Title: All small things considered: the diversity of fungi, bacteria and oomycota isolated from the seagrass, Zostera marina

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Abstract

Fungi in the marine environment are often neglected as a research topic, despite that fungi having critical roles on land as decomposers, pathogens or beneficial endophytes. Here we used culture-dependent methods to survey the fungi associated with the seagrass, Zostera marina, also obtaining bacteria and oomycete isolates in the process. A total of 108 fungi, 41 bacteria and 2 oomycetes were isolated. These isolates were then taxonomically identified using a combination of molecular and phylogenetic methods. The majority of the fungal isolates were classified as belonging to the classes Eurotiomycetes, Dothideomycetes, and Sordariomycetes. Most fungal isolates were habitat generalists like Penicillium sp. and Cladosporium sp., but we also cultured a diverse set of rare taxa including possible habitat specialists like Colletotrichum sp. which may preferentially associate with Z. marina leaf tissue. Although the bulk of bacterial isolates were identified as being from known ubiquitous marine lineages, we also obtained several Actinomycetes isolates which might produce interesting secondary metabolites and a Phyllobacterium sp. which may be involved in nitrogen cycling in the seagrass ecosystem. We identified two oomycetes, another understudied group of marine microbial eukaryotes, as Halophytophthora sp. which may be opportunistic pathogens of Z. marina. Overall, this study generates a culture collection of fungi, bacteria and oomycetes which expands knowledge of the diversity of Z. marina associated microbes and highlights a need for more investigation into the functional and evolutionary roles of microbial eukaryotes associated with seagrasses.

Introduction

Despite their global importance in terrestrial systems, the diversity, function, evolution, and global importance of fungi in the marine environment remains understudied. There are only ~1100 currently accepted species of marine fungi despite estimates that true diversity is much
higher, at 10,000 or more species (Jones, 2011; Jones et al., 2015). It is well known that fungi play vital roles in land plant health and fitness (e.g. as pathogens or beneficial endophytes), and although much less is known about fungi in aquatic ecosystems, it is thought they have important roles in organic matter degradation and food web dynamics (Grossart et al., 2019). Thus, it is likely that fungi engage in similarly vital functional roles when associated with marine plants, like seagrasses.

Seagrasses are fully submerged marine angiosperms and are foundation species in coastal ecosystems. Seagrass beds perform important ecosystem services and can store carbon over very long timescales in their above and below ground tissues and in surrounding sediments (e.g. "blue carbon") (Fourqurean et al., 2012). Unfortunately, seagrass beds are threatened by human-related activities such as pollution, climate change and coastal development, and restoration efforts thus far have been mostly ineffective (Orth et al., 2006). In addition to their global ecological importance, seagrasses also have a unique evolutionary history. Sometimes referred to as the “whales of the plant world”, seagrasses are a paraphyletic group of multiple lineages that convergently adapted to the marine environment between 70 and 100 million years ago (Les et al., 1997; Wissler et al., 2011). There are only ~60 species of seagrass compared to the ~250,000 species of terrestrial flowering plants, a testament to the strict selective pressure posed by re-entry to the marine environment. This work focuses on one widespread seagrass species, Zostera marina, which occurs throughout much of the Northern Hemisphere.

Culture-based studies have found fungi associated with leaves, roots and rhizomes of seagrasses, but there is little agreement between studies about the taxonomic composition of these communities within and between seagrass species (Newell, 1981; Kuo, 1984; Cuomo et al., 1985; Devarajan & Suryanarayanan, 2002; Sakayaroj et al., 2010; Mata & Cebrián, 2013; Shoemaker & Wyllie-Echeverria, 2013; Supaphon et al., 2013, 2014, 2017; Panno et al., 2013; Kirichuk & Pivkin, 2015; Venkatachalam, 2015; Venkatachalam et al., 2015; Torta et al., 2015; Ling et al., 2015; Vohník et al., 2016). Recently culture-independent studies of seagrass-associated fungi have more thoroughly investigated the diversity of these microorganisms and highlighted a need to further understand factors affecting their biogeography and community dynamics (Wainwright et al., 2018, 2019; Hurtado-McCormick et al., 2019; Ettinger & Eisen, 2019). However, these studies were severely hampered by a lack of representation of fungal sequences from the marine environment in public databases and found that taxonomic assignments could not be made for many fungal sequences associated with seagrasses. This suggests both a need to expand molecular knowledge of marine and seagrass-associated fungi in public databases and that seagrasses may harbor diverse and understudied fungal lineages.

Here we used a culture-dependent survey followed by molecular and phylogenetic identification to (i) obtain and identify a diverse collection of fungi associated with Z. marina, (ii) place this fungal collection in the phylogenetic context of isolates obtained from other seagrass surveys, and (iii) compare and contrast the composition of this fungal collection to high throughput sequencing results of the composition of the fungal community associated with Z. marina from the same location.
Methods

Sample collection and isolation

Individual *Zostera marina* plants and associated sediment were collected opportunistically from Westside Point (GPS: 38°19'10.67"N, 123° 3'13.71"W) in Bodega Bay, CA during several sampling trips between summer 2017 and summer 2018 using a 2.375 inch diameter modified PVC pipe as described in Ettinger & Eisen (2019). Bulk leaf tissue was also collected during these trips using gloves and placed in sterile plastic bags. Seawater was also collected in autoclaved 1 L nalgene bottles. All samples were kept cold on ice in a dark cooler for transport back to the lab.

