petiole infected with *Tetramyxa marina*. **a** Enlarged infected cells filled with cleaved sporogenic plasmodia. **b** Clusters of infected cells filled with mature resting spores. Scale bars: **a** = 100  $\mu\text{m}$ ; **b** = 200  $\mu\text{m}$ . Photo © Martin Vohník

### Distribution of the *H. stipulacea* phytomyxid parasite

The recorded global distribution of the symbiosis is shown in Fig. 3. In the Red Sea, five new localities with infected *H. stipulacea* populations were described in the Gulf of Aquaba. The infection rates at four of them reached 4% and they were found when looked for, regardless of the season (Table 1). In the Mediterranean, one new locality at Samos Island, Greece was added to the ones already known in Turkey and Sicily (Vohník et al. 2017, Chapter 1) with the infection rate reaching up to 3%. In agreement with Chapter 1, the symbiosis was observed during the fall months at this locality and seemed to be absent when checked for in February and March. In the Caribbean Sea, the phytomyxid was found at two relatively distant islands – Bonaire and Martinique, with repeated observations independent on the season in the former. All the infected plants were growing on a sandy substrate, usually forming dense meadows.





**Fig. 3** The geographical distribution of the phytomyxid parasite found in the invasive seagrass *Halophila stipulacea*. The distribution area of *H. stipulacea* is outlined by the green dashed line, the recorded occurrence of its phytomyxid parasite is represented by red dots

### **Stereomicroscopy, light microscopy**

In terms of both anatomy and morphology, all the infected specimens examined were identical to each other and to the description provided in Vohník et al. (2017). No visible differences were found among the samples coming from the Red, the Mediterranean and the Caribbean Sea-s.

### **DNA extraction, amplification and sequencing**

The DNA isolation and amplification of the 18S rRNA gene provided 5 partial sequences of this gene identical to the sequence obtained in Chapter 1, deposited to GenBank under the accession number MN128644. Hence, no further molecular analyses were carried out at this point.

### **Discussion**

Based on the findings presented in this chapter, it is clear that the distribution range of the phytomyxid inspected is closely linked to the one of its seagrass host, including the newest invaded region, the Caribbean Sea. We strongly suspect that the symbiosis might be in fact quite common in the *H. stipulacea* populations worldwide and is simply being overlooked due to its positioning at the base of the seagrass leaves, which are often covered with sediment. This suspicion is also supported by the fact that when properly searched, at seven out of the nine newly described localities the infection rate reached at least 3% (Table 1). In Chapter 1, an interesting seasonal pattern at a locality in Messina, Sicily is stated as another possible reason for the sporadic findings of this symbiosis. This seems to be plausible mainly for the Mediterranean Sea, perhaps for its seasonality and low winter temperatures, but less for the tropical waters of the Caribbean and the Red Sea, where the parasite can be observed independently on the part of the year (Table 1).

The omnipresence of a microbial parasite in seagrass populations is not an uncommon phenomenon, the ecology of these symbioses however remains greatly understudied. For example, *Labyrinthula zosterae*, the causative agent of the eelgrass wasting disease (Muehlstein 1991), was confirmed to be present in as much as 89% of the northern European *Zostera* populations (Bockelmann et al. 2013). Labyrinthulomycetes in general seem to be associated with many genera of marine phanerogams and under normal conditions might play a part in the senescence of their leaves (Vergeer & den Hartog 1993). Yet to this day, we still do not fully understand the causes of their pathogenic outbreaks. Other marine phytomyxid species (such as *Plasmodiophora diplantherae* or *P. bicaudata*) also seem to be present throughout the worldwide distribution of their hosts (den Hartog 1965, 1989). However, even less is known about their pathogenic potential. A crucial question therefore arises, how and whether at all *Tetramyxa marina* alters the fitness of its host. Experimental research on the viability of infected

plants would be of use for solving this matter and assessing the threat this phytomyxid does or does not represent for the seagrass populations affected.

The chronological order of all the published reports on infected *H. stipulacea* specimens (Table 1) correlates with its gradual migration through the Suez Canal and further to the western Atlantic and offers a foundation for the host-parasite co-introduction hypothesis to the both newly colonized seas. Although a note exists on the presence of a phytomyxid infection in *Halophila baillonis* in Guadeloupe long before the *H. stipulacea*'s arrival to the region (see Feldmann 1936), the 18S rRNA gene sequence uniformity of the samples analyzed here suggests a close relation of all our findings. Greater gene variability would be expected if a phytomyxid examined was already long established and evolving in the Caribbean seagrasses. Moreover, in the Mediterranean there had been no suitable hosts until the connection with the Red Sea was built. One could object, based on the theory of Beijerinck (1913), that the marine microbial organisms are ubiquitous and that there are no geographical barriers for their dispersal; this view has been nevertheless already questioned by many and phylogeographic patterns were described for plenty of the marine protist groups (Dolan 2005, Foissner 2006, Weisse 2008).

