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## FUNGAL ENDOPHYTES VARY BY SPECIES, TISSUE TYPE, AND LIFE CYCLE STAGE IN INTERTIDAL MACROALGAE<sup>1</sup>

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Fungal symbionts of terrestrial plants are among the most widespread and well-studied symbioses, relatively little is known about fungi that are associated with macroalgae. To fill the gap in marine fungal taxonomy, we combined simple culture methods with amplicon sequencing to characterize the fungal communities associated with three brown (*Sargassum muticum*, *Pelvetia canaliculata*, and *Himantalia elongata*) and two red (*Mastocarpus stellatus* and *Chondrus crispus*) macroalgae from one intertidal zone. In addition to characterizing novel fungal diversity, we tested three hypotheses: fungal diversity and community composition vary (i) among species distributed at different tidal heights, (ii) among tissue types (apices, mid-thallus, and stipe), and (iii) among “isomorphic” *C. crispus* life cycle stages. Almost 70% of our reads were classified as Ascomycota, 29% as Basidiomycota, and 1% that could not be classified to a phylum. Thirty fungal isolates were obtained, 18 of which were also detected with amplicon sequencing. Fungal communities differed by host and tissue type. Interestingly, *P. canaliculata*, a furoid at the extreme high intertidal,

did not show differences in fungal diversity across the thallus. As found in filamentous algal endophytes, fungal diversity varied among the three life cycle stages in *C. crispus*. Female gametophytes were also compositionally more dispersed as compared to the fewer variable tetrasporophytes and male gametophytes. We demonstrate the utility of combining relatively simple cultivation and sequencing approaches to characterize and study macroalgal–fungal associations and highlight the need to understand the role of fungi in near-shore marine ecosystems.

**Key index words:** evolutionary ecology; fungi; holobiont; life cycle; marine; mycology; seaweed; symbiosis

**Abbreviations:** ASV, amplicon sequence variant; CTAB, cetrimonium bromide; GLMM, generalized linear mixed model; LSD, log-transformed sequencing depth; MEA, malt agar extract; mGLM, multivariate generalized linear model; nMDS, non-metric dimensional scaling; OTU, operational taxonomic unit; PDA, potato dextrose agar; PIE, probability of interspecific encounter; WYM, Wickerham’s yeast medium

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Fungi play key ecological and evolutionary roles in terrestrial and aquatic ecosystems (reviewed in Peay et al. 2016). Nearly all plants interact with fungi in the soil, which support the absorption of

water, acquisition of minerals, and disease resistance (Behie and Bidochka 2014). At the same time, fungi represent the largest source of plant pathogens (Blackwell 2011) and an important group of decomposers in terrestrial (Baldrian 2017) and in freshwater systems (Gessner et al. 2007). While fungal ecology has been widely studied in terrestrial ecosystems (O'Brien et al. 2021), recent studies have shown that marine angiosperms—seagrasses (e.g., Ettinger and Eisen 2019) and mangroves (Lee et al. 2019)—also harbor diverse fungal communities in different tissues (e.g., leaves vs. roots). Many of these fungi cannot be assigned taxonomically and are likely undescribed species. Nevertheless, the diversity and ubiquity of fungi in nearshore marine ecosystems (Kohlmeyer and Kohlmeyer 1979, Richards et al. 2012) suggests they may fulfill functional roles in marine angiosperms, similar to their terrestrial counterparts (Ettinger and Eisen 2019).