Once back in the lab, plant tissues, sediment and seawater were plated on a variety of different media types. Generally for seagrass tissues (leaf, root or rhizome) this involved, (1) rinsing the tissue with autoclaved nanopure water, (2) using flame sterilized scissors to cut ~1 cm pieces of tissue, (3) placing a subset of these tissue segments directly on plates using flame sterilized tweezers, (4) taking another subset of tissue segments and placing these segments into 1.5 mL centrifuge tubes with 1000 µl of autoclaved nanopure water, (5) vortexing the 1.5 mL centrifuge tubes for ~30 sec, (6) either smashing tissue segments using a sterile pestle or leaving the segments intact, and (7) directly plating intact tissue segments on media using flame sterilized tweezers and pipetting 350 µl of wash liquid or of smashed tissue directly on plates. For sediment this involved, (1) placing sediment into 1.5 mL centrifuge tubes with 1000 µl of autoclaved nanopure water, (2) vortexing the tubes for ~30 sec, and (3) then pipetting 350 µl of sediment suspension directly onto plates. For seawater this involved pipetting 350 µl of seawater directly onto plates.

A variety of media recipes were used to try to obtain a diverse collection of fungal isolates. These media included 1% tryptone agar (10 g tryptone, 10 g agar, 1 L distilled water), potato dextrose agar (PDA), potato carrot agar (PCA), palm oil media (12 g agar, 10 g dextrose, 10 g yeast extract, 3 g peptone, 2 g L-arginine, 10 mL Tween80,10 mL palm oil, 1 L distilled water, final pH: 8.0), lecithin media (12 g agar, 10 g dextrose, 10 g yeast extract, 3 g peptone, 2 g L-arginine, 10 mL Tween80, 0.7 g lecithin, 1 L distilled water, final pH: 8.0), malt extract agar (MEA; 30 g malt extract, 15 g agar, 1 L distilled water, final pH: 5.5), and glucose yeast peptone agar (GYPA; 15 g agar, 5 g yeast extract, 5 g peptone, 40 g glucose,1 L distilled water). A variety of salt amendments were used including: adding no salt, adding varying amounts of instant ocean (8 g, 16 g, or 32 g) or substituting distilled water for 0.45 µM Millipore filtered natural aged seawater. All media was amended with 50 mg/mL ampicillin, with some media batches also amended with 50 mg/mL trimethoprim or 50 mg/mL streptomycin. Additionally, some media batches also included the addition of 5 g/L dehydrated crushed *Z. marina* leaf tissue. For the exact media conditions each isolate was grown on see Table S1.

Plates were wrapped in parafilm and incubated at room temperature in the dark in a cabinet drawer for 4 weeks and observed every 2-3 days for fungal growth. Fungal isolates were then
sterilely subcultured onto new plates and the process repeated until we were confident we had a single isolate.

**DNA extraction, polymerase chain reaction (PCR) and Sanger sequencing**

DNA was extracted from the majority of isolates using the MoBio PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) with minor changes to the manufacturer's protocol as follows. To improve fungal lysis, samples were heated at 70 °C for 10 minutes between steps 4 and 5. For step 5, samples were bead beaten on the homogenize setting for 2 minutes using a mini-bead beater (BioSpec Products). For a subset of isolates DNA was extracted with either the Qiagen Plant DNeasy (QIAGEN, Hilden, Germany), the Qiagen DNeasy PowerSoil Pro Kit (QIAGEN, Hildren, Germany) or the Zymo Xpedition Fungal/Bacterial DNA Mini Prep (Zymo Research Inc, Irvine, CA, United States) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed using Taq DNA Polymerase (QIAGEN, Hilden, Germany). The fungal ITS-28S rRNA gene region was obtained using the ITS5 (White et al., 1990) and LR3 (Vilgalys & Hester, 1990) primer set, the bacterial 16S rRNA gene was obtained using the 27F (Lane, 1991) and 1391R (Turner et al., 1999) primer set, and the oomycete 28S rRNA gene was obtained using the LR0R (Cubeta et al., 1991) and Un-Lo28S1220 (Bala et al., 2010) primer set. When amplifying the fungal ITS-28S rRNA gene region, PCR was performed with the following conditions: 95°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 15 seconds, 72°C for 1 minute, and a final extension at 72°C for 8 minutes (Kress & Erickson, 2012). When amplifying the bacterial 16S rRNA gene, PCR was performed with the following protocol: 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, 54°C for 30 seconds, 72°C for 1 minute and 30 seconds, and a final extension at 72°C for 5 minutes (modified from Dunitz et al., 2015)). When amplifying the oomycete 28S rRNA gene, PCR was performed with the following protocol: 94°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 sec, and a final extension at 72°C for 10 minutes (adapted from Bourret et al. (2018)).

PCR products were visualized on 2% agarose E-gels (Invitrogen, Carlsbad, CA, United States). PCR products were then purified using the Nucleospin Gel and PCR kit (QIAGEN, Hilden, Germany) and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, United States). The PCR products were sequenced using the Sanger method by the UC Davis College of Biological Sciences UC DNA Sequencing Facility (http://dnaseq.ucdavis.edu/). The resulting ABI files were visualized and consensus sequences were generated using seqtrace v. 0.9.0 (2018) following the Swabs to Genomes workflow (Dunitz et al., 2015). Consensus sequences for the PCR products were deposited at NCBI Genbank under the following accession no. MN543905-MN544012 for the fungal ITS-28S rRNA gene region, MN931878-MN931917 for the bacterial 16S rRNA gene, and MN944508-MN944509 for the oomycete 28S rRNA gene.