All the above-mentioned seagrass-associated microbes are suspected to be genus-specific symbionts (den Hartog 1965, den Hartog 1989, Vergeer & den Hartog 1991). If this holds true also for *Tetramyxa marina*, then its suggested co-migration might have further ecological impacts to the other native Caribbean *Halophila* species. To fully comprehend the host-specificity of this protist, a comparison with other *Halophila* species infected with a phytomyxid and preferably also *Plasmodiophora halophilae* is necessary.

## Personal credits

Three foreign researchers contributed to the knowledge summarized in this chapter. Razy Hoffman (School of Plant Sciences and Food Security, Tel Aviv University) discovered four of the Israeli localities and helped with the search of *Tetramyxa marina* type material, Fee Smulders (Aquatic Ecology and Water Quality Management Group, Wageningen University & Research) was the first person to observe the symbiosis in the Caribbean Sea (Bonaire) and Emma Ward (School of Biological Sciences, University of Essex) found the infected specimens in Samos Island, Greece. The locality in Martinique was discovered by Martin Vohník.

I would also like to thank Dr. Yaacov Lipkin for his lifetime work in the seagrass research that led us to a deeper understanding of the described symbiosis and the Steinhardt Museum of Natural History and National Research, Tel Aviv University, Israel for the access to their collection.

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## CHAPTER 3

### COMPARISON OF THE PHYTOMYXID PARASITE FOUND IN *H. STIPULACEA* WITH *TETRAMYXA PARASITICA* GOEBEL, 1884



*Tetramyxa parasitica* (from den Hartog 1963)

## Introduction

The research of marine phytomyxids experienced its peak mainly in the 20<sup>th</sup> century with the era of exceptional aquatic botanists, such as Carl Ferdinandsen, Jean Feldmann or Cornelis den Hartog, and has not really been in the center of attention ever since. To this day, seven phytomyxid species associated with the marine or coastal saltmarsh phanerogams have been described in total (Table 1), many still with limited to no notes on their anatomy, distribution, life cycle or effect on the host's fitness. In GenBank, the publicly accessible sequence database, there are only records of three of these species at the moment, comprising four 18S rRNA gene sequences (Table 1). The nomenclature and phylogeny of these Phytomyxea representatives are therefore based mainly on the methods used before the boom of molecular analyses, i.e. light and transmission electron microscopy, and there are many apparent flaws in their current states.

The phytomyxid found in the invasive seagrass *H. stipulacea* was hypothesized by many (Chapter 1) to be closely related to the morphologically similar organism (*Plasmodiophora halophilae*) in the petioles of *H. ovalis* collected by Hjalmar Jensen in Java (Ferdinandsen & Winge 1913). Because there is no direct evidence of this protist species (as the type and only material has been most likely lost, see Chapter 1) it seems impossible to further investigate the validity of this theory until a new infected specimen of *H. ovalis* is discovered. It is however obvious, that even if the two represent the same entity, the phytomyxid found in *H. stipulacea* is phylogenetically very distinct from the genus *Plasmodiophora* (Fig. 6 in Chapter 1).

Furthermore, the organism examined in this thesis was, independently of each other, determined as *Tetramyxa* by two groups of researchers (Lipkin & Avidor 1974, Marziano et al. 1995). In both cases, the determination leaned on the observation of resting spores arranged in tetrads, a supposed identifier for the given genus (Cook 1933). This assessment was later