Apart from pioneering studies on fucoids and their associated ascomycetes (Kohlmeyer and Kohlmeyer 1979, Stanley 1992), even less is known about the occurrence and functional roles of fungi as algal symbionts (Richards et al. 2012). Macroalgae are critical ecosystem engineers in inter- and subtidal ecosystems (Lüning 1990) and form the basis of a productive global aquaculture industry valued at \$6 billion USD per annum (Ferdouse et al. 2018). We are now beginning to appreciate the complex interactions between macroalgae and the associated communities of prokaryotes and eukaryotes (i.e., the holobiont; Meyer-Abich 1934, Margulis 1990, Bordenstein and Theis 2015). The relationship between macroalgal hosts and fungal symbionts—or algalicolous fungi—can range from mutualistic to pathogenic (Correa 1994, Potin 2012). Most of the research on the macroalgal holobiont has focused on the relationships between macroalgae and bacteria (reviewed in Egan et al. 2013, van der Loos et al. 2019) or on filamentous algae living as endophytes in macroalgal hosts (e.g., Correa and McLachlan 1992, Schoenrock et al. 2015), often from the perspective of disease. Nevertheless, macroalgal–fungal relationships have been recognized. For example, the brown alga *Ascophyllum nodosum* and the ascomycete *Stigmidium ascophylli* (basonym *Mycosphaerella ascophylli*) have synchronized reproduction cycles (Kohlmeyer and Kohlmeyer 1979, Stanley 1992). *Stigmidium ascophylli* is also an endophyte of the brown alga *Pelvetia canaliculata* and both brown macroalgae do not occur naturally without the fungus (Webber 1967, Kohlmeyer and Kohlmeyer 1979). The fungus may help the algal host withstand desiccation at low tide (Garbary and London 1995). Similarly, *Lautitia danica* is a parasitic fungus specialized to attack the reproductive tissues of the red alga *Chondrus crispus* (Schatz 1984). Due to limited interest in algal-associated fungi, these detailed examples are rather exceptions in the literature and we know comparatively little

about the role fungi play in the ecology and evolution of macroalgae.

More recently, algalicolous fungal diversity has been explored using high-throughput amplicon sequencing (Wainwright et al. 2017, 2019). Similar to amplicon sequencing studies of fungal diversity associated with marine animals (e.g., Amend et al. 2012, Bonthond et al. 2018) or angiosperms (e.g., Ettinger and Eisen 2019), these studies corroborate that a high proportion of the detected fungal taxa are either novel or absent in available reference databases, such as GenBank and UNITE (Abarenkov et al. 2010), which hampers amplicon sequencing-based studies of marine fungi, including algalicolous groups. Both a lack of sequence data for described species and poor availability of species descriptions of marine fungi more generally contribute to this lack of reference data. Therefore, conducting new collection efforts is imperative to begin filling in taxonomic gaps and develop a baseline understanding of the diversity and functional roles of algalicolous fungi. Culture-independent approaches, such as ITS rDNA amplicon sequencing, should be combined with traditional cultivation efforts by means of capturing the observed novelty partially in new cultures that can be preserved as reference material and used for subsequent experimental manipulation.

In this study, we combined culture-independent amplicon sequencing with a cultivation assay to explore the cultivability of detected novel taxa—a first step to characterizing algalicolous fungi. We targeted two red (*Mastocarpus stellatus* and *Chondrus crispus*) and three brown (*Sargassum muticum*, *Pelvetia canaliculata*, and *Himanthalia elongata*) benthic macroalgal species that are common across the coastlines of the Northeastern Atlantic (Fig. 1) to place the detected fungal diversity in a broader ecological context for future work.

First, we tested whether fungal assemblages associated with intertidal macroalgae vary among macroalgal hosts from the same locality. To the best of our knowledge, such specific associations for red algae have not been described. *Mastocarpus stellatus* and *Chondrus crispus* both belong to the Order Gigartinales (Rhodophyta) and are observed submerged in tide pools or exposed to rocks throughout the mid to low-intertidal (Bunker et al. 2017). Brown algae, however, are thought to have the highest fungal diversity, possibly due to greater thallus complexity (Raghukumar 2017), but there have been few efforts to document fungal diversity more broadly across algal taxa. *Sargassum muticum*, *Pelvetia canaliculata*, and *Himanthalia elongata* belong to the order Fucales (Ochrophyta), with fungal symbionts already recorded in *P. canaliculata* (Kohlmeyer and Kohlmeyer 1979, Seloisse and Le Tacon 1998). *Sargassum muticum* is a globally invasive species and therefore a novel host in the intertidal zone of the Northeast Atlantic (Engelen et al. 2015). Moreover, at low tide, both *S. muticum* and *H. elongata* inhabit tide