**Taxonomic analyses**
Preliminary taxonomic assignment of sequences from the PCR products generated above were obtained by comparing the best results (or “top match”) across three methods to obtain a consensus assignment. The three methods included (1) using NCBI’s Standard Nucleotide BLAST’s megablast option with default settings, (2) using the Ribosomal Database Project (RDP) classifier with the appropriate respective database (e.g. the 16S rRNA training set for bacteria, the Fungal LSU, WARCUP and UNITE datasets for fungi) and default settings, (3) using the SILVA Alignment, Classification and Tree (ACT) service with the appropriate database (SSU for bacteria, LSU for fungi and oomycetes) and default settings (Altschul et al., 1990; Wang et al., 2007; Pruesse et al., 2012; Quast et al., 2013; Cole et al., 2014). Taxonomic assignments for isolates and associated isolation conditions were then imported into R (v. 3.6.0) for visualization and analysis using the following packages: ggplot2 (v. 3.2.1), dplyr (v. 0.8.4), reshape (v. 0.8.8), patchwork (v. 1.0.0), and tidyverse (v. 1.3.0) (Wickham, 2016; Zhang, 2016; Pedersen, 2019; R Core Team, 2019; Wickham et al., 2020) (File S1).

**Phylogenetic analyses of fungal isolates**

Sequences closely related to the fungal ITS-28S rRNA gene PCR products generated above were identified using NCBI’s Standard Nucleotide BLAST’s megablast option with default settings to further confirm fungal taxonomy through phylogenetic placement (Table S2). Additionally, we wanted to place the Z. marina associated fungal isolates in the context of the phylogenetic diversity of available other seagrass-associated fungal isolates. To this end, we performed a literature search to obtain, to our knowledge at the time of the search, all available 28S rRNA sequences obtained from seagrass associated fungal isolates for inclusion in phylogenetic analyses (Table S3) (Sakayaroj et al., 2010; Gnavi et al., 2014; Vohník et al., 2016, 2019; Supaphon et al., 2017). Finally, to provide a further framework for these phylogenies, as well as appropriate outgroup taxa, we downloaded the available 28S rRNA sequences previously used in James et al. (2006a,b) (Table S4).

Using the sequences listed in Table 1 and Tables S2-S4, we generated four different sequence alignments, (1) an alignment to investigate seagrass isolates in the Basidiomycota and Zygomycota phyla, (2) an alignment to investigate seagrass isolates in the Eurotiomycetes class in the Ascomycota phylum, (3) an alignment to investigate seagrass isolates in the Sordariomycetes class in the Ascomycota phylum, and (4) an alignment to investigate seagrass isolates in the Dothideomycetes class in the Ascomycota phylum.

Each of the four sequence alignments was generated using MAFFT (v. 7.402) (Katoh et al., 2002) with default parameters on the CIPRES Science Gateway web server (Miller et al., 2010). The alignments were trimmed using trimAl (v.1.2) with the -gappyout method (Capella-Gutierrez et al., 2009) and then manually inspected with JalView (Waterhouse et al., 2009). Sequence alignments were then further trimmed to the D1/D2 regions of the 28S rRNA gene with trimAl using the select option (e.g. Basidiomycota / Zygomycota alignment { 614-2899 }, Eurotiomycetes alignment { 0-569 }, Sordariomycetes alignment { 501-1224 }, and Dothideomycetes alignment { 0-429, 993-1755 }). Spurious sequences (e.g. sequences which contained few or no nucleotides after trimming) were then removed with trimAl using -resoverlap.
The resulting alignments contained: 80 sequences with 614 positions (Basidiomycota / Zygomycota), 91 sequences with 509 positions (Eurotiomycetes), 96 sequences with 501 positions (Sordariomycetes), and 107 sequences with 563 positions (Dothideomycetes).

JModelTest2 (v. 2.1.10) was run with the number of substitution schemes (-s) set to 3 (JC/F81, K80/HKY, SYM/GTR) and then otherwise default parameters on the CIPRES Science Gateway web server to select a best-fit model of nucleotide substitution for use in phylogenetic analyses for each alignment (Guindon & Gascuel, 2003; Darriba et al., 2012). The best-fit model based on the Akaike Information Criterion values for all alignments was the GTR + I + G evolutionary model.

Using the CIPRES Science Gateway web server, Bayesian phylogenetic inference for each alignment was performed using MrBayes (v. 3.2.2) with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run over 10,000,000 generations. The analysis stopped early if the optimal number of generations to reach a stop value of 0.01 or less for the convergence diagnostic was achieved (Huelsenbeck & Ronquist, 2001). This occurred for the Eurotiomycetes, Sordariomycetes and Dothideomycetes alignments at 2,150,000 generations, 1,375,000 generations and 2,140,000 generations, respectively. The Basidiomycota / Zygomycota alignment ran for the full 10,000,000 generations, only achieving an average standard deviation of split frequencies of 0.049. The first 25% of trees generated for each alignment were discarded as burn-in and for the remaining trees, a majority rule consensus tree was generated and used to calculate the Bayesian Posterior Probabilities. The resulting phylogenies were then visualized with the ggtree (v. 2.0.1), treeio(v. 1.11.2), ggplot2 (v. 3.2.1), and tidyverse (v. 1.3.0) packages in R (v. 3.6.0) and clade labels were added in Adobe Photoshop CS6 (Wickham, 2016; Yu et al., 2017, 2018; R Core Team, 2019; Wickham et al., 2019; Wang et al., 2020) (File S1). Alignments and phylogenies generated here were deposited to Dryad (Ettinger & Eisen, 2020).