**Table 1** Species of Phytomyxea described from marine or salt-tolerant phanerogams

species	plant host	status	GenBank sequence
<i>Tetramyxa parasitica</i> (Goebel 1884)	<i>Ruppia</i> spp., <i>Potamogeton</i> spp., <i>Zanichellia</i> spp.	cosmopolitan, commonly found	N/A
<i>Plasmodiophora halophilae</i> (Ferdinandsen & Winge 1913)	<i>Halophila ovalis</i>	single record	N/A
<i>Plasmodiophora diplantherae</i> (Ferdinandsen & Winge 1914)	<i>Halodule</i> spp.	cosmopolitan, commonly found	KF111238 (Neuhauser et al. 2014)
<i>Plasmodiophora bicaudata</i> (Feldmann 1940)	<i>Zostera</i> spp.	cosmopolitan, commonly found	N/A
<i>Plasmodiophora maritima</i> (Feldmann 1958)	<i>Triglochin maritima</i>	found on multiple occasions	N/A
<i>Tetramyxa marina</i> (Lipkin & Avidor 1974)	<i>Halophila stipulacea</i>	found on multiple occasions	MN128644 (Chapter 1)
phagomyxid in eelgrass root hairs (Elliot et al. 2019)	<i>Zostera marina</i>	single record	MG847140, MG991824 (Elliot et al. 2019)

reviewed by Bulman & Braselton (2014), who expressed their doubts and suggested further study of the phytomyxids associated with marine phanerogams. Moreover, the recent works dealing with this particular symbiosis give evidence of prevalence of resting spores arranged in dyads (Vohník et al. 2017, Chapter 1), with tetrads being totally absent in some cases (Vohník et al. 2017). Based on the phylogenetic analyses presented in Chapter 1, it is clear that the phytomyxid species colonizing *H. stipulacea* petioles represents a member of a former environmental clade “TAGIRI-5”. Whether this clade also includes the other described *Tetramyxa* species is still a matter of conjecture.

To resolve the phylogenetic placement of the genus *Tetramyxa* and its supposed affiliation to the phytomyxid examined, a comparison of the latter and the type species *Tetramyxa parasitica* was carried out on morphological, anatomical and molecular level.

## Materials and methods

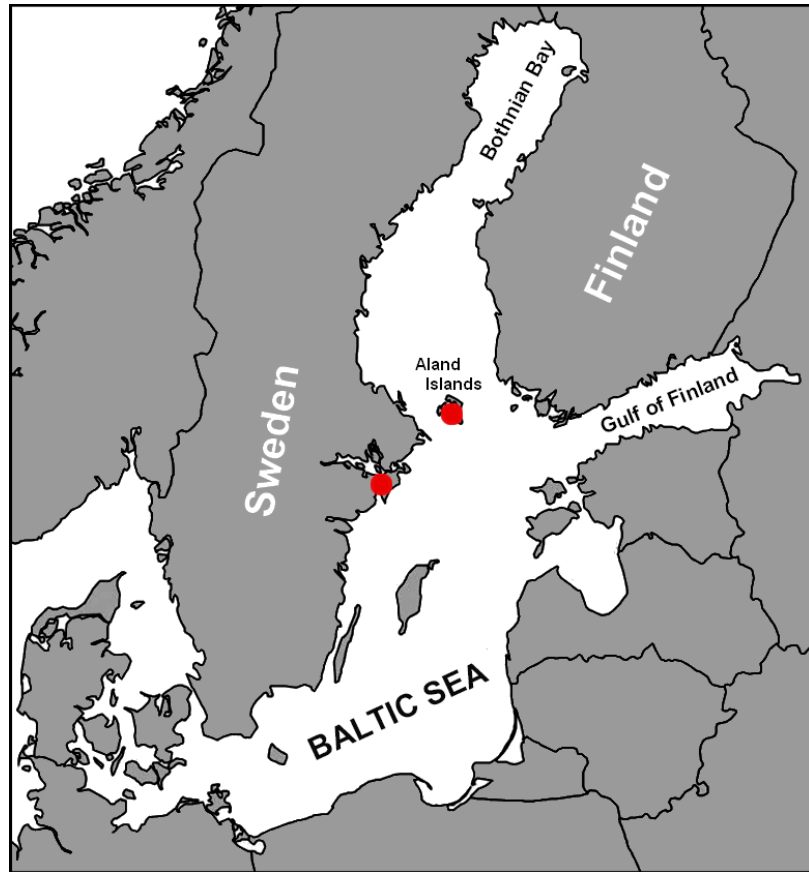
### Material collection

***H. stipulacea* phytomyxid parasite** The *H. stipulacea* galls collected during the samplings in Israel, Martinique, Bonaire and Egypt, described in detail in Chapter 2, were used for the purposes of this comparison.