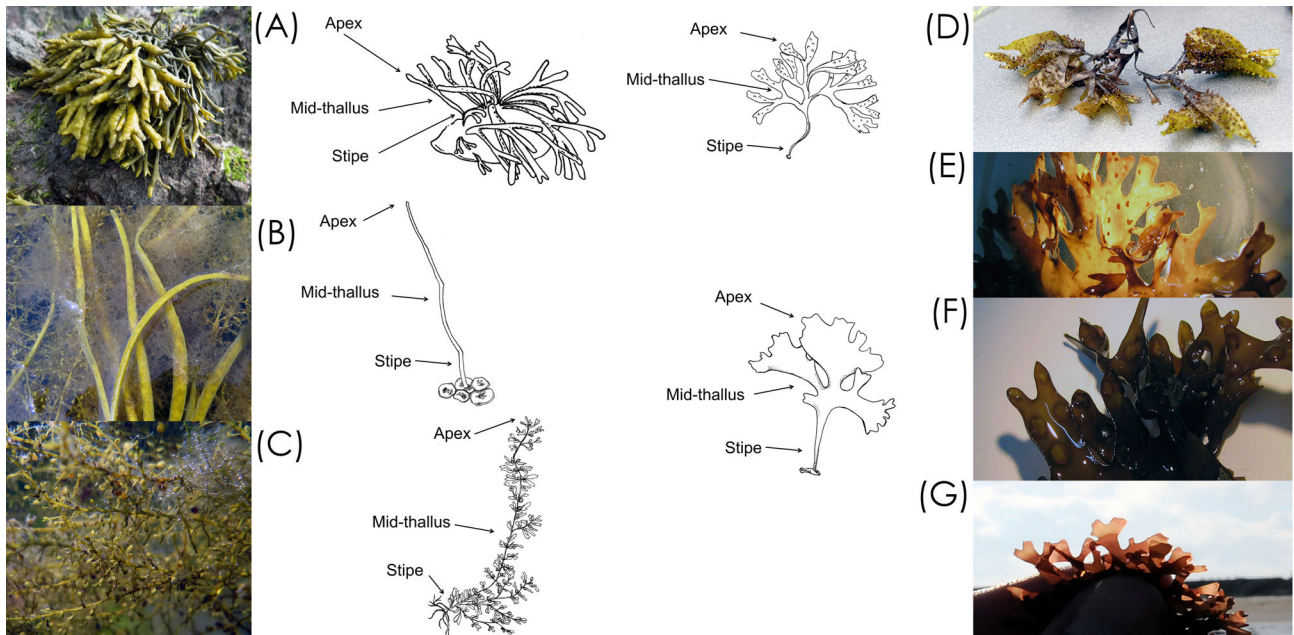


FIG. 1. Photographs and thallus drawings of macroalgal species sampled at Wembury. (a) *Pelvetia canaliculata*, (b) *Himanthalia elongata*, (c) *Sargassum muticum*, (d) *Mastocarpus stellatus*, (e) *Chondrus crispus* tetrasporophyte, (f) *C. crispus* female gametophyte, and (g) *C. crispus* male gametophyte. Drawings depict each species and how we classified the thallus for our study with the apex, mid-thallus, and stipe. Photos by S.A. Krueger-Hadfield and drawings by K.M. Schoenrock.

pools lower on the shore, whereas *P. canaliculata* is found at the uppermost limit of the intertidal in its own discrete zone (Bunker et al. 2017). These five species were selected to compare the abundance and distribution of fungal endophytes across algal taxa.