Comparisons to ITS amplicon data from Ettinger & Eisen (2019)

To compare to high throughput sequencing data associated with Z. marina from the same location (Westside Point, Bodega Bay, CA), we utilized an amplicon sequence variant (ASV) dataset previously analysed in Ettinger & Eisen (2019). Specifically, we are using the subset ASV table that was used to investigate differences between bulk sample types. Briefly, this ASV table was previously subset to a depth of 10,000 sequences and included 49 samples from four sample types: leaf epiphytes (n = 13), root epiphytes (n = 14), rhizome epiphytes (n = 7), and sediment (n = 15). We then used this ASV table to make comparisons to the fungal taxa isolated in this study. To investigate whether fungal genera isolated in this study were also detected in the high throughput sequencing data, we generated a list of the unique genera found in the ASV table and compared it to the list of fungal genera isolated here. To investigate whether the fungal genera isolated in this study were detected from the same Z. marina tissues, we collapsed the ASV table to the genus level using the tax_glom function in phyloseq. We then subsampled the ASV table to only include the genera of fungi isolated in this study, transformed this ASV table represent presence / absence and visualized a comparison of the distribution of
these genera across sample types (leaf, root, rhizome, sediment) to the distribution of these genera across isolation sources (leaf, root, rhizome, sediment). To investigate the mean relative abundance of the fungal orders isolated in this study in the high throughput sequencing data, we collapsed the ASV table to the order level using the tax_glom function in phyloseq. We then subsampled the ASV table to only include the orders of fungi isolated in this study and visualized the distribution of these orders across sample types (leaf, root, rhizome, sediment). These analyses were performed in R (v. 3.6.0) using the ggplot2 (v. 3.2.1), dplyr (v. 0.8.4), reshape (v. 0.8.8), patchwork (v. 1.0.0), phyloseq (v. 1.30.0) and tidyverse (v. 1.3.0) packages (Wickham, 2007, 2016; McMurdie & Holmes, 2013; Pedersen, 2019; R Core Team, 2019; Wickham et al., 2019, 2020) (File S1).

Results

**Taxonomic diversity of fungi isolated from Z. marina**

In an attempt to cultivate a wide diversity of fungal isolates, we used a variety of media types including several which had been used previously to isolate fungi from seagrasses (e.g. PDA (Sakayaroj et al., 2010; Vohník et al., 2016; Supaphon et al., 2017), GPYA (Panno et al., 2013), MEA (Torta et al., 2015)). A total of 108 fungal isolates were obtained, with the majority cultured from *Z. marina* leaf tissue (n = 50), resulting in a range of morphological diversity (Figure 1). The rest of isolates were cultured from rhizome tissue (n = 22), root tissue (n = 16), associated sediment (n = 13), seawater (n = 5), combined leaf and root tissues (n = 1), and combined root and rhizome tissues (n = 1) (Figure 2, Figure S1).

Almost all of the fungal isolates were taxonomically classified as belonging to the Ascomycota (n = 103), with the remaining five isolates classified as Basidiomycota (n = 4) and Zygomycota (n = 1), respectively (Table 1). Within the Ascomycota, isolates were further identified as being in three classes: the Eurotiomycetes (n = 62), Dothideomycetes (n = 30), and Sordariomycetes (n = 11).

Based on the molecular identifications, Eurotiomycetes isolates were classified as *Penicillium* sp. (n = 59) and *Talaromyces* sp. (n = 3). Sordariomycetes isolates were putatively classified as *Colletotrichum* sp. (n = 4), *Acrostalagmus* sp. (n = 1), *Emericellopsis* sp. (n = 1), *Sarocladium* sp. (n = 1), *Trichoderma* sp. (n = 1), and unidentified *Hypocreales* sp. (n = 3). Dothideomycetes isolates were classified as *Cladosporium* sp. (n = 11), *Ramularia* sp. (n = 11), *Aureobasidium* sp. (n = 1), and unidentified *Pleosporales* sp. (n = 7). Basidiomycota isolates were putatively classified as *Pseudozyma* sp. (n = 2), *Rhodotorula* sp. (n = 1), and *Naganishia* sp. (n = 1). The single Zygomycota isolate was putatively classified as *Absidia cylindrospora*.

We observed a positive relationship between the number of tissue types and number of media types a fungal genus was isolated from ($R^2$ = 0.86, Figure S2) which we hypothesize may indicate that some fungal genera are habitat generalists. A similar positive relationship is also observed between the number of tissue types and the number of salt sources ($R^2$ = 0.93) as well as between the number of media types and number of salt sources ($R^2$ = 0.87). We note
that we did not perform an experiment to confirm this pattern or attempt to control for effort or isolation frequency in this study and thus, this positive relationship must be interpreted with caution.

**Taxonomic diversity of bacteria and oomycota isolated from Z. marina**

Although our intent here was to isolate fungi, we also cultivated and identified 41 bacteria and 2 oomycetes. As with the fungal cultivation results, the majority of bacterial isolates were obtained from *Z. marina* leaf tissue (n = 16). The rest of the bacterial isolates were cultured from rhizome tissue (n = 9), root tissue (n = 7), associated sediment (n = 5), seawater (n = 2) and combined leaf and root tissues (n = 1) (Figure S3).