***Tetramyxa parasitica* in *Ruppia maritima*** A substantial effort was put into obtaining samples of *Tetramyxa parasitica* from four different herbarium collections, where the infected specimens of *Ruppia spp.* were supposed to be stored. However, at three of them (the Floyd Bartley Herbarium of Ohio University, the former Father Reader Herbarium now located in the collections of the University of Bristol, the Herbarium of the Université de Montpellier) no galls with *Tetramyxa parasitica* or even no *Ruppia spp.* plant material were to be found. Moreover, at the Herbarium of the University of California, Berkeley, where the specimen of *Ruppia maritima* infected with a plasmodiophorid was stored by Setchell (1924) (who had apparently mistaken *Tetramyxa parasitica* for a new species which he named *Thecaphora ruppiae*) there is a lack of sufficient material for sampling necessary to carry out any molecular analyses, as we were informed. A sampling campaign to Germany and Scandinavia was therefore organized in August 2018 in search for a fresh material of *Tetramyxa parasitica*, based on the available literature (Luther 1949, Kornaś 1958, Braselton 1990).

During this campaign, two infected populations of *Ruppia maritima* were discovered in the Baltic Sea, one situated near Tunnggrund island, Åland (Finland; N 60.05893°, E 19.95996°) and another one near Järna (Sweden; N 59.09213°, E 17.65390°) (Fig. 1). The plants showing an evidence of a plasmodiophorid infestation were collected in very shallow waters (depths of 0.1–0.2 m) both times, few meters from the shore. They were immediately put into plastic tubes filled

**Fig.1** Collection sites of *Tetramyxa parasitica* in the Baltic Sea (red dots)



with 30% ethanol diluted in local seawater for preservation. The samples were then transported to the laboratory, where they were stored in a refrigerator (5–7° C) until used for further analyses.

### **Stereomicroscopy, light microscopy**

The stereomicroscopy and light microscopy procedures were identical to those described in Chapter 2.

### **DNA extraction, amplification and sequencing**

Samples of *Tetramyxa parasitica* from both localities (Järna, Sweden and Tunngrund island, Åland, Finland) were used for DNA isolation and amplification. The laboratory procedures followed those described in Chapter 1, with a minor alternation in the purification kit used (i.e. Zymoclean Gel DNA Recovery Kit (Zymo Research Corp., California, the USA).

### **Molecular phylogenetic analyses**

Due to a low in-group variability of the 18S rRNA gene within the Phytomyxea, the same dataset of aligned 18S rRNA gene sequences as described in Chapter 1, including the newly determined sequence of the phytomyxid associated with *H. stipulacea*, was used for the analyses carried out here. The sequences were aligned using MAFFT (Kato et al. 2002) on the MAFFT 7 server