Second, we tested whether fungal assemblages vary among tissue types within the host: apices (newest tissue), mid-thallus, and stipe/basal thallus (oldest tissue; Fig. 1). Seagrass-associated fungi vary on leaves, roots, and rhizomes (Ettinger and Eisen 2019). Wainwright et al. (2019) found differences in fungal communities on leaves, vesicles, and the holdfast in *Sargassum ilicifolium*. Apart from *Lautitia danica* infecting *Chondrus crispus* reproductive tissues (Schatz 1984), the location within the thallus of fungal endophytes is unknown. By focusing on distinct thallus regions across the five species, we aimed to investigate the specificity with which fungal endophytes may associate macroalgal hosts.

Third, we tested whether fungal assemblages differed among “isomorphic” male gametophytes, female gametophytes, and tetrasporophytes in *Chondrus crispus*. Eukaryotes display tremendous life cycle variation (Beukeboom and Perrin 2014), but we do not understand the drivers of this diversity (Mable and Otto 1998, Krueger-Hadfield 2020). However, heteromorphic stages, such as the crustose tetrasporophytes and foliose gametophytes found in *Mastocarpus stellatus*, are phenotypically distinct (Lubchenco and Cubit 1980), and harbor different pro- and eukaryotic microbial communities (Lemay et al.

2018). Isomorphic stages, on the other hand, continue to puzzle evolutionary biologists because the stages are often assumed to be ecologically equivalent and therefore cannot exploit different niches (Valero et al. 1992). Combining genetic and ecological predictions, Hughes and Otto (1999) demonstrated that even subtle differentiation between “isomorphic” stages was sufficient to generate evolutionarily stable and morphologically similar stages in the same life cycle. While ecologically relevant phenotypes differ between stages, empirical tests of differentiation remain rare (Thorner 2006, Krueger-Hadfield 2020). In haploid-diploid algae, one stage may serve as an escape from pathogens (e.g., viruses in the coccolithophore *Emiliania huxleyi*; Frada et al. 2008). This may occur in *C. crispus* (Bouarab et al. 2001) in which a pathogenic algal endophyte preferentially infects tetrasporophytes and to a lesser extent male gametophytes, whereas female gametophytes are rarely infected (Correa et al. 1987, Plumb 1999, Krueger-Hadfield 2011). This stage-specific infection is manifest at the shore-level in which most *C. crispus* populations are heavily gametophyte biased (Krueger-Hadfield 2011, Krueger-Hadfield et al. 2013b). Fungal diversity may also vary among life cycle stages (but see Mathieson and Prince 1973). This might be an important component to understanding the evolutionary stability of life cycles with morphologically similar stages as well as the ecological consequences of fungal symbioses in algae. However, we do not know if fungi are differentially abundant in different life cycle stages.

Here, we tested the effect of the macroalgal host, tissue type, and, for *Chondrus crispus*, life cycle stage on fungal community composition. This study explores the diversity of fungal endophytes in macroalgae, combining culture-dependent and culture-independent approaches, using working hypotheses that may be useful for future studies addressing the role of fungi in the macroalgal holobiont.

#### MATERIALS AND METHODS

**Sample collection.** Macroalgal thalli and seawater samples were collected from Wembury, Devon, UK (GPS: 50.317620, -4.084406) on July 12, 2018. All thalli were reproductive at the time of sampling and all species were identified based on morphology. *Pelvetia canaliculata* has the highest intertidal distribution among the species sampled, occupying a distinct upper intertidal zone. *Himanthalia elongata* and *Sargassum muticum* were sampled from mid-intertidal pools. Both *P. canaliculata* and *S. muticum* are hermaphroditic, but *H. elongata* is dioecious with separate sexes. We did not distinguish between male and female *H. elongata* individuals. We sampled female gametophytic *Mastocarpus stellatus* (papillate) thalli and all three life cycle stages in *C. crispus* from within tide-pools or on exposed rocks in the mid-intertidal zone where both species overlap. *Mastocarpus stellatus* females may be sexual or apomictic (asexual; reviewed in Dudgeon et al. 2017) in this part of the species range, but we did not distinguish among females as this requires subsequent culturing of thalli to determine whether a female frond is sexual or asexual (Guiry and West 1983, Krueger-Hadfield et al. 2013b).