Bacterial isolates were identified as belonging to the Actinobacteria (n = 4), Firmicutes (n = 2), Bacteroidetes (n = 2), and Proteobacteria (n = 33) (Table 2). The two Firmicute isolates were further classified as *Bacillus* sp., the two Bacteroidetes isolates as *Joostella* sp., and the Actinobacteria isolates as *Streptomycetes* sp. (n = 2), *Rhodococcus* sp. (n = 1), and *Isopericola* sp. (n = 1). The Proteobacteria isolates were classified as *Vibrio* sp. (n = 18), *Pseudoalteromonas* sp. (n = 8), *Hafnia* sp. (n = 2), *Pseudomonas* sp. (n = 1), *Shewanella* sp. (n = 1), *Marinomonas* sp. (n = 1), and *Phyllobacterium* sp. (n = 1).

The two oomycete isolates were obtained from combined *Z. marina* leaf and root tissues and were both identified as *Halophytophthora* sp. (Table 3).

**Phylogenetic comparison of fungal isolates across seagrass species**

To confirm fungal isolate identity and investigate if *Z. marina* fungal isolates were closely related to fungal isolates obtained from other seagrass species, we built four phylogenetic trees, 1) a phylogeny of seagrass isolates in the Basidiomycota and Zygomycota phyla (Figure 3), (2) a phylogeny of seagrass isolates in the Eurotiomycetes class in the Ascomycota phylum (Figure 4), (3) a phylogeny of seagrass isolates in the Sordariomycetes class in the Ascomycota phylum (Figure 5), and (4) a phylogeny of seagrass isolates in the Dothideomycetes class in the Ascomycota phylum (Figure 6). The placements of isolates in these phylogenies were consistent with the taxonomic identities previously determined by molecular methods.

We expected to see more shared taxonomic groups and phylogenetic clustering between the fungal isolates of *Z. marina* and those of other seagrass species than was observed in Figures 3-6. Many of the fungal isolates from *Z. marina* did not cluster with fungal isolates that had been previously cultivated in association with other seagrass species. However, this could be the result of the limits of culture-dependent studies generally or the use of different media recipes and methods for isolating fungi in other studies.

The fungal taxa that did have close relatives that were associated with other species included *Penicillium* sp. (Figure 4), *Trichoderma* sp. (Figure 5), *Cladosporium* sp. and *Ramularia* sp. (Figure 6). We note that *Penicillium* sp., *Cladosporium* sp. and *Ramularia* sp. are drivers of the
positive relationship observed previously between the number of tissue types and number of media types a fungal genus was isolated from (Figure S2).

Comparisons to ITS amplicon sequencing data from Ettinger & Eisen (2019)

We compared the diversity of the fungi isolated here to high throughput sequencing data associated with Z. marina from the same location (as previously analysed in Ettinger & Eisen (2019). We found that the fungal genera isolated in this study were generally also detected in the sequencing data (Table 1). Only two genera were not detected in the sequencing data, Pseudozyma sp. and Absidia cylindrospora.

We then investigated whether the fungal genera isolated in this study were also detected in association with the same types of samples (e.g. leaf, root, rhizome, sediment) in the sequencing data. We observed that many of rare (e.g. not as frequently isolated) genera were not consistently detected on the same sample type with both methods, whereas many of the abundant (e.g. more frequently isolated) genera were detected with both methods (Figure 7). When we looked at the mean relative abundance of the fungal orders isolated in this study in the high throughput sequencing data, we observed that the genera detected using both methods generally were in orders that had higher mean relative abundances in the seagrass ecosystem (Figure S4). We also observed that some orders such as the Eurotiales (e.g. Penicillium sp.) and Capnodiales (e.g. Cladosporium sp. and Ramularia sp.) had similar mean relative abundances across all sample types. While other orders such as Glomerellales (e.g. Colletotrichum sp.) had a higher mean relative abundance on one sample type (e.g. leaves).

Discussion

Here, we generated a fungal collection of 108 isolates expanding understanding of the diversity of Z. marina associated fungi, while also underscoring how little we know about these understudied microorganisms. Generally, the taxonomic diversity observed in our cultivation efforts is consistent with that of other culture-dependent studies which found Eurotiomycetes, Dothideomycetes, and Sordariomycetes to be the main classes of fungi associated with seagrasses (Sakayaroj et al., 2010; Supaphon et al., 2017). This is also consistent with what is known of the diversity of fungal associations with terrestrial plants, Members of the Sordariomycetes and Dothideomycetes have been found to be the predominant members of land plant fungal endophyte communities (U’Ren et al., 2012), while Eurotiomycetes have been found to be the dominant members of freshwater plant communities (Sandberg et al., 2014).

Dark septate endophytes (DSE), particularly members of the Pleosporales within the Dothideomycetes (Figure 6), have been observed to form associations with several seagrass species (Panno et al., 2013; Gnavi et al., 2014; Vohník et al., 2015, 2016, 2017, 2019; Borovec & Vohník, 2018; Hurtado-McCormick et al., 2019). DSE are a morphological, not phylogenetic (e.g. not each other's closest relatives) group of plant associated fungi, and are largely uncharacterized. The most well described of these DSE associations is between the Mediterranean seagrass, Posidonia oceanica, and its dominant root-associated fungus,
Posidoniomyces atricolor. This Pleosporales member has been found associated with changes in root hair morphology and can form ecto-mycorrhizal-like structures (Vohník et al., 2016, 2017, 2019; Borovec & Vohník, 2018). Here, although we isolated seven members of the Pleosporales, none appeared to be close relatives to Posidoniomyces atricolor.