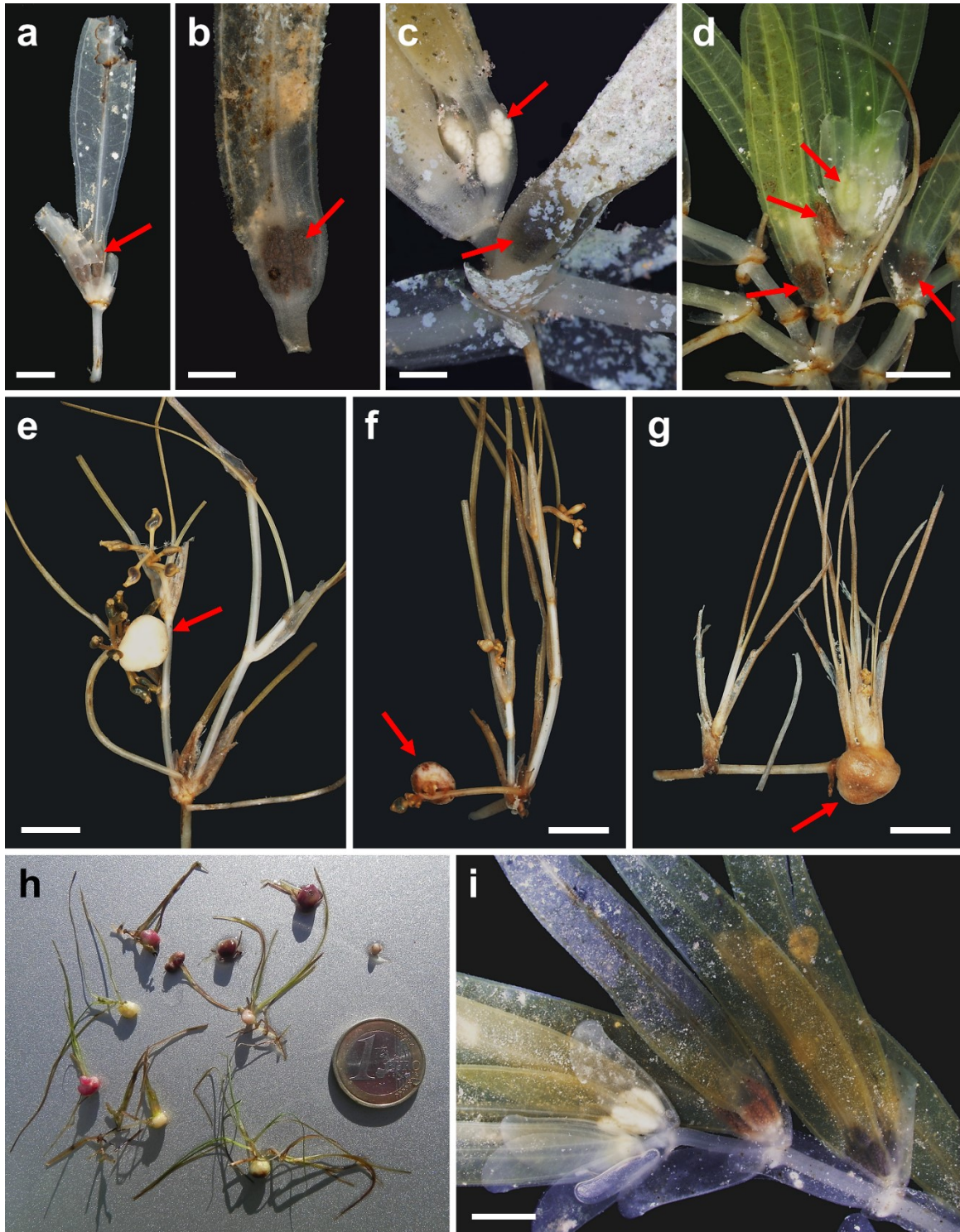
(<https://mafft.cbrc.jp/alignment/server/>) with the G-INS-i algorithm at default settings, followed by manual correction and exclusion of poorly aligned sites in BioEdit 7.0.4.1 (Hall 1999). The final dataset contained 1725 aligned sites and is available upon request. Phylogenetic tree was constructed by maximum likelihood (ML) method. The ML analysis was performed using RAxML 8.0.0 (Stamatakis 2014) under the GTRGAMMAI model, with 10 random taxon additions to generate starting trees. Bootstrap support was inferred from 500 pseudoreplicate datasets.

## **Results**

### **Morphology and anatomy**

Both phytomyxid species examined in this chapter caused easily visible morphological changes to the affected plants, comprising of hypertrophied tissue forming specific galls (Fig. 2). While the phytomyxid found in *H. stipulacea* only infected the leaf petioles without exception (Fig. 2a–d), galls formed by *Tetramyxa parasitica* could be observed almost anywhere along the *Ruppia* body (including the stem, leaves and inflorescence) creating striking deformations (Fig. 2e–g). The coloration of the galls varied from white to darker pink/red/brown in *Tetramyxa parasitica* (Fig. 2h) and brown/black in the *H. stipulacea* phytomyxid (Fig. 2i), depending on its maturity stage, which is in agreement with previous reports on both species (den Hartog 1963, Braselton 1990, Vohník et al. 2017).



