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A Novel Phagomyxid Parasite Produces Sporangia in Root Hair Galls of Eelgrass (*Zostera marina*)

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The objective of this study was to identify the parasite causing the formation of root hair galls on eelgrass (*Zostera marina*) in Puget Sound, WA. Microscopic and molecular analyses revealed that a novel protist formed plasmodia that developed into sporangia in root hair tip galls and released biflagellate swimming zoospores. Root hair galls were also observed in the basal section of root hairs, and contained plasmodia or formed thick-walled structures filled with cells (resting spores). Phylogenetic analyses of 18S rDNA sequence data obtained from cells in sporangia indicated that the closest relative of the parasite with a known taxonomic identification was *Plasmodiophora diplantherae* (86.9% sequence similarity), a phagomyxid parasite that infects the seagrass *Halodule* spp. To determine the local geographic distribution of the parasite, root and soil samples were taken from four eelgrass populations in Puget Sound and analyzed for root hair galls and parasite DNA using a newly designed qPCR protocol. The percent of root hairs with galls and amount of parasite DNA in roots and sediment varied among the four eelgrass populations. Future studies are needed to establish the taxonomy of the parasite, its effects on *Z. marina,* and the factors that determine its distribution and abundance.

Key words: Plasmodiophora; *Zostera marina*; Parasite; Zoospores; Root hair; rDNA.

Introduction

Seagrasses are important foundational species in coastal environments where they provide a variety of ecosystem services (Dewsbury et al. 2016; Nordlund et al. 2016): e.g., high productivity and 3-dimensional structure supports a wide diversity of organisms (Duffy 2006; Heck and Thoman 1984), carbon storage (Fourqurean et al. 2012), stabilization of sediments (Koch et al. 2009), and nutrient and pathogen filtration (Lamb et al. 2017). However, seagrasses are in decline worldwide because they are highly vulnerable to environmental stressors and anthropogenic influences in shallow coastal habitats where the majority of humans live (Grech et al. 2012; Short and Wyllie-Echeverria 1996; Waycott et al. 2009). For example, a recently published long-term study of eelgrass (*Zostera marina*) throughout Chesapeake

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Bay showed that eelgrass area has declined by 29% and that decreasing water clarity and warming ocean temperatures are likely the main drivers of the decline (Lefcheck et al. 2017). A similar long-term study of *Z. marina* in Puget Sound (Shelton et al. 2016) found that at the whole estuary scale (100s of km), eelgrass populations were stable and resilient, but significant changes had occurred at the subsite scale (0.1 km). The study did not identify any specific factors that were responsible for the changes, but a recent study suggests that environmental stressors may cause immunosuppression in eelgrass, making them more susceptible to wasting disease at specific sites (Groner et al. 2016). Wasting disease is caused by the slime mold *Labyrinthula zosterae* (Martin et al. 2016), and environmental factors (e.g., temperature, salinity, sulfide) have been shown to influence pathogenicity and virulence (Bishop et al. 2017; Olsen et al. 2014). Local changes in environmental conditions could stress eelgrass populations and lead to an outbreak of wasting disease and rapid population decline. Increased sea surface temperatures have been shown to be correlated with increased pathogen activity in marine systems (Eisenlord et al. 2016; Harvell et al. 1999; Maynard et al. 2016), and it is predicted that pathogen outbreaks will increase in the future.

Other pathogens and endobionts have also been reported for *Z. marina* and they could be involved in population declines (Sullivan et al. 2018). Recently, *Phytophthora* spp. and *Halophytophthora* sp. have been shown to be prevalent in *Z. marina* plants and seeds (99% infected) at a variety of geographic locations, and these pathogens can reduce sexual reproduction and seed germination (Govers et al. 2016). Another study (Shoemaker and Wyllie-Echeverria 2013) found 35 endophytic taxa (fungi and bacteria) living in the rhizomes of *Z. marina* in Puget Sound, but nothing is known about how these endobionts influence their hosts. A variety of recent studies have also described the microbiome of seagrasses (Cúcio et al. 2016; Ettinger et al. 2017; Ugarelli et al. 2017), but little is known about their effects on eelgrass. Symbioses involving plant hosts can change from being mutualistic to parasitic depending on environmental conditions (Johnson et al. 1997; Kniskern and Rausher 2006; Newton et al. 2010). Sediment conditions (e.g., oxygen and sulfide concentrations, salinity) in the vicinity of the rhizome can vary dramatically over small spatial and temporal scales and may be important stressors causing microbes to alter their interactions with their hosts (Sullivan et al. 2018).

A variety of phytomyxean parasites have been reported from seagrasses (Neuhauser et al. 2012). For example, *Plasmodiophora bicaudata,* has been reported to form hypertrophied galls on the shoots of *Zostera* spp. and reduce root growth and increase the chances of plants being uprooted (den Hartog 1989; Karling 1968). Very little is known about this pathogen, but it is thought to have a worldwide distribution (den Hartog 1989). It has been reported from *Z. japonica* in British Columbia, Canada, but whether it occurs in *Z. marina* at other locations is unknown. The class Phytomyxea includes parasites that have major effects on terrestrial crops and are vectors for the transmission of viruses that cause diseases in plants (Bulman and Braselton 2014; Neuhauser et al. 2014; Sullivan et al. 2018). Very little is known about phytomyxeans because their characteristics of small size, complex life cycle, and obligate association with their host, making them difficult to observe and identify morphologically and nonculturable. There have been no extensive marine biodiversity studies of this group (Neuhauser et al. 2011), and information is lacking on their distribution, abundance, and effects on hosts in the marine environment. Clearly more research is needed to examine the diversity of seagrass parasites (Neuhauser et al. 2011, 2014; Sullivan et al. 2018), especially those living in plant structures such as the roots and rhizomes that are in direct contact with protists and other microbes living in the sediments.

As part of a study of *Z. marina* and rhizosphere microbes in Commencement Bay, Washington (Elliott et al. 2006), we observed that some of the root hairs of plants had enlarged tips that looked like galls (Fig. 1B–D). To the best of our knowledge, these root hair galls have not been described in the literature for *Z. marina*. In this study, we describe normal root hairs of *Z. marina* and root hairs with galls (Fig. 1A,B) using light microscopy, scanning electron microscopy, and epifluorescence microscopy. To determine the identity of the cells observed in root hair galls we used standard DNA barcoding protocols and qPCR. Our results indicate that a novel phagomyxid parasite forms plasmodia in root hair cells that develop into sporangia in galls at the tips of root hairs and resting spores in galls at the base of root hairs. A qPCR protocol was developed for detecting and quantifying this organism. We provide the first description of this protist parasite in *Z. marina*, and quantify the occurrence of root hair galls and the prevalence of the phagomyxid in eelgrass beds at four locations that represent 4