Chytridiomycota were found to be prevalent members of the Z. marina leaf microbiome in Ettinger & Eisen (2019), however, no chytrids were cultured in this study. This is likely because the isolation methods used here favor cultivation of Dikarya. Alternative methods and media recipes should be utilized (e.g. baiting) to isolate representatives of these important members of the fungal community. Similarly we note that the methods used here would fail to culture fungi involved in obligate associations with seagrasses. In these cases, a combination of microscopy and/or cell sorting for directed cultivation or sequencing might prove valuable for assessing the functional roles of these organisms to the seagrass ecosystem.

Previous work on the fungal community associated with the seagrass, Enhalus acoroides, identified a pattern of distance decay, where the fungal community was more similar between seagrass that were closer together geographically than between seagrass that were distant from each other (Wainwright et al., 2019). This suggests that dispersal limitation and/or habitat specialization are playing important roles in structuring the fungal community associated with seagrasses. In this study, we opportunistically sampled fungi associated with a single seagrass species, Z. marina, from a single seagrass patch in Bodega Bay, CA. We did not investigate the fungal community of this seagrass species at other locations and thus, we cannot test for a pattern of distance decay here. However, we do see evidence supportive of a role of habitat specificity and/or dispersal efficiency at a local level in the fungal genera isolated here from Z. marina.

A pattern observed across culture-based studies of seagrass-associated fungi is that ubiquitous fungi are the dominant members of the communities, but that seagrasses also consistently host a diverse set of rare taxa. For example, ubiquitous fungi like Penicillium sp. and Cladosporium sp. have been previously reported as the dominant fungi of leaves in other culture-based studies of Z. marina (Shoemaker & Wyllie-Echeverria, 2013; Kirichuk & Pivkin, 2015; Petersen et al., 2019), other seagrass species (Alva et al., 2002; Devarajan & Suryanarayanan, 2002; Rodriguez, 2008; Sakayaraj et al., 2010; Mata & Cebrián, 2013; Venkatachalam et al., 2015) and freshwater aquatic plants (Sandberg et al., 2014). Additionally, Penicillium sp. and Cladosporium sp. were some of the only fungi in this study which were found to have close relatives associated with different seagrass species. We hypothesize that these fungal genera may be habitat generalists (taxa that occur evenly distributed across a wide range of habitats, (Pandit et al., 2009; Székely & Langenheder, 2014)) in the seagrass (and potentially larger marine) ecosystem as they were isolated from multiple media types, detected from most sample types (Figure 7) and found to have similar mean relative abundances across sample types (Figure S4). However, just because these fungi are ubiquitous, does not reflect negatively on their potential importance. These habitat generalists have been shown to be highly adaptable with the innate ability to survive in wide range of extreme conditions (e.g. high salinity), are known to perform ecologically important functions (e.g. degradation of organic matter) and
represent sources of biologically interesting and active secondary metabolites (Imhoff et al., 2011; Panno et al., 2013; Petersen et al., 2019).

We hypothesize that some fungi associated with Z. marina may be habitat specialists (taxa that are more restricted to a specific habitat range, (Pandit et al., 2009; Székely & Langenheder, 2014)). For example, some Colletotrichum sp. may be habitat specialists that preferentially associate with Z. marina leaf tissue. A Colletotrichum sp. ASV (SV10) was found to be dominant on leaves in Ettinger & Eisen (2019) and a Colletotrichum spp. isolate was previously reported from another seagrass species as a leaf endophyte (Rodriguez, 2008). However, in this study, we are unable to decouple the contribution of environmental factors (e.g. habitat or niche specialization) from life history strategies (e.g. dispersal, growth rate). For example, although we hypothesize that some genera may be habitat generalists, it is possible that these patterns may also reflect that some genera have more efficient dispersal mechanisms or faster-growth rates and are able to outcompete slower-growing taxa. We realize these ideas may not be unconnected and that habitat generalists, by their nature, may be assembled by dispersal related mechanisms, and specialists by species sorting (Pandit et al., 2009). Regardless, future studies could use alternative approaches such as adding different combinations and concentrations of fungicides in order to fully survey rare and slow-growing fungi in this system.

Many of the fungal taxa isolated here are known to have complex life history strategies when associated with land plants. For example, the genus Ramularia includes species that are pathogens of a variety of important agricultural plants including barley and sugar beets (Cromey et al., 2002; Videira et al., 2016) and the genus Colletotrichum includes members that can form endophytic or pathogenic associations with land plants (De Silva et al., 2017). Additionally there is mounting support for a multi-niche view of fungi, with many phyto-pathogens now being found able to form benign or even beneficial endophytic associations with plants (Selosse et al., 2018). Thus, future research should endeavour to investigate the true functional roles these fungal genera may have when associated with Z. marina and whether these functional roles shift when Z. marina is stressed or challenged.