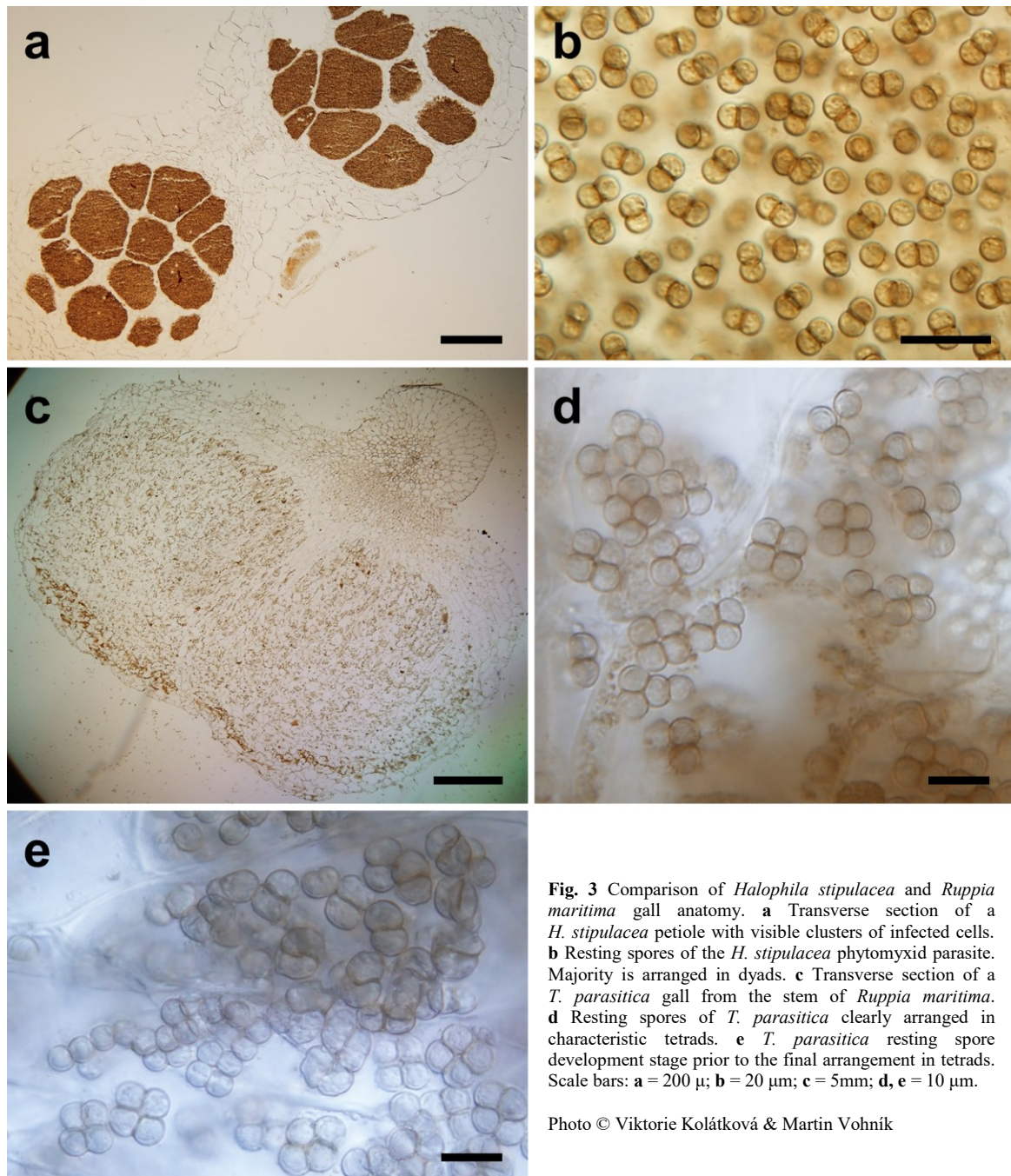


**Fig. 2** Morphological features of *Halophila stipulacea* and *Ruppia maritima* infected by a phytomyxid. **a–d** Galls in the petioles of *H. stipulacea*. **e–g** Galls in the inflorescence and stems of *Ruppia maritima* caused by the presence of *Tetramyxa parasitica*. **h** Coloration range of the *T. parasitica* galls in different stages of maturity. **i** Coloration range of the *H. stipulacea* phytomyxid parasite galls in different stages of maturity. Samples originating from: **a, b** Martinique; **c** Nuweiba; **d** Bonaire; **e, f** Åland; **g, h** Järna; **i** Eilat. Scale bars: **a** = 4mm; **b, c** = 2mm; **d** = 5mm; **e–g** = 4mm; **i** = 5 mm. Photo © Viktorie Kolátková & Martin Vohník

In terms of anatomy, typical clusters of infected parenchymatic cells (Fig. 3a) and predominant resting spore arrangement in dyads (Fig. 3b) were visible in the thin sections of the *H. stipulacea* galls. Whereas in the case of *Ruppia maritima*, the gall anatomy was quite distinct and corresponded with the description published by den Hartog (1963). The gall comprised of



parasite-free cortex surrounding infected cells (Fig. 3c) filled with resting spores arranged in tetrads (Fig. 3d). In some sections, what seemed to be a formation of these tetrads, was captured (Fig. 3f).