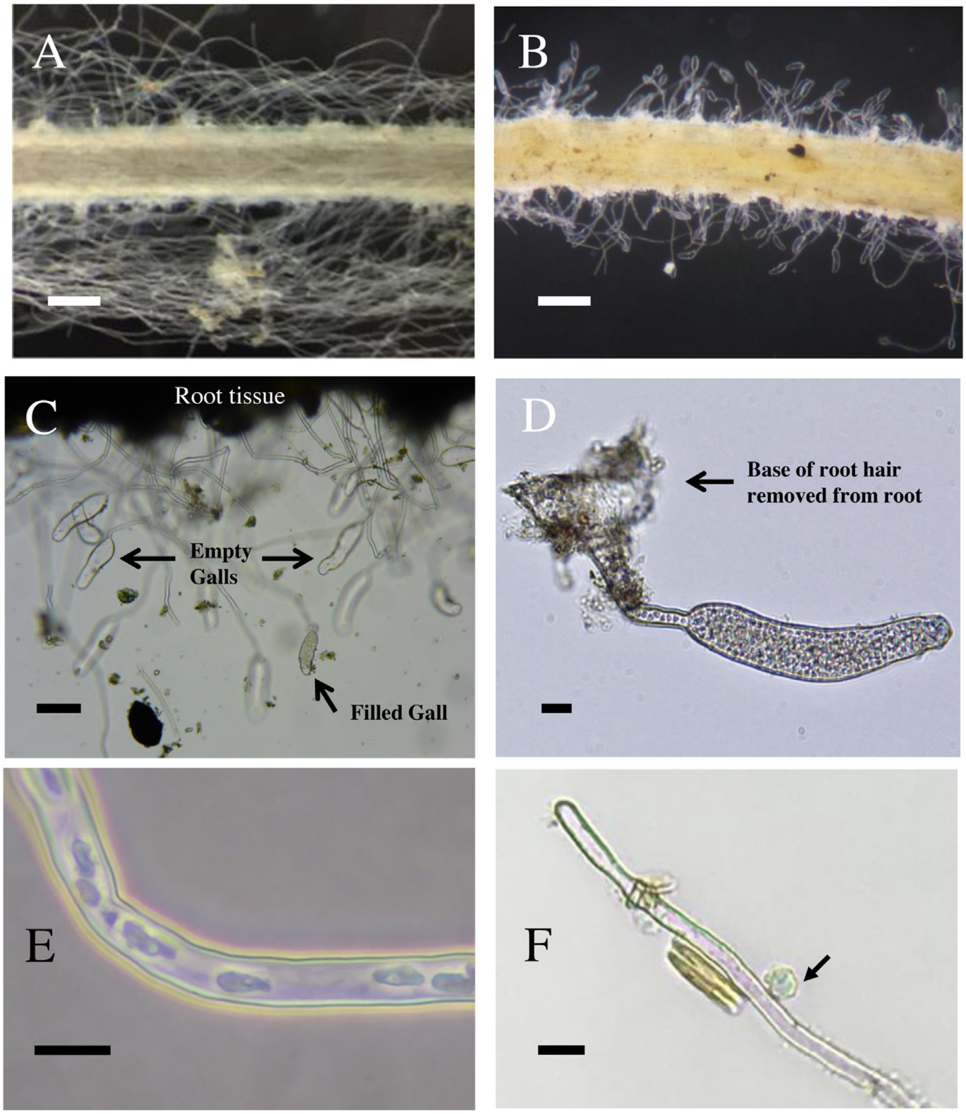

Figure 1. Typical eelgrass root with normal root hairs (**A**), root with root hair tip galls (**B**), root hair tips with empty and filled galls (**C**), root hair removed from root with filled gall (**D**), zoospores moving in root hair (**E**), and light microscopy of Trypan blue stained root hair with attached zoospore (arrow) (**F**). Scale bars: A–B are 250 µm, C is 50 µm, D–F are 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

geographic regions of Puget Sound (south, central, north, and Hood Canal) (Fig. 2).

Results

Microscopy

Galls were commonly observed on the tips of root hairs of *Z. marina* (Fig. 1) collected from Ruston Way and Chambers Bay (>50% of root hairs), but they were found in significantly lower abundance (<25% of root hairs) on the roots from Lynch Cove and Padilla Bay (Kruskal–Wallis test, Chi-squared = 50.045, df = 3, $p < 0.0001$, Fig. 3A). There was significant variation in the number of root hair tip galls observed on different roots, with some roots on the same rhizome having many root hair tip galls and other roots with few root hair tip galls or none at all. There was an average of 6.6 root hairs per mm of root (±2.7 SD, n = 131), with a relatively even distribution of root hairs along the root (C.V. = 0.41), but no root hairs within a few cm of the growing root tip.

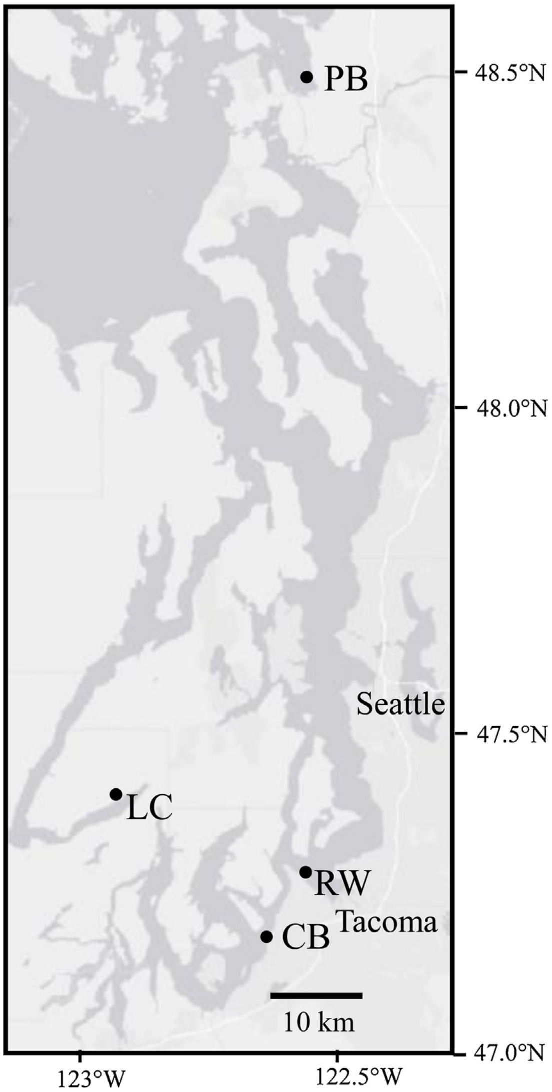

Figure 2. Map of study locations in Puget Sound. Ruston Way (RW), Chambers Bay (CB), Padilla Bay (PB), and Lynch Cove (LC).

Root hair tip galls occurred at an average density of 0.92 per mm of root (±1.37 SD, n = 131), and they had a clumped distribution. Root hair tip galls typically occurred in clusters at random locations along a root (C.V. = 1.49). Root hair tip galls were commonly found on relatively short root hairs (Fig. 1B) and they varied in size and shape from small spherical structures to large allantoid-shaped structures (Figs 1 and 5) with an average length (124.7 μm ± 55 SD, n = 199) and width (29.6 μm ± 5.3 SD, n = 199).

Most of the root hair tip galls were transparent and devoid of cellular material (empty), but a small percentage (<1%) of the galls were filled with cellular material (filled) (Figs 1 and 5). When roots were left in a petri dish with seawater at room temperature, the filled root hair tip galls developed a mass of swimming zoospores after a few hours (Supplementary Material Videos 1, 2). The zoospores typically began moving in the root hair tip galls and then within a few more hours they eventually moved down the root hair (Fig. 1E, Supplementary Material Video 3) using amoeboid locomotion or swimming using their two flagella. Eventually the zoospores reached the base of the root hair cell, where they swam out into the surrounding water if the root hair was removed from the root (Fig. 1D, Supplementary Material Video 4). For attached root hairs, we observed zoospores in the surrounding water, but are unsure of the exact mechanism employed by the zoospores to exit the root. Light microscopy and SEM did not reveal any distinct openings in the root hair tip galls for the zoospores to escape (Fig. 4A,B) . However, broken root hairs and galls were observed on plants and they could provide a route for zoospores to exit the roots (Fig. 4C,D). Observations of zoospores (diameter = 4.15 μm ± 0.45 μm, N = 12) showed that they swam to the tissues of the plants where they appeared to encyst on the root hairs (Fig. 1F) and epidermal cells.

When roots were stained with Trypan blue, the cells in the filled root hair galls stained dark blue but the empty root hair galls did not stain (Fig. 5A). Trypan blue is known to stain chitin, which indicates that the inner wall of these root hairs was parasite material and the outer wall that was not stained was root material. SEM images of root hairs with galls showed that they were double-walled (Fig. 4D), which was also observed in some broken root hairs (Fig. 4C). Root hairs with galls had enlarged basal cells, and the material in some of these cells stained dark blue (Fig. 5D). Staining of fixed plant material with DAPI showed many nuclei in filled root hair tip galls (Fig. 5C) and in the basal part of the root hair cells that had filled galls (Fig. 5E). These cells could be zoospores or multinucleate plasmodia.

Plasmodia with distinct vacuoles inside of their cells (arrow) were observed in the basal part of some root hair cells (Fig. 6A) . DAPI staining revealed that the plasmodia were multinucleate (Fig. 6B). The basal part of some root hair cells contained thick-walled structures that typically filled the base of the root hairs (average length = 41 μm ± 10.5 SD, n = 11; width = 26.1 μm ± 4.5 SD, n = 11) and they contained individual cells. The cells were on average 3.46 μm ± 0.34 SD in diameter, n = 14 (Fig. 6C) and they stained with DAPI (Fig. 6D). The thick-walled structures were very resistant to degradation and were observed in decaying eelgrass root samples and root hairs that had detached from the roots (Fig. 6E–H). We interpret these structures to be resting spores because of their thick walls and resistance to degradation over time in samples that were kept for over 6 months. We observed some of the thick-walled structures to be empty with openings in them (Fig. 6H), but did not observe the release of zoospores.