Ten thalli of *Sargassum muticum*, *Pelvetia canaliculata*, *Himanthalia elongata*, and *Mastocarpus stellatus* were sampled with gloves and stored in separate polyethylene bags. We sampled fifty *Chondrus crispus* thalli in the same manner to obtain at least five male gametophytes, female gametophytes, and tetrasporophytes (Krueger-Hadfield and Hoban 2016). We determined the ploidy and sex of *C. crispus* thalli in the lab based on reproductive structures (Tveter-Gallagher et al. 1980, Krueger-Hadfield et al. 2013a, 2015) and randomly selected five of each stage for subsequent analyses.

Before further processing, all algae were surface sterilized by washing the thallus for 10 s in absolute ethanol followed by 10 s in autoclaved MilliQ water. For all algae, we separated thalli into three tissue types: stipe, mid-thallus, and apex (Fig. 1; see also Correa et al. 1987). All apices bore reproductive structures. The carposporophytes for both *Chondrus crispus* and *Mastocarpus stellatus* as well as the tetrasporangial sori in *C. crispus* could also be found for some tissue in the mid-thallus. We divided the long, strap-like thalli of *Himanthalia elongata* into these same subsections, but considered the stipe as the basal portion of the thallus that emerged from the characteristic buttons. We did not sample the button (Fig. 1).

**Cultivation-dependent approach.** Five to ten fragments of approximately 1–2 cm of each tissue type from each thallus were placed on three different culture media: malt agar extract, potato dextrose agar, and Wickerham's yeast medium. All media were prepared with artificial seawater and supplemented with 10 mg · L<sup>-1</sup> chloramphenicol to inhibit bacterial growth. Plates were incubated at 15°C under low light conditions, 12 h · d<sup>-1</sup> and repeatedly inspected for fungal growth over the following days/weeks. Fragments of mycelia or yeast cells were transferred to new plates with the corresponding culture medium when growth was observed. To reduce the number of isolates for subsequent processing, *Penicillium*- and *Aspergillus*-like fungi were identified based on

visual inspection. Species from these two genera likely result from environmental sources and grow and sporulate extremely fast on common culture media. Therefore, we decided to exclude them from the subsequent isolation efforts. Therefore, it should be noted that algicolous *Aspergillus* spp. and/or *Penicillium* spp. may exist, but were not targeted in the cultivation assay in this study.

Fungal biomass was collected from agar plates either with a sterile loop (yeast) or using a sterile scalpel blade and transferred to a lysis tube. DNA was extracted from the biomass using the Qiagen DNeasy Blood and Tissue Kit with minor modifications. Samples were initially lysed using a beadbeater before following the manufacturer's protocol. Extracted DNA quantity was determined using a Nanodrop spectrophotometer and stored at -20°C. For PCR, the internal transcribed spacer (ITS) region was amplified with primers ITS1F (5'-CTTGGCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') from the extracted DNA using GoTaq<sup>®</sup> DNA Polymerase (Promega) and the following temperature regime: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final 72°C for 5 min. PCR products were verified using agarose gel electrophoresis before submission for Sanger sequencing in both directions (Source Biosciences, Nottingham, UK). Merged and quality-filtered sequences were deposited in GenBank (Accession numbers: MZ081427–MZ081458, see also Table S1 in the Supporting Information). Full ITS rDNA sequences (including ITS1, 5.8S, and ITS2) were classified in Mothur v.1.43.0 (Schloss et al. 2009), using the UNITE reference database (Abarenkov et al. 2010) and the naïve Bayesian classification method using 100 bootstrap iterations (Wang et al. 2007). In addition, they were compared in a quick BLAST search with settings adjusted to include only ex-type sequence data.