**Fig. 3** Comparison of *Halophila stipulacea* and *Ruppia maritima* gall anatomy. **a** Transverse section of a *H. stipulacea* petiole with visible clusters of infected cells. **b** Resting spores of the *H. stipulacea* phytomyxid parasite. Majority is arranged in dyads. **c** Transverse section of a *T. parasitica* gall from the stem of *Ruppia maritima*. **d** Resting spores of *T. parasitica* clearly arranged in characteristic tetrads. **e** *T. parasitica* resting spore development stage prior to the final arrangement in tetrads. Scale bars: **a** = 200  $\mu$ ; **b** = 20  $\mu$ m; **c** = 5mm; **d**, **e** = 10  $\mu$ m.

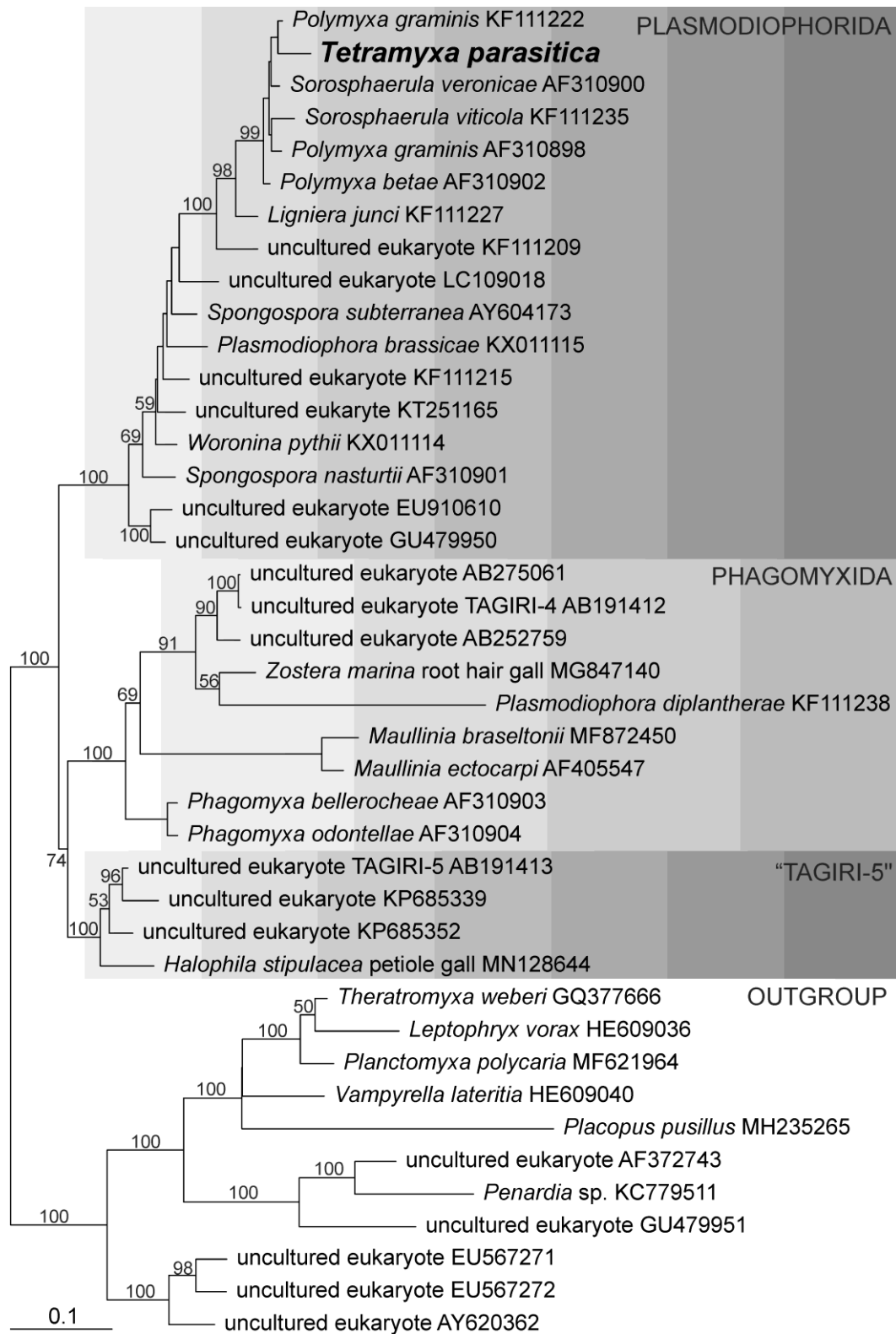
Photo © Viktorie Kolátková & Martin Vohník

### Molecular phylogenetic analyses

The amplification and sequencing of the 18S rRNA gene from both *T. parasitica* samplings resulted in two identical sequences, thus only one of them was included in the final analyses.