Molecular Phylogenetic Analyses

PCR using 18S primers with cells from filled root hair tip galls resulted in a 1772 bp PCR product. A BLAST search of the consensus sequence for the 18S gene indicated that it was most similar (92.6%) to the environmental clone sequence NAMAKO-19 (AB252759) that was isolated from a sediment core taken at 22 m in an anoxic location in a saline meromictic lake on the shore of Kamikoshiki Island, Japan (Takishita et al. 2007b). A phylogeny constructed with various protist sequences used in other phylogenetic studies of phagomyxids (Murúa et al. 2017; Neuhauser et al. 2014) showed that the root hair gall 18S sequence clustered in a well-supported clade with other phagomyxids (Fig. 7)

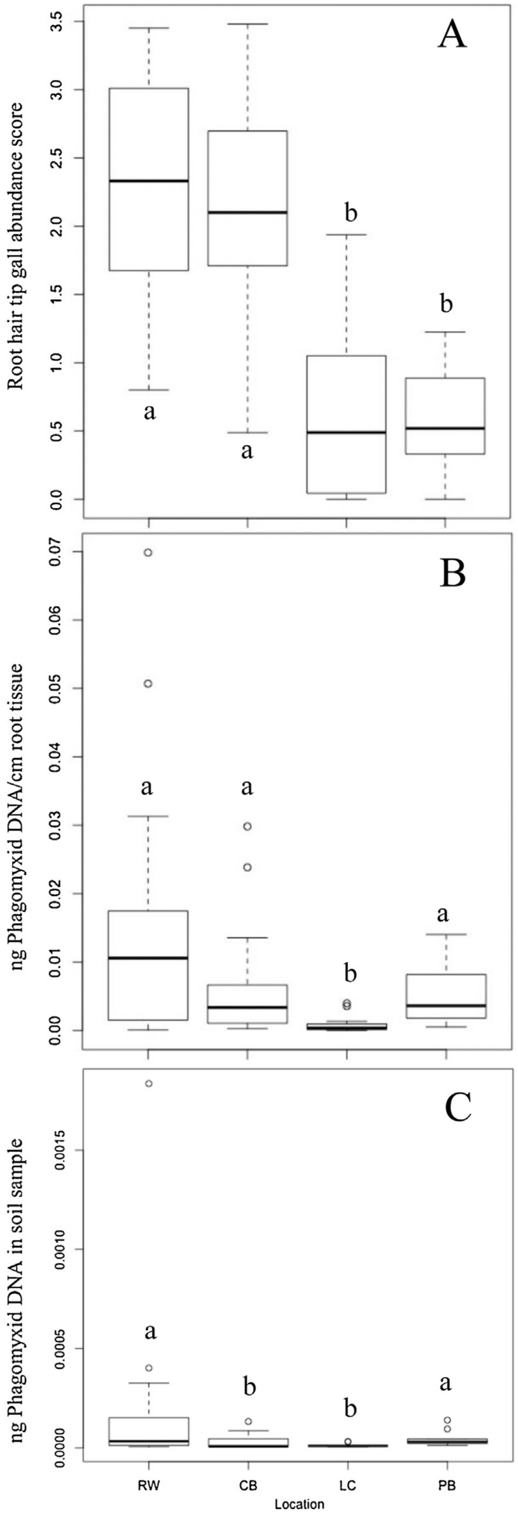

Figure 3. (**A**) Box plot for root hair gall abundance on 40 cm root samples (20 samples per location, rating from 0–4 for the percent root hairs with galls: 0 = no galls, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) from 4 locations (RW = Ruston Way, CB = Chambers Bay, LC = Lynch Cove, PB = Padilla Bay). Variances were unequal among locations, and a Kruskal-Wallis test indicated there were significant differences in root hair gall abundance score among locations (Chi-squared = 50.045, df = 3, $p < 0.0001$), and significant differences in paired comparisons indicated by different letters (Dunn test for multiple comparisons). (**B**) Box plot of the amount of phagomyxid DNA per cm root tissue in root samples (20 per location) from four different locations determined using qPCR. Variances were unequal among locations, and a Kruskal–Wallis test indicated there were significant differences among locations (Chi-squared = 25.645, df = 3, $p < 0.0001$). Significant differences among locations are indicated with letters (Dunn test for multiple comparisons). (**C**) Box plot of the amount of phagomyxid DNA in 0.25 g samples of sediment collected in rhizosphere from four different locations determined using qPCR. Variances were unequal among locations, and a Kruskal–Wallis test indicated there were significant differences among locations (Chi-squared = 9.86, df = 3, $p < 0.02$). Significant differences among locations are indicated with letters (Dunn test for multiple comparisons).

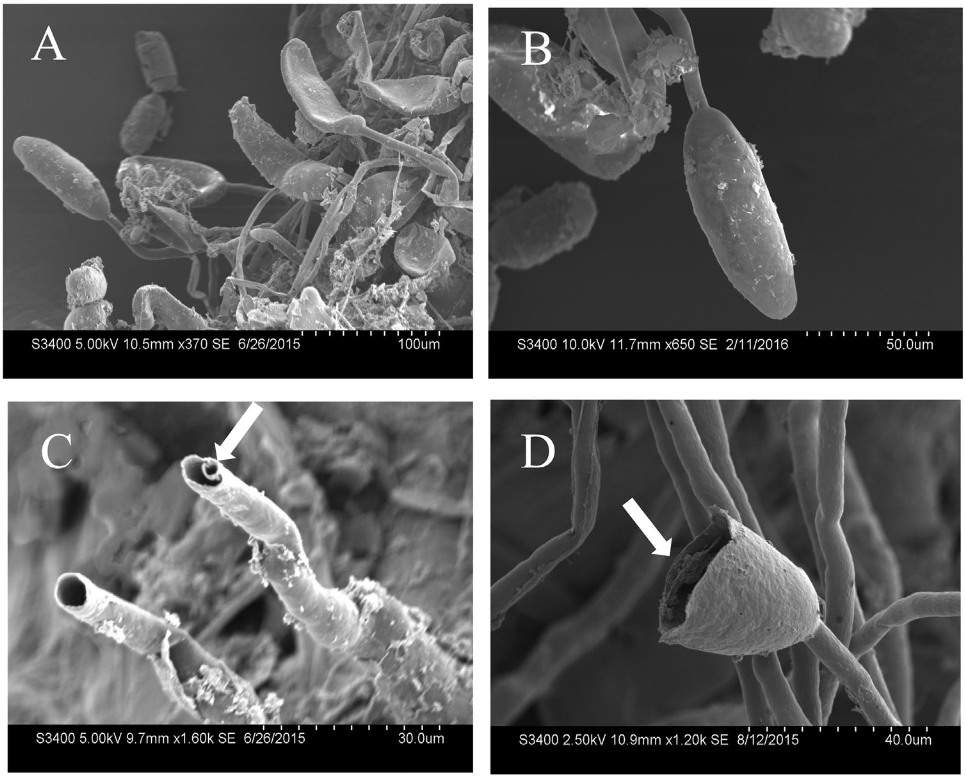

Figure 4. Scanning electron microscope images of many root hair tip galls (**A**), single root hair tip gall (**B**), broken root hairs with arrow showing double-wall where inner layer is parasite and outer layer is root tissue (**C**), broken root hair tip gall with arrow showing double-wall (**D**).

, and specifically with the environmental clade “Tagiri4” (Murúa et al. 2017) with members from anoxic marine sediments in Japan (Takishita et al. 2005, 2007a, 2007b). The topology of the phylogenetic trees constructed were similar to those in other recent studies of phagomyxids (Murúa et al. 2017; Neuhauser et al. 2014). Other well supported clades in the phylogeny were for two species of *Maullinia* that parasitize bull kelp (Murúa et al. 2017), and *Phagomyxa bellerocheae* and *P. odontellae*, parasites of diatoms (Schnepf et al. 2000).