**High-throughput amplicon sequencing.** From the *Sargassum muticum*, *Pelvetia canaliculata*, *Himanthalia elongata*, and *Mastocarpus stellatus* individuals, five thalli per species were haphazardly selected and small fragments of each tissue type (i.e., stipe and mid-thallus, apex) were preserved in absolute ethanol for DNA extraction. We preserved the same three tissues for five thalli of each life cycle stage in *Chondrus crispus*. DNA was extracted using an adapted phenol:chloroform protocol. Algal tissue was cut with sterile scissors to small fragments inside 2 mL tubes and suspended in 900 µL 2% cetrimerium bromide (CTAB) buffer. All samples were vortexed and incubated on a shaker with 1 µL of 20 mg · mL<sup>-1</sup> proteinase K at 56°C for 1 h. Then, 900 µL phenol:chloroform:isoamylalcohol (25:24:1) was added and all tubes were vortexed and centrifuged for 10 min at 12,000g. For each tube, the upper aqueous phase was transferred to a new 2 mL containing 900 µL phenol:chloroform:isoamylalcohol solution and the centrifugation step was repeated. The subsequent aqueous phases were transferred to 2 mL tubes with 700 µL chloroform:isoamylalcohol, vortexed and centrifuged at 12,000g for 10 min. The aqueous phases were transferred to new 2 mL tubes with 700 µL chloroform:isoamylalcohol and the centrifugation step was repeated. The upper phases were transferred to 1.5 mL tubes and precipitated with 600 µL cold (4°C) isopropanol at 12,000g for 10 min. After removing the isopropanol, the pellets were washed with 2 rounds of absolute ethanol and two rounds of 70% ethanol solution, centrifuging at 12,000g. DNA pellets were dissolved in nuclease-free water and shipped to the Integrated Microbiome Resource (Dalhousie University, Halifax, Canada) for sequencing the ITS2 rDNA region using the primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3', Turenne et al. 1999) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White et al. 1990) on the Illumina MiSeq platform using the 600 cycle MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA).

Demultiplexed fastq files were quality processed in Mothur v.1.43.0 (Schloss et al. 2009). To compare the amplicon reads with the Sanger sequences from cultures, we chose to generate a community matrix based on amplicon sequence variants (ASVs; i.e., unique sequence reads) instead of using a 3% clustering criterion to generate operational taxonomic units (OTUs). The UNITE reference database (Abarenkov et al. 2010) was used to classify all ASVs. The community matrix was purged from sequences not classified to the fungal kingdom, singletons, and samples with <100 reads. *Himanthalia elongata* were excluded from downstream analyses as only a few samples yielded more than 100 reads after quality filtering. Raw sequencing reads were deposited in the sequence read archive (SRA) under the accession number PRJNA727013.

**Analyses.** To analyze differences in fungal community composition between macroalgae, multivariate generalized linear models were fitted using the mvabund package (Wang et al. 2012). The models included the factor species in which the factor tissue type (levels: stipe, mid-thallus, and apex) was nested. To account for differences in sequencing depth across samples, the natural log of the sequencing depth (LSD) was included as an offset. The same model was fitted on the ASV community matrix with and without the four water samples. To analyze differences in community composition between *Chondrus crispus* life cycle stages, a third mGLM was fitted on the ASV matrix trimmed to *C. crispus* samples only. Besides the natural log of the sequence read count as offset, the factors tissue type (stipe, mid-thallus, and apex), life cycle stage (tetrasporophyte, female, and male), and the corresponding interaction term were included. For the multivariate output, using the anova.manyglm function, the univariate models were resampled within individuals (to account for the dependence of the stipe, mid-thallus, and apex samples from the same individual) with 500 bootstrap iterations and a Likelihood-Ratio-Test (LR). Explained deviances were extracted for each ASV for the factor genus and the factor life cycle stage and converted to percentages. To visualize patterns in community composition, we used the residuals from an mGLM including only the LSD as an offset for non-metric dimensional scaling (nMDS), with the vegan package (Oksanen et al. 2020). Group centroids and their 95% confidence regions were obtained by dividing the standard error with the  $\chi^2_{0.95}$  confidence value with 2 degrees of freedom, as implemented in the vegan package.