The phylogenetic tree of Phytomyxea inferred from the 18S rRNA gene sequences is shown in Fig. 4. The newly determined sequence of *Tetramyxa parasitica* clearly belongs to the

order Plasmodiophorida and appears to be related to *Polymyxa graminis*, a plasmodiophorid species known for parasitizing on plants and crops from the family Poaceae (Ledingham 1939).



**Fig. 4** Phylogenetic tree of Phytomyxea, based on the 18S rRNA gene sequences, constructed by the maximum likelihood in RAxML (GTRGAMMAI model). Shaded boxes highlight the three major clades of Phytomyxea. Values at branches represent statistical support in bootstrap values (RAxML); support values below 50 are not shown. Newly determined sequence in bold

## Discussion

The results provided in this chapter indicate that despite the observations of some resting spore tetrads in the galls found on *H. stipulacea* leaves (Marziano et al. 1995, Chapter 1), the phytomyxid infecting the invasive seagrass does not belong in the genus *Tetramyxa*, as it is morphologically, anatomically and phylogenetically distinct from its type species - *Tetramyxa parasitica*. To this day, *T. parasitica* has only been observed in brackish/freshwater angiosperms (genera: *Zanichellia*, *Potamogeton* and *Ruppia*) and clues exist that it does not form galls in the conditions of increased salinity (den Hartog 1963). Following the work presented in Chapter 1 and Braselton (2019)'s speculations, we disprove the so far single published assumption of its occurrence in the genuine marine environment made by Marziano et al. 1995.

The phylogenetic analyses place *T. parasitica* in a close relation with the terrestrial/freshwater phytomyxid representants (particularly the genus *Polymyxa*), which group in the clade of Phytomyxea labeled as Plasmodiophorida (Fig. 4). The *H. stipulacea* phytomyxid parasite, on the contrary, falls within a distant "TAGIRI-5" clade (Fig. 4), which is closely related to the Phagomyxida – a branch within the Phytomyxea comprising of species described from marine hosts (seagrasses, diatoms and brown-algae) and deep sea environmental clone sequences. We therefore agree that the environment inhabited might be a more important lead to the evolution and phylogenetic relations within this group than the phylogenetic affinity of their hosts (Neuhauser et al. 2014).

By excluding the phytomyxid associated to *H. stipulacea* from the genus *Tetramyxa*, a need arises for the establishment of a new genus, possibly even an order, with the given organism representing its type species. However, to avoid another confusion and further changes in the nomenclature, its relationship to *Plasmodiophora halophilae* (Ferdinandsen & Winge 1913) should be resolved first. A settlement of this issue would moreover lead to a better understanding of the phytomyxid's host-range and shed even more light on its global distribution.

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## CONCLUSIONS

The findings regarding the *H. stipulacea* phytomyxid parasite, presented in this thesis, represent a valuable piece of knowledge both for the research of protist diversity and the seagrass ecology field.

We have proven that the organism examined can spread effectively throughout its host populations and colonize them repetitively at least at certain localities. Once established, it remains present for many years/decades in the affected regions, indicating that (similarly to *Labyrinthula zosterae*) it probably does not cause its host's wipeout under regular conditions. Considering its seasonal occurrence in the Mediterranean Sea (which also strikingly resembles the observations reported for *L. zosterae*) and the year-round presence in both the Red Sea and the Caribbean, it is possible that the completion of the phytomyxid's life cycle might be inhibited by the decreasing ambient water temperature.

Given the fact, that Phytomyxea are obligate biotrophs and need their host to produce the environmentally resistant resting spores, we presume that the organism reached both the Mediterranean and the Caribbean Sea accompanying the invasive seagrass. The uniformity of the 18S rRNA gene sequences obtained supports this view, by our opinion. To understand, whether the phytomyxid represents any threat to the indigenous seagrass species in the invaded regions is a matter of further research and should be investigated in the nearest future.

Based on the phylogenetic analyses and the comparison to *Tetramyxa parasitica*, it is now clear that the first member of a new clade within the class Phytomyxea (at this moment labeled as "TAGIRI-5") is described here. Further clarifications of phylogenetic relations among the marine Phytomyxea representants and adjustments of their nomenclature are highly encouraged. To resolve the nomenclature of this particular organism a comparison with *Plasmodiophora halophilae* is however required first.