The closest relative of the root hair gall parasite with a known taxonomic identification was *Plasmodiophora diplantherae* (86.9% similarity) which infects the seagrass *Halodule* (Walker and Campbell 2009). The Bayesian analysis showed weak support for this species being included in a clade with the root hair gall 18S sequence and “Tagiri4” environmental sequences (Fig. 7), but this was not strongly supported in the PhyML or RaxML analyses (Supplementary Material Figs S1, S2). The 18S sequence for *Plasmodiophora diplantherae* and some of the other species in the phylogeny presented by Murúa et al. (2017) are relatively short (<831 nt), which reduced the phylogenetic signal. When only species with 18S sequences >1664 nt were included in the phylogenetic analysis, there was exceptionally strong support for all of the major clades in the Phagomyxida identified in Fig. 7 using all tree building methods (Supplementary Material Fig. S3).

PCR using ITS primers with cells from filled root hair galls resulted in 399 bp PCR products. A BLAST search of the ITS consensus sequence indicated that it was most similar (92.8%) to *Phagomyxa bellerocheae* (AF310906) and *P. odontellae* (AF310905), two of the few phagomyxids with ITS sequences in the database. The 5.8S rRNA region for the gall parasite was similar to that of these two *Phagomyxa* species (95.6% similar), but the ITS1 and ITS2 regions were very different (46.5% similarity). A phylogeny produced with sequences of the 5.8S rRNA region with other related protist species (Murúa et al. 2017; Neuhauser et al. 2014), indicated that sequences from the gall parasite were in a clade with other phagomyxids (Supplementary Material Fig. S4).

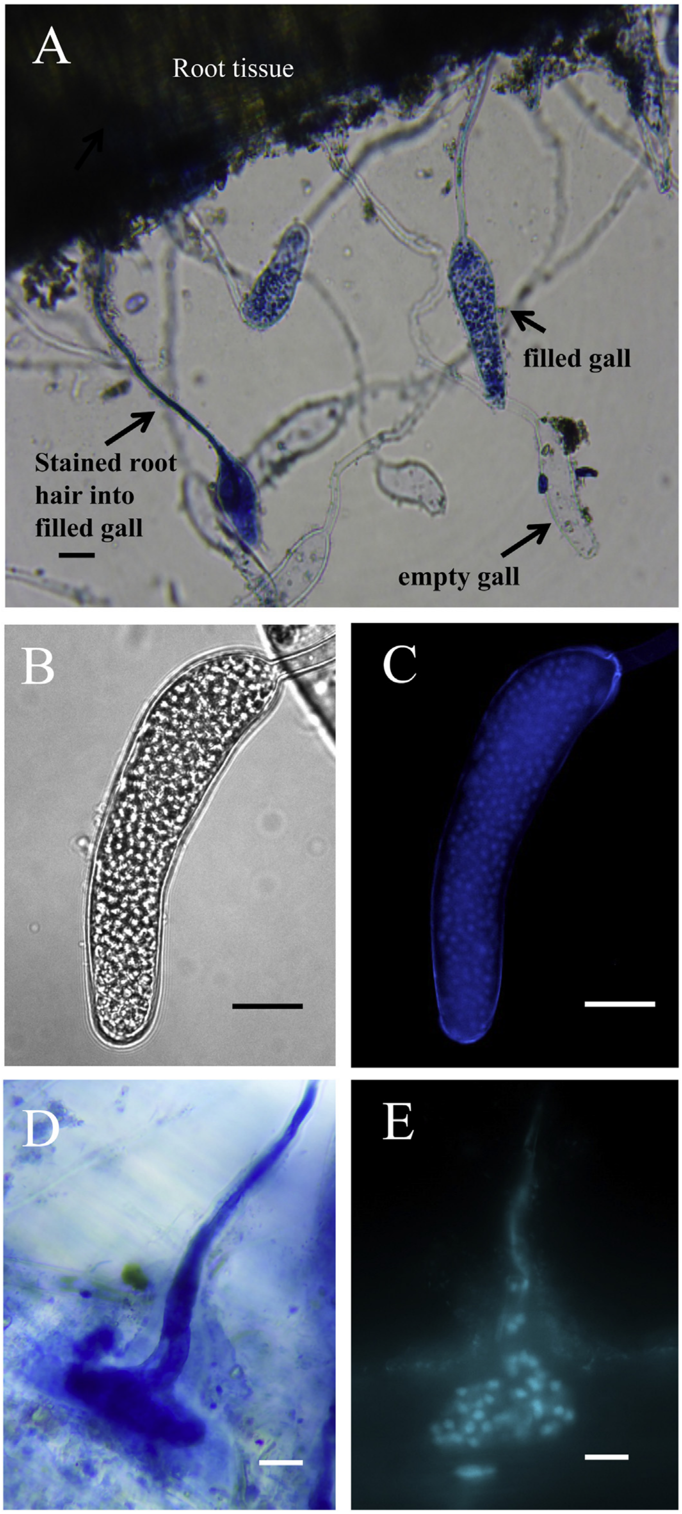

Figure 5. Brightfield image of Trypan blue stained root with filled and empty root hair tip galls (**A**), bright field image of DAPI stained filled root hair tip gall (**B**), UV epifluorescence image of DAPI stained filled root hair tip gall showing nuclei (**C**), Brightfield image Trypan blue stained root hair showing plasmodia in base and extending up into the root hair tip gall (**D**), UV epifluorescence microscopy image of DAPI stained root hair showing staining from base up into the root tip gall (**E**). Scale bars: A–C are 20 µm, D-E are 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

qPCR of Samples from Different Locations

A dilution series of the phagomyxid 18S PCR product (1772 bp) described above resulted in a standard curve with an $R^2 > 0.99$, efficiency = 98%, and a melt curve with a single peak. Sequencing of the qPCR products from the Ruston Way samples resulted in the expected phagomyxid DNA sequence, confirming that a single product was amplified in the qPCR reaction. DNA from root samples taken at all sites resulted in positive amplifications, indicating that amplification was not inhibited by products in the DNA extractions. This was verified by independent qPCR analyses of a plant internal control (CoxI) (Bilodeau et al. 2014) that resulted in similar C_t values for samples from the different sites (One-way ANOVA, $F = 1.107$ $df = 3,16$, $p = 0.375$).

A standard curve was run with each qPCR analysis and was used to calculate the amount of phagomyxid DNA in each root and soil sample. The amount of phagomyxid DNA varied greatly among root samples from the same location (Fig. 3B), but there was significantly higher amounts in root samples from Ruston Way, Chambers Bay and Padilla Bay than Lynch Cove (Kruskal–Wallis test, Chi-squared = 25.645, $df = 3$, $p < 0.0001$). For all 80 samples, there was a significant correlation between the amount of phagomyxid DNA in each root sample and the number of root hair galls observed on the roots (Spearman rank correlation, $rho = 0.304$, $p = 0.007$). This reflected the variation among the four sites. However, when data from each of the four sites ($n = 20$) was analyzed independently, there was no relationship between the amount of phagomyxid DNA in each root sample and the number of root hair galls observed ($p > 0.05$).

The individual empty root hair tip galls tested in qPCR assays did not have any measureable amount of phagomyxid DNA in them. However, individual filled root hair tips galls run in qPCR assays always had a measureable amount of phagomyxid DNA (mean = 4.27×10^{-4} ng/µl, SD = 4.73×10^{-4}, $n = 5$ each), and one filled root hair gall had as much 12% of the DNA present in a typical sample from Ruston Way (comprised of 40 cm of root with at least hundreds of root hair galls).

Soil samples collected near the roots of eelgrass had very low levels of phagomyxid DNA in comparison to roots (Fig. 3C). The highest levels of phagomyxid DNA were in soil samples from Ruston Way, and there were significant differences among sites (Kruskal–Wallis test, Chi-squared = 9.86, $df = 3$, $p = 0.02$), with Ruston Way and Padilla Bay having higher levels of phagomyxid DNA than Chambers Bay and Lynch Cove.