Although our goal here was to isolate seagrass-associated fungi, we also identified 41 bacterial isolates associated with Z. marina. Since we were using antibiotics, we do not expect these isolates to be representative of the true diversity of the culturable bacterial community associated with Z. marina. Most of the bacterial isolates we obtained are from known ubiquitous marine lineages (Vibrio, Pseudoalteromonas, Pseudomonas, Shewanella, and Bacillus) which are likely habitat generalists and have all been previously cultured from Z. marina from Bodega Bay, CA (Lee et al., 2015a,b, 2016a,b; Alexiev et al., 2016; Lujan et al., 2017). We also isolated several bacterial isolates that may represent rare or slow-growing taxa with interesting ecological implications for the seagrass ecosystem. This includes several Actinomycetes (Streptomyces sp., Rhodococcus sp. and Isoptericola) which are known to produce a variety of antibiotics and interesting secondary metabolites (Singh & Dubey, 2018) and members of the genus, Isoptericola, have been previously isolated as endophytes of mangrove plants (Yang et al., 2015). We also isolated a Phyllobacterium sp. and representatives of this genus are slow-growing N₂-fixing plant-growth promoting bacteria which have been previously isolated from
mangrove rhizosphere and the roots of land plants (Holguin et al., 1992; Rojas et al., 2001; Mantelin et al., 2006). Land plants often overcome nitrogen limitation through beneficial relationships with N$_2$-fixing bacteria and similar associations have been observed between N$_2$-fixing bacteria and Zostera (Capone & Budin, 1982; Welsh, 2000; Bagwell et al., 2002; Adhitya et al., 2007; Sun et al., 2015). Based on their role as established N$_2$-fixers in other plant systems, it is possible *Phyllobacterium* sp. are involved in fixing nitrogen for seagrasses and this possibility should be further investigated.

Just like marine fungi, oomycetes are neglected in marine systems even though they are often implicated as important pathogens of land plants. In the course of this study, we isolated two members of the *Halophytophthora*. Representatives of *Halophytophthora* have been previously isolated associated with *Z. marina* (Man In 't Veld et al., 2019) and this genus includes known saprophytes (organisms living on organic matter) and are thought to be important decomposers in mangrove ecosystems (Nakagiri, 2000). Recently, Govers et al. suggested that *Halophytophthora* sp. Zostera may be common in *Z. marina* beds, and that this oomycete may serve as an opportunistic pathogen by decreasing seed germination in *Z. marina* populations under certain environmental conditions (Govers et al., 2016). More work is needed to understand the possible implications of these oomycetes in the seagrass ecosystem.

**Conclusion**

Overall, this study generated a fungal culture collection which broadens understanding of the diversity of *Z. marina* associated fungi and highlights a need for further investigation into the functional and evolutionary roles of fungi and microbial eukaryotes (e.g. oomycetes) associating with seagrasses more generally. We placed this fungal collection in the phylogenetic context of isolates obtained from other seagrass surveys and found that only habitat generalists were isolated in association with multiple species. We then compared the composition of this fungal collection to high throughput sequencing results of the fungal community associated with *Z. marina* from Ettinger & Eisen (2019) and found that taxa isolated here were generally present in the sequencing data, but that they were not prevalent, with the exception of the Glomerellales (e.g. *Colletotrichum* sp.) on the leaves. Although this study greatly expands general knowledge of the diversity of *Z. marina* associated fungi, there are still many unanswered questions to be addressed related to the life history strategies, functional roles, and dispersal mechanisms of marine and seagrass-associated fungi. One of the biggest challenges in marine mycology is assessing whether the fungal taxa observed are actively growing in the marine ecosystem (Amend et al., 2019). For our study here we could ask - are many of the proposed habitat generalists actively growing in the seagrass ecosystem or merely passing through as spores? Additionally, many of the fungi cultured here in association with *Z. marina* have close relatives that are also known to be opportunistic pathogens of land plants. Are these fungi *Z. marina* pathogens or do they serve some other function in the marine environment? Ultimately this work serves as a necessary first step towards experimental and comparative genomic studies investigating the functional roles of these understudied microorganisms and which may lead to important discoveries related to molecular biology, natural product discovery, fungal diversity and evolution, and global importance of marine fungi.
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Figures and Tables

**Figure 1:** Microbes isolated from the seagrass, *Zostera marina*
An example of the morphological diversity of microbial isolates (bacteria, fungi and oomycota) associated with the seagrass, *Z. marina.*
Figure 2: Distribution of counts of fungal isolates across isolation sources

A histogram representing the number of fungal isolates grouped by order and colored by isolation source (leaf, leaves and roots, rhizome, root, roots and rhizome, seawater or sediment). The numbers included on each bar represent the count of isolates obtained from that particular isolation source.
Figure 3: Phylogenetic placement of seagrass fungal isolates in the Basidiomycota and Zygomycota.

A molecular phylogeny of 28S rRNA genes for isolates in the Basidiomycota and Zygomycota was constructed using Bayesian inference. This alignment was generated using MAFFT (v. 7.402) on the CIPRES Science Gateway web server, trimmed using trimAl (v.1.2) and a phylogenetic tree was inferred on the trimmed alignment with a GTR + I + G model using MrBayes (v. 3.2.2) (Huelsenbeck & Ronquist, 2001; Katoh et al., 2002; Capella-Gutierrez et al., 2009; Miller et al., 2010). Displayed at each node as a circle in the tree are the Bayesian posterior probabilities (e.g. a black circle represents probabilities greater or equal to 90%, a grey circle represents probabilities greater or equal to 70%, a white circle represents probabilities less than 70%). The names of fungi isolated from Z. marina are shown in green, fungi isolated from other seagrass species are shown in black, and all other fungi are shown in grey. The GenBank accession numbers of the sequences used to build this phylogeny can be found in Table 1 and Tables S2-S4.
**Figure 4:** Phylogenetic placement of seagrass fungal isolates in the Eurotiomycetes. A molecular phylogeny of 28S rRNA genes for isolates in the Eurotiomycetes was constructed using Bayesian inference. This alignment was generated using MAFFT (v. 7.402) on the CIPRES Science Gateway web server, trimmed using trimAl (v.1.2) and a phylogenetic tree was inferred on the trimmed alignment with a GTR + I + G model using MrBayes (v. 3.2.2) (Huelsenbeck & Ronquist, 2001; Katoh et al., 2002; Capella-Gutierrez et al., 2009; Miller et al., 2010). Displayed at each node as a circle in the tree are the Bayesian posterior probabilities (e.g. a black circle represents probabilities greater or equal to 90%, a grey circle represents probabilities greater or equal to 70%, a white circle represents probabilities less than 70%). The names of fungi isolated from *Z. marina* are shown in green, fungi isolated from other seagrass species are shown in black, and all other fungi are shown in grey. The GenBank accession numbers of the sequences used to build this phylogeny can be found in Table 1 and Tables S2-S4.
**Figure 5:** Phylogenetic placement of seagrass fungal isolates in the Sordariomycetes.