We analyzed asymptotic richness based on the chao1 estimator (Chao and Bunge 2002) and the probability of interspecific encounter (PIE) as a measure of evenness (McGlinn et al. 2019) with generalized linear mixed models. Models for asymptotic richness assumed a Poisson distribution with a natural logarithm in the link function and for PIE, which was logit-transformed, a Gaussian distribution in the original scale. To compare diversity parameters among species, the factor tissue type (stipe, mid-thallus, and apex) was nested under the factor species and individual identity was included as a random intercept. For the life cycle stages of *Chondrus crispus*, the factors life cycle stage, tissue type, and their interaction were included as fixed variables and individual identity as a random intercept. The 95% confidence intervals of all displayed estimates were obtained by bootstrapping the models with 1000 iterations. Models for richness and evenness were fitted using the lme4 package (Bates et al. 2015).

## RESULTS

*Fungal endophyte amplicon sequencing summary.* After processing and quality trimming of the

sequences, 74,310 reads remained that were classified to the fungal kingdom from 72 samples. Read counts per sample ranged from 105 to 7811 with a median of 579.5. Of all reads, 69.3% were classified to Ascomycota, 28.7% to Basidiomycota, and 1.5% were not classified to phylum level. Reads classified to other phyla (Mortierellomycota, Chytridiomycota, Glomeromycota, Mucoromycota, Kickxellomycota, and Entomophthoromycota) were all detected below 1% of the total read count. The most abundant ASV in *Chondrus crispus* was classified to the genus *Didymella*. *Mastocarpus stellatus* and *Sargassum muticum* were dominated by ASV7 which was classified to the genus *Mycosphaerella*—this was also one of the more abundant ASVs detected in the seawater. The dominant ASV in *Pelvetia canaliculata* was not classified beyond the phylum Ascomycota. It was similar to the *Didymella* ASV in *C. crispus* and was either absent or at very low abundance in seawater samples (Fig. 2).

**Comparisons between cultures and ASVs.** In total, 30 fungi were isolated from the five algal hosts (Tables 1, S1). Based on the ITS sequence, all isolates could be classified at least to the genus level with the UNITE reference database and belonged with two exceptions to Ascomycota. One isolate from *Chondrus crispus* was classified as a Basidiomycete (*Rhodotorula muscilaginosa*) and an isolate from *Mastocarpus stellatus* as Mucoromycota (*Mucor laxor-rhizus*). For 18 isolates, an identical ASV was found and in some cases, these corresponded to more abundant (>5% relative count) ASVs, including ASV7 (*Mycosphaerella tassiana*), ASV12 (*Cladosporium cladosporioides*), ASV16 (*Alternaria metachromatica*), and ASV57 (*Didymella glomerata*). The other isolates were identical or similar to rarer taxa and nine isolates were not detected with high-throughput amplicon sequencing (Table 1). Similarity scores to ex-type sequences in Genbank obtained with BLAST ranged from 90.2% to 100% (median 99.4%). The strain with the least similar BLAST hit was resolved with *Sodiomyces tronii* (Glomerellales) and was classified with UNITE to *Acrostalagmus luteoalbus* (Glomerellales).

**Amplified sequence variation among macroalgal species and tissue type.** In terms of ASV composition, differences were found among macroalgal species (LR, Deviance<sub>4