Discussion

This study used a combination of microscopy and modern molecular techniques to show that *Z. marina* was infected with a novel protist parasite that produces plasmodia and eventually sporangia in root hair tip galls and thick-walled structures (i.e., resting spores) in root hair base galls. The sporangia released biflagellate zoospores, indicating that they were a type of eukaryotic protist. Molecular analysis of cells from filled root hair tip galls using 18S and ITS gene sequences identified them as a unique member of the Class Phytomyxea, which is a group of obligate endobiotic parasites that are part of the eukaryote supergroup Rhizaria (Bulman and Neuhauser 2016; Neuhauser et al. 2014). The Phytomyxea is comprised of two orders, the Plasmodiophora, which are a group of well-known parasites of land plants and oomycetes, and the Phagomyxida, a very poorly known group of marine parasites of angiosperms, diatoms and brown algae, as well as morphologically uncharacterized environmental sequences from benthic marine sediments (Neuhauser et al. 2014; Sullivan et al. 2018). The plasmodiophorids have been intensively studied because they are parasites of crops, and cause diseases that result in major losses for brassicas, potatoes, and grain crops such as maize, rice, and wheat (Neuhauser et al. 2014). They are also known to serve as vectors for viruses that cause diseases of plants. The Order Phagomyxida is a monophyletic group comprised of five species that are biotrophic parasites (they do not kill their host) of seagrasses, and algae including diatoms. In addition, there are two clades containing environmental sequences isolated from anoxic marine sediments (Fig. 7).

Molecular Phylogenetic Analyses

The 18S gene sequence of the parasites in the root hair tip galls was found to be most closely related to members of the Environmental clade “Tagiri 4” (Murúa et al. 2017), a well-supported clade in the 18S molecular phylogeny (Fig. 7). The most closely related member to the root hair gall parasite sequence was environmental sequence Namako-19 (92.6% similar) from clone libraries of DNA extracted from sediments (Takishita et al. 2007b), which has not been linked to morphologically char-

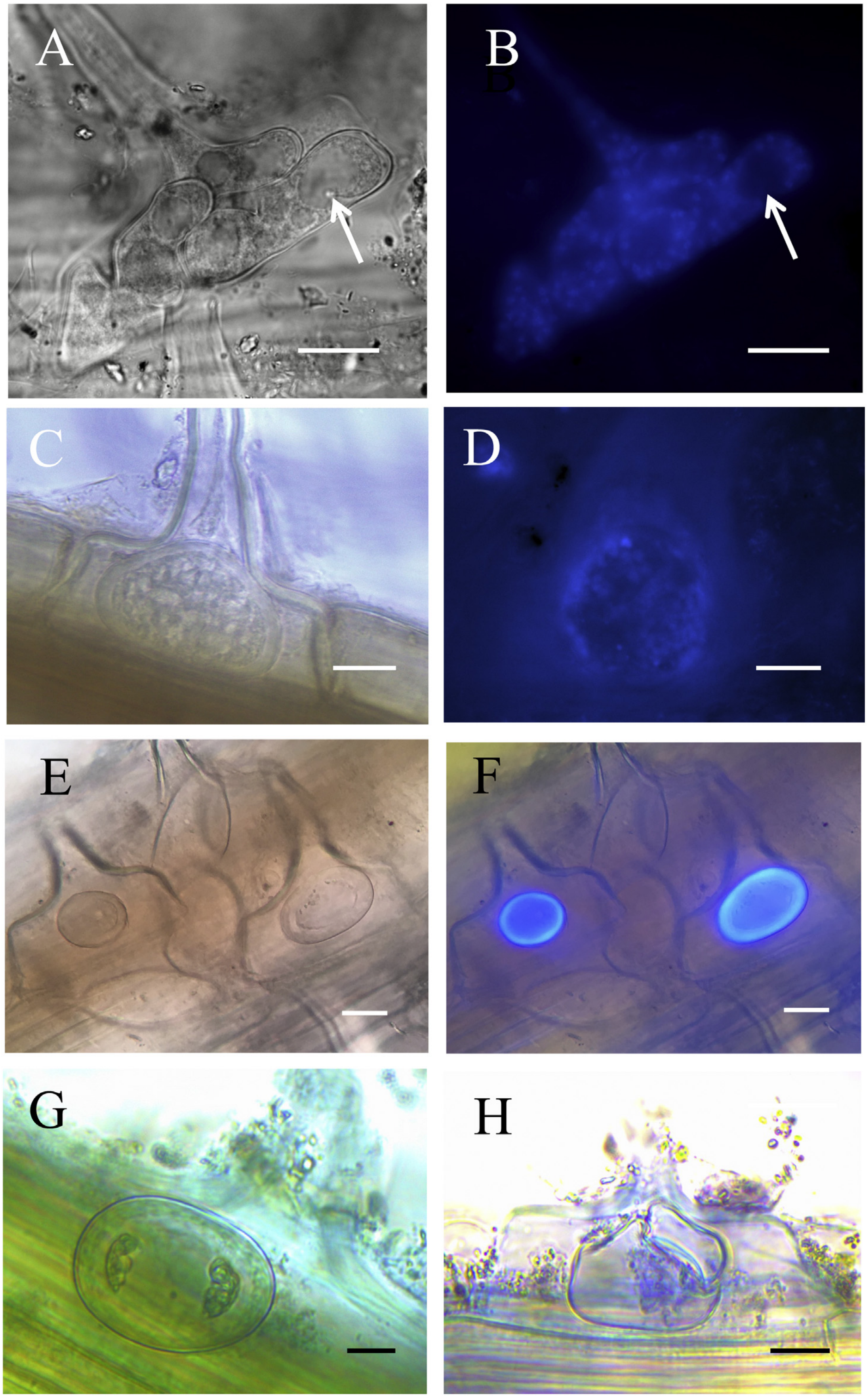

Figure 6. Brightfield image of root hair base filled with multinucleate plasmodium that contains large vacuoles (arrow) (**A**), UV epifluorescence microscopy of root hair base filled with multinucleate plasmodium showing brightly stained nucleii around vacuoles (arrow) (**B**), Brightfield image of root hair base showing thick-walled structure filled with cells (**C**), UV epifluorescence microscopy of root hair base with thick-walled structure (**D**), Brightfield microscopy of thick-walled structures in basal part of root hair cells (**E**), a merged image of brightfield

acterized DNA-lineages or to any particular host species. We hypothesize that the root hair gall parasite has a close affinity to this morphologically uncharacterized DNA lineage because members of the Tagiri 4 clade are related parasites infecting seagrasses in Japan. All of the study sites where sediment samples were collected in Japan were either in marine lakes or nearshore areas where infected plant material from shallow waters could sink and decompose on the bottom (Takishita et al. 2005, 2007a, 2007b). The plant material could either be in a state of decomposition and contain the parasite, or resting spores of the parasite could remain in the sediments at the site for long periods of time. The sites were all anoxic, which could assist in reducing the rate of decomposition. We found evidence of root hair gall parasite DNA in sediment samples taken near the roots of eelgrass, which suggests that phagomyxid zoospores or resting spores can be present in sediments. Further research in seagrass beds and shallow water habitats near the collection sites in Japan may provide an answer to whether members of the Tagiri 4 clade are related parasites infecting seagrasses in Japan.

The most closely related described species to the root hair gall parasite of *Z. marina* is *Plasmodiophora diplantherae* (86.9% similarity) which infects the tropical seagrass *Halodule* (Walker and Campbell 2009). This species produces large galls on the shoots of *Halodule* and has spherical resting spores that are 4 μm in diameter. The low 18S sequence similarity and different morphological characters of *P. diplantherae* suggests that the root hair gall parasite of *Z. marina* is not a closely related species. The only other described species in the same genus, *Plasmodiophora bicaudata,* forms hypertrophied galls on the shoots of *Zostera* spp. and has ovoid or slightly spindle-shaped resting spores (6–7 μm by 3–4 μm) with characteristic filiform appendages (den Hartog 1989; Karling 1968). *P. bicaudata* has been reported from *Z. japonica* in British Columbia, Canada, but no DNA sequence data is available for this species. Given that the galls of *P. bicaudata* have been described on the shoots of *Zostera* spp. (not the roots) and the resting spores are different in size and shape, suggests that the root hair gall parasite described here is a different species. DNA sequence data is needed from galls of plants exhibiting morphological characteristics described for *P. bicaudata* in order to determine its relationship to the root hair gall parasite described here.