A molecular phylogeny of 28S rRNA genes for isolates in the Sordariomycetes was constructed using Bayesian inference. This alignment was generated using MAFFT (v. 7.402) on the CIPRES Science Gateway web server, trimmed using trimAl (v.1.2) and a phylogenetic tree was inferred on the trimmed alignment with a GTR + I + G model using MrBayes (v. 3.2.2) (Huelsenbeck & Ronquist, 2001; Katoh et al., 2002; Capella-Gutierrez et al., 2009; Miller et al., 2010). Displayed at each node as a circle in the tree are the Bayesian posterior probabilities (e.g. a black circle represents probabilities greater or equal to 90%, a grey circle represents probabilities greater or equal to 70%, a white circle represents probabilities less than 70%). The names of fungi isolated from *Z. marina* are shown in green, fungi isolated from other seagrass species are shown in black, and all other fungi are shown in grey. The GenBank accession numbers of the sequences used to build this phylogeny can be found in Table 1 and Tables S2-S4.

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**Bayesian Probability (BP)**
- BP > 90
- 70 ≤ BP < 90
- BP < 70
**Figure 6:** Phylogenetic placement of seagrass fungal isolates in the Dothideomycetes.

A molecular phylogeny of 28S rRNA genes for isolates in the Dothideomycetes was constructed using Bayesian inference. This alignment was generated using MAFFT (v. 7.402) on the CIPRES Science Gateway web server, trimmed using trimAl (v.1.2) and a phylogenetic tree was inferred on the trimmed alignment with a GTR + I + G model using MrBayes (v. 3.2.2) (Huelsenbeck & Ronquist, 2001; Katoh et al., 2002; Capella-Gutierrez et al., 2009; Miller et al., 2010). Displayed at each node as a circle in the tree are the Bayesian posterior probabilities (e.g. a black circle represents probabilities greater or equal to 90%, a grey circle represents probabilities greater or equal to 70%, a white circle represents probabilities less than 70%). The names of fungi isolated from *Z. marina* are shown in green, fungi isolated from other seagrass species are shown in black, and all other fungi are shown in grey. The GenBank accession numbers of the sequences used to build this phylogeny can be found in Table 1 and Tables S2-S4.
Figure 7: Comparison of the detection fungal genera across methods
A heatmap representing a comparison of the detection of the presence / absence of fungal genera isolated in this study and fungal genera identified in high throughput sequencing data from Ettinger & Eisen (2019). For each fungal genera, we visualize if it was not detected (light grey), detected in one method (dark grey) or detected by both methods (black) for each sample type / isolation source (leaf, root, rhizome, sediment).
Table 1: Fungi isolated from the seagrass, Zostera marina
Here we report the taxonomic information for each fungal isolate (Class, Order, Molecular ID), provide the GenBank accession number for the ITS-28S rRNA gene sequence for each isolate, and report on the tissue the isolate was obtained from (e.g. leaf, leaves and roots, rhizome, root, roots and rhizome, seawater or sediment). We also report on whether the genus of each isolate includes marine fungal representatives based on the consensus compiled in Jones et al. (2015) and whether the genus of each isolate was detected in the ITS amplicon data in Ettinger & Eisen (2019). Organisms for which a taxonomic identification below the order level was not possible, have a “NA” value for these columns.

Table 2: Bacteria isolated from the seagrass, Zostera marina
Here we report the taxonomic information for each bacterial isolate (Class, Order, Molecular ID), provide the GenBank accession number for the 16S rRNA gene sequence for each isolate, and report on the tissue the isolate was obtained from (e.g. leaf, leaves and roots, rhizome, root, roots and rhizome, seawater or sediment). We also report the molecular ID of the top BLAST match, the BLAST % identity to the bacterial isolate and the GenBank accession number for the 16S rRNA gene sequence for the top BLAST match.

Table 3: Oomycota isolated from the seagrass, Zostera marina
Here we report the taxonomic information for each oomycete isolate (Class, Order, Molecular ID), provide the GenBank accession number for the 28S rRNA gene sequence for each isolate, and report on the tissue the isolate was obtained from (e.g. leaf, leaves and roots, rhizome, root, roots and rhizome, seawater or sediment). We also report the molecular ID of the top BLAST match, the BLAST % identity to the oomycete isolate and the GenBank accession number for the 28S rRNA gene sequence for the top BLAST match.
Author Contributions
CLE conceived and designed the experiments, performed sampling, analyzed the data, prepared figures and/or tables, wrote and reviewed drafts of the paper. JAE advised on data analysis, edited and reviewed drafts of the paper.

DNA Deposition
The consensus sequences were deposited at GenBank for the fungal ITS2-28S rRNA gene regions under accession no. MN543905-MN544012, for the bacterial 16S rRNA gene under accession no. MN931878-MN931917, and for the oomycete 28S rRNA gene under accession no. MN944508-MN944509.

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