It is unknown whether the root hair gall parasite of *Z. marina* is an introduced species from another host and/or geographic area that evolved the ability to infect *Z. marina*. Studies of this symbiosis from other geographic locations and with other seagrass species (e.g., *Z. japonica)* may provide an answer to this question. Phytomyxids are known to have the ability to infect a wide variety of hosts, and host shifts are suggested to be a major cause of speciation (Neuhauser et al. 2014). The root hair gall parasite may infect other species of marine hosts and have shifted to *Z. marina* over evolutionary time. Further work on the phagomyxid parasites of other seagrasses and potential hosts (e.g., algae, diatoms) is needed to address this question.

Microscopic Observations and Life History

Traditionally, the taxonomy of plasmodiophorids is based on morphological characteristics of resting spores and the species of host plant (Bulman and Braselton 2014). Morphological observations of the root hair gall parasite and its effects on *Z. marina* roots were consistent with the life history of other Phytomyxea (see diagrams of life cycles in Neuhauser et al. 2014; Bulman and Neuhauser 2016). These organisms have a complex biphasic life history with primary (sporangial) and secondary (sporogenic) zoosporic stages. Each stage produces zoospores that infect their hosts and produce multinucleate plasmodia inside host cells. The primary plasmodia produce zoosporangia and the secondary plasmodia produce resting spores. Both stages release biflagellate zoospores. Given this general life history, the filled root hair tip galls observed in this study are likely the result of primary plasmodia in root hair cells that produce thin-walled zoosporangia in galls at the tips of the root hairs. Plasmodia of other phytomyxeans are known to cause host tissues to hypertrophy or cells to swell in order to create space for the for-

and UV epifluorescence microscopy of Calcofluor white stained root showing cyst-like structures in basal part of root hair cells (**F**), Brightfield image of Trypan blue stained thick-walled structure in basal part of root hair cell (**G**), Brightfield image of Trypan blue stained thick-walled structure in basal part of root hair cell (**H**). Scale bars: A–B and E–F are 20 μm, C–D and G–H are 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

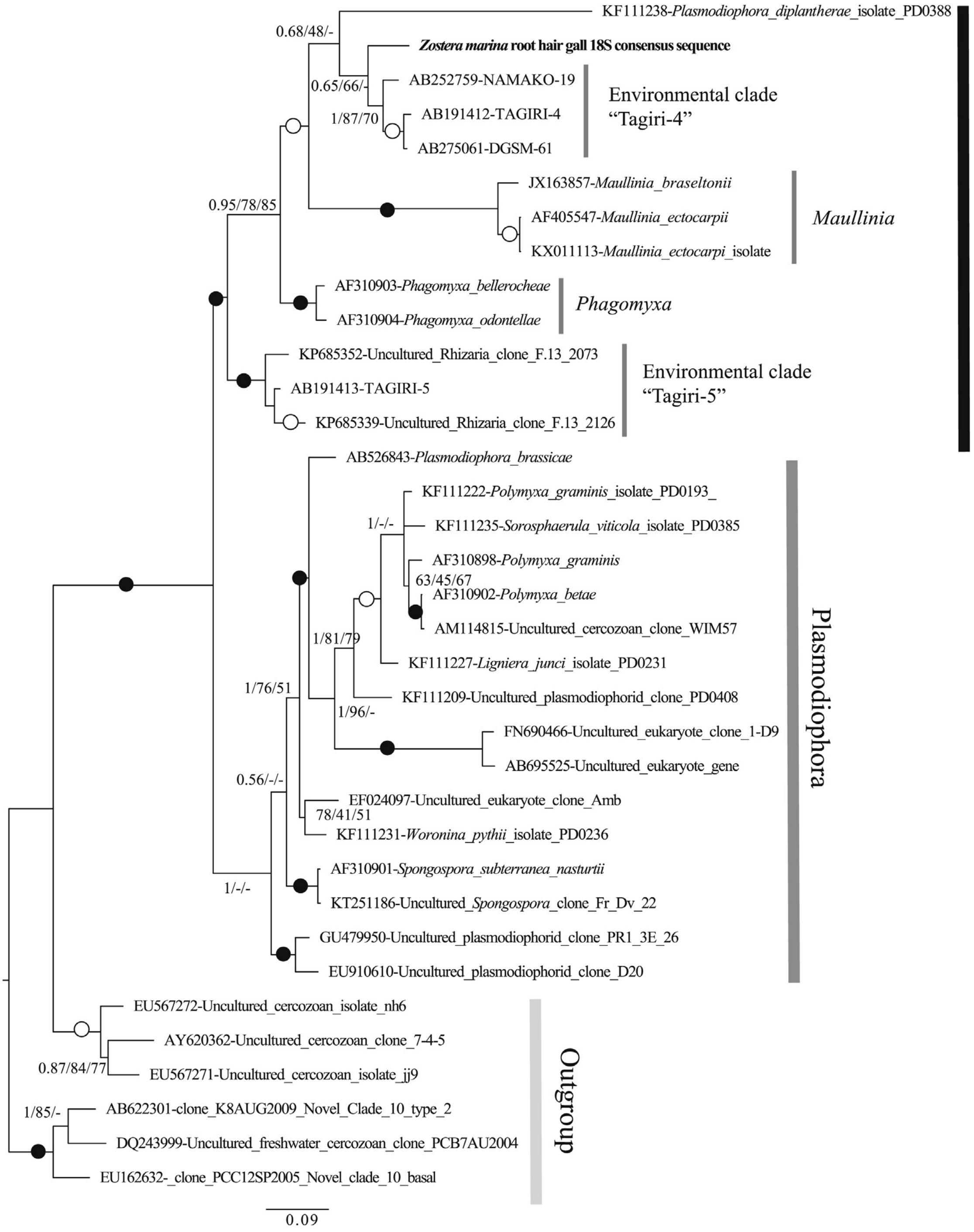

Figure 7. Bayesian analysis of 18S rDNA sequences of Phytomyxea and outgroup taxa (based on Neuhauser et al. 2014 and Murúa et al. 2017), showing placement of *Zostera marina* root hair tip gall 18S consensus sequence. Alignment length of 1860 nt, 35 sequences, 1329 informative sites. MrBayes run parameters: Generations 1,000,000, sample frequency 10, burnin 25%. Scale bar indicates number of substitutions per site and numbers at nodes are posterior probabilities (MrBayes)/bootstrap support (PhyML)/bootstrap support (RaxML). Filled circles indicate 1/100/100. Open circles indicate 1/100/ > 96.

mation of zoosporangia (Neuhauser et al. 2011). Other species of plasmodiophorids (e.g., *Plasmodiophora brassicae*, *Ligneria* spp.) commonly infect the root hairs of plants and cause hypertrophies, but DNA sequence data indicates that they are in the Plasmodiophorida lineage and not the Phagomyxida lineage (Fig. 7). The phagomyxid root hair gall parasite described here is unique in causing distinct hypertrophies in root hair tips of *Z. marina* that contain thin-walled sporangia. Trypan blue staining and electron microscopy demonstrated that the sporangia were separated from the plant cell wall by a single membrane containing chitin. This is characteristic of some members of the Phagomyxida (Braselton and Miller 1975).

The secondary zoospores released from the root hair tip galls were biflagellate, as is known for phagomyxids. The free-swimming zoospores attached to the root hairs and epidermal cells of *Z. marina* where they appeared to retract their flagella and form an encystment structure. Secondary zoospores infect the cells of plants and result in the formation of plasmodia. We observed lobose plasmodia and thick-walled spherical structures in the basal part of root hair cells that were likely infected by secondary zoospores. The plasmodia had internal lipoidal globules that are considered to be characteristic of sporogenic plasmodia (Miller and Dylewski 1983). These plasmodia likely develop into the thick-walled structures that we observed containing individual cells (Fig. 6). We consider the thick-walled structures to be resting spores, given that they contain cells and are thick-walled and resistant to degradation. We observed the resting spores in decomposing plants, and the cells may germinate and be released from the root hairs when they detach from the root in the sediment or in plants that have been uprooted and are transported to a different location. Resting spores allow the parasite to persist under adverse conditions in the marine environment and they could disperse with currents in uprooted plant material. We observed resting spores that appeared to be ruptured that did not contain cells, which suggests that the thick walls of resting spores eventually break open releasing the cells. Further research is needed to demonstrate that the resting spores produce zoospores and investigate the environmental conditions under which this process occurs. Very little is known about the factors that determine whether a plasmodium develops into resting spores or sporangia (Bulman and Braselton 2014), and studies of eelgrass roots may be useful in examining this question.

Traditionally, the primary morphological traits used to identify phytomyxids were the morphological characteristics of the resting spores (size, shape, arrangement), but molecular phylogenetic studies have radically altered the placement of some groups (Bulman and Neuhauser 2016). For example, DNA sequence data for the seagrass parasite *Plasmodiophora diplantherae* demonstrated that it is a member of the Phagomyxida and not the Plasmodiophorida (Neuhauser et al. 2014). Resting spores have not been observed in the phagomyxid parasites of diatoms (*Phagomyxa* spp.) (Schnepf et al. 2000), and molecular phylogenetic data have shown that they are a unique group of phagomyxid parasites. *Plasmodiophora diplantherae* has been described as having globose resting spores of 4–4.6 μm in diameter, and the thick-walled structures observed in this study contain cells of approximately the same size, but multiple cells are contained in one thick-walled resistant structure. The resting spores of phytomyxeans are known to vary in morphology and arrangement in different host species, and the unique thick-walled structures observed in *Z. marina* may be a response to this particular host. The parasite is most similar morphologically and ecologically to members of the genus *Ligneria*, which are characterized by resting spores that are indefinite in size, shape, and structure. Resting spores can occur in cylindrical solid masses or hollow aggregates. Members of this genus infect grasses and plasmodia cause enlargement of root hairs and result in sporangia and groups of resting spores. However, Karling (1968) suggests that the genus *Ligneria* is “scarcely more than a convenient dumping ground for species which cause little or no hypertrophy and develop cystosori of indefinite shape, size, and structure.” Also, *Ligneria* is a member of the order Plasmodiophorida and not the Phagomyxida. Thus, the placement of the eelgrass parasite within an existing genus of the Phagomyxida is problematic, and further taxonomic work on the parasite and other members of the Phytomyxea is needed to firmly establish its taxonomic position within the Phagomyxida.

We assume that primary zoospores are released from the thick-walled resting spores and they would go on to infect plants and cause the formation of plasmodia that would lead to the development of root hair tip galls. We observed most filled root hair tip galls in the spring, which suggests that this is when these infections primarily occur. The thick-walled structures in the base of root hairs were mostly observed in plants collected in the fall. Further quantitative work is needed to characterize the full life history of this phagomyxid parasite to determine if there are any seasonal patterns in the formation of sporangia and resting spores. Whether this parasite is host specific to *Z. marina* is unknown, and its life history may involve infection of other organisms (e.g., *Z. japonica*). Relatively few studies have examined interactions of phagomyxids with alternative hosts (Neuhauser et al. 2014), and surveys of other plants and algae in Puget Sound are needed to examine this question.

Distribution and Abundance

At the scale of the population, the number of copies of phagomyxid DNA in eelgrass roots varied among the four study sites (Fig. 3). Our results indicate that the differences in phagomyxid DNA concentrations among sites was not a result of amplification bias. We used a DNeasy Powersoil DNA extraction kit (Qiagen) on our samples, which is designed to eliminate PCR inhibitors present in soils and root tissue. Phagomyxid DNA was detected in every sample analyzed using qPCR from the four study sites, which indicates that there was successful amplification. In addition, an independent qPCR analysis using CoxI plant control primers (Bilodeau et al. 2014) verified that the plant DNA in the samples from the different sites was equally amplifiable. Thus, we conclude that the relatively low phagomyxid DNA concentrations from sediment samples and root samples at the Lynch Cove site were not the result of PCR amplification inhibition by components in DNA extractions.

The number of copies of phagomyxid DNA in eelgrass roots was highest in plants from locations that had a high percentage of galls (>50%) on their root hair tips (Ruston Way, Chambers Bay). However, parasite DNA concentrations were also high in plants from Padilla Bay that had relatively few root hair tip galls (<25%). The lowest parasite DNA concentrations occurred in plants from Lynch Cove that had few root hair tip galls (<25%). This suggests that the presence of the parasite is related to the formation of root hair galls in eelgrass, but other factors are involved. We found that filled root hair tip galls had high concentrations of phagomyxid DNA in qPCR analyses, whereas phagomyxid DNA was not detected in empty root hair galls. Phagomyxid DNA concentrations in filled root hair galls were up to 12% of the average concentration in samples from Ruston Way. Thus, it may only take a few filled root hair tip galls on a root to cause large increases in phagomyxid DNA concentrations. We observed filled root hair tip galls most frequently on the roots of plants from the Ruston Way site, which may explain why some plants at this site had very high phagomyxid DNA concentrations. This may also explain why the plants at Padilla Bay with few root hair galls had high concentrations of phagomyxid DNA if only a few of the galls were filled in some samples. Alternatively, the phagomyxid may also be infecting the basal part of the root hair cells and forming resting spores, or infecting other cells in the plant roots that did not result in the formation of root hair tip galls. Further research using qPCR and fluorescence *in situ* hybridization (FISH) should be conducted to demonstrate the location of the parasite in different plant tissues (e.g., epidermal and cortical cells of roots, rhizome and shoot).

There are many other potential causes of the geographic variation in root hair gall abundance at the different locations. It may be related to the resistance of the different eelgrass populations to the parasite or to environmental differences among the four locations. There may be differences in genetic variation of eelgrass populations among the different locations that could provide resistance to the parasite and formation of galls. A variety of studies have demonstrated that *Z. marina* populations vary in their genetic structure at small spatial scales (Kim et al. 2017; Talbot et al. 2016), but we are not aware of studies in Puget Sound that show there is significant genetic variation among the four populations studied. A major environmental difference among the four locations in this study is the grain size of the sediment, with the Ruston Way and Chambers Bay sites being coarse sand and Padilla Bay and Lynch Cove being mud. This could influence a variety of other environmental variables (e.g., oxygen, sulfide, organic content) that may affect the host-parasite relationship or bacterial community living in the rhizosphere (Cúcio et al. 2016). Further observational studies and experiments need to be conducted to examine the genetics of host eelgrass plants and associated environmental variables (e.g., temperature, salinity, sulfide, grain size, nutrients, microbiome, etc.) at each location to answer this question.

Conclusions

Very little is known about phagomyxids because their characteristics of small size, complex life cycle, and being non-culturable, make them difficult to observe and identify morphologically. Thus, there have been no extensive marine biodiversity studies of this group (Neuhauser et al. 2011, 2014; Sullivan et al. 2018). The biodiversity of parasites is thought to be underestimated in the oceans (Rohde

2016), and it is suggested that there are many more species of phagomyxids to be discovered (Neuhauser et al. 2011). The effects of phagomyxids on marine primary producers have not been examined, but it has been suggested that the hypertrophied structures that cause stunted roots on *Zostera* species by *P. caudata* may increase their susceptibility to being uprooted (den Hartog 1989). Similarly, the large galls on *Halodule wrightii* have also been reported to be associated with increased uprooting (Walker and Campbell 2009). The root hair galls described in this study may also make *Z. marina* more susceptible to uprooting because of the stunted root hair growth. Uprooting and subsequent release of zoospores into the water column may increase the dispersal of the parasite. This should be examined in future studies. In addition, the loss of the normally long root hairs on the plants with root hair tip galls may limit nutrient uptake. Whether root hairs with galls are functional or not also needs to be examined, but we assume that infection with the phagomyxid kills the root hair cell because empty root hairs appeared to be devoid of cellular material. Another negative effect of the phagomyxid parasite may be that it makes plants more susceptible to infection by other microbes or be vectors for viruses. Given that many species of phytomyxeans have known potential to cause diseases in crops and transmit viruses (Neuhauser et al. 2014), they have the potential to have significant effects on natural populations of eelgrass. Whether infection with phagomyxids make eelgrass more susceptible to other known pathogens such as *Labyrinthula* or *Phytophthora* needs to be examined in future studies.

Methods

Field sampling: The rhizome, roots, and shoots of eelgrass (*Z. marina*) were collected during low tides at four locations in Puget Sound, WA (Fig. 2). During initial exploratory studies in spring/summer/fall of 2015–2018, plants were haphazardly collected at the Ruston Way site. Systematic sampling was conducted during low tides in May/June of 2017, when twenty plants were randomly selected along 30 m transects at all four sites. The rhizome and roots of the plants were dug up with a trowel or coring device (12 cm diameter) and then the sediment and plants were put into plastic zip-lock bags and kept on ice in a cooler until returned to the laboratory.

Microscopy: Within 24 h after collection from the field, the rhizome and roots were carefully extracted from the sediment and washed in artificial seawater (32 psu, Instant Ocean®) to remove as much sediment as possible. Individual roots were cut off at the base next to the rhizome with scissors and put into petri dishes with sterile seawater (0.45 μm filtered). For a subset of plants collected from the Ruston Way site in spring/summer 2015, the number of normal root hairs and root hairs with galls were counted along the length of the roots at 5 mm intervals under a dissecting microscope at 40× magnification. Images of individual root hair galls were taken with a Leica camera attached to either a dissecting microscope (40×), Leica inverted compound microscope (200×), or compound microscope (1000×). The length and width of the root hair galls were measured in ImageJ (Schneider et al. 2012).

At all four collection locations in 2017, a total of 40 cm of root material was collected from each plant and each root was examined under a dissecting microscope and was given a rating from 0 to 4 for the percent root hairs with galls at the root hair tips: 0 = no galls, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%. We did not differentiate between the numbers of filled vs empty root hair galls because identifying filled root hair galls was very time consuming. After measuring and analyzing roots for root hair galls, the root samples were stored in cryovials at −20 °C. An average rating of percent root hairs with galls was calculated for the roots making up each sample. A Kruskal–Wallis test (variances were unequal among samples) with the Dunn test for multiple comparisons was used to compare median values of ratings for percent root hairs with galls among locations using R (R Core Team 2015).

To determine if there was material of non-plant origin in the roots or root hairs, individual roots were stained with 0.1% Trypan blue (which stains chitin) (Scholz et al. 2014) in lactophenol for 15–30 min. Roots were also fixed in 4% paraformaldehyde overnight at 4 °C and then stored in 70% ethanol. These roots were examined using DAPI (4’, 6-diamidino-2-phenylindole) staining under a Nikon D-Eclipse C1 Confocal/Epifluorescence microscope using NIS-Elements BR 3.10.

Roots were prepared for scanning electron microscopy (SEM) by fixing them in 4% glutaraldehyde in sterile seawater overnight at 4 °C. The roots were then put through a dehydration series (30, 50, 70, 90, 100% ethanol) and critical point drying using HMDS (hexamethyldisilazane) (Bray et al. 1993). The dried roots were mounted on stubs and sputter coated with gold/palladium using a Denton Vacuum, Inc. sputter coater. The prepared samples were examined on a Hitachi S-3400N scanning electron microscope.

Molecular phylogenetic analyses: Root samples from the Ruston Way site were observed in detail at 40× under a dissecting microscope for the presence of root hair tip galls that contained cellular material (Fig. 1). Using sterile fine forceps and metal probes, individual root hair cells that contained cellular material (filled root hair galls) were separated from the roots. The filled root hair galls were then transferred using a micropipette to another petri dish containing sterile seawater for 1 min, and then transferred to another petri dish filled with nuclease free distilled water for 1 min. A fine pin was then used to transfer the root hair galls to a 1 μl drop of nuclease free distilled water in a sterile petri dish, where it was subsequently crushed to release the cellular contents inside the root hair galls. The 1 μl drop of cell suspension was then transferred to a PCR tube and frozen at −20 °C.

The 1 μl cell suspension from the root hair galls was used as template in a 25 μl PCR reaction using universal eukaryotic 18S rDNA primers MoonA (ACCTGGTTGATCCTGCCAG) and MoonB (TGATCCTTCYGCAGGTTCAC) (Moon-van der Staay et al. 2000; Takishita et al. 2010) or ITS primers ITS6 (GAAGGTGAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990; Cooke et al. 2000). The reaction mixture contained the 1 μl cell suspension, 22.5 μl SuperMix (Invitrogen), 0.5 μl of each primer (25 μM), and 0.5 μl nuclease-free water. PCR conditions for

18S were as follows: an initial denaturation step of 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 2 min, and a final elongation step of 72 °C for 7 min. PCR conditions for ITS were as follows: an initial denaturation step of 94 °C for 3 min, 35 cycles of 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. The amplicons were visualized by gel electrophoresis (1% agarose gel), and then purified using a MoBio Ultraclean PCR clean-up kit. The purified PCR products were sequenced at GeneWiz using the original primers and internal primers for the 18S gene (F-566: CAGCAGCCGCGGTAATAC, R-563: ACCAGACTTGCCCTCC, R-1200: CCCGTGTTGAGTCAAAT-TAAGC, F-1247: TTCTATGGGTGGTGGTGCAT) (Hadziavdic et al. 2014; Hugerth et al. 2014). The sequences were submitted to GenBank under the accession numbers MG847140 and MG991824.

Consensus sequences of the forward and reverse reads were constructed in Geneious 6.0 (https://www.geneious.com) (Kearse et al. 2012), and the sequences were chimera checked using USEARCH 6.0 implemented in RDP (Cole et al. 2014). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify similar sequences (Altschul et al. 1990). A subset of the 18S and ITS sequences used in the phylogenies by Neuhauser et al. (2014) and Murúa et al. (2017) were included in phylogenetic analyses. Sequences were aligned using MAFFT (Katoh and Standley 2013) and GUIDANCE2 (Sela et al. 2015) was used to identify and remove unreliable columns (score <0.375). Phylogenetic analyses were conducted using MrBayes, PhyML, and RaxML in Topali2 (Milne et al. 2009).

qPCR of root and soil samples from different locations: A DNeasy Powersoil DNA extraction kit (Qiagen) was used to extract DNA from 40 cm (approximately 0.1 g wet wt. or 5 mg dry wt.) samples of root tissue from each of 20 plants from the four sampling locations. The same protocol was used to extract DNA from 0.25 g samples of sediment taken near the roots. This is the recommended protocol for extraction of DNA from soil and plant roots (Lear et al. 2018). qPCR primers were designed for the 18S gene based on the sequence from the PCR products for DNA from the filled root hair galls described above. Primers were designed using the program Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012), with forward primer (TTCTATGGGTGGTGGTGCAT) and reverse primer (CCTCAAACTTCCCTCGGCTA), producing a product of 163 bp. The specificity of the primers was determined using Primer BLAST and the primers and resulting sequences were also compared to sequences of other related Phytomyxean taxa (Murúa et al. 2017). The forward primer matched the 18S sequence of all described phagomyxids in Murúa et al. (2017), but the reverse primer differed by at least 1 bp from 18S sequences of other phagomyxids.

PCR amplification was performed using a Bio-Rad CFX96 real-time PCR machine with Bio-Rad CFX Manager software, version 2.0. Duplicate qPCR reactions were prepared for each sample with SsoAdvanced Uni Inh-Tol SYBR Green Supermix (Bio-Rad) containing the following: 10 μl SYBR Green, 0.5 μl forward and reverse primers (10 μM), 1 μl of 1/10 diluted template, and 8 μl nuclease free water. qPCR conditions involved an initial denaturation step of 98 °C for 3 min, followed by 40 cycles of 98 °C for 10 s and 60 °C for 30 s. A melt curve was added at the end of amplification to check for specificity of qPCR products. A temperature gradient (57–63 °C) was used to optimize the annealing temperature. The standard curves for phagomyxid DNA were constructed using serially diluted DNA from a purified phagomyxid PCR product (1772 bp). The amount of phagomyxid DNA per cm of root tissue or in soil sample was then calculated. qPCR products were sequenced using the forward and reverse qPCR primers to verify that the consensus sequence matched the target sequence. Also, identical qPCR analyses were conducted with crushed individual filled or empty root hair galls in 1 μl of water as the DNA template. To verify that inhibitors were not affecting quantification of DNA, a plant internal control (CoxI) was run in an additional qPCR using the primers and conditions described in Bilodeau et al. (2014). A Kruskal–Wallis test (variances were unequal among samples) with the Dunn test for multiple comparisons was used to compare the amount of phagomyxid DNA in root and soil samples from the four different locations (R Core Team 2015).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2018.12.001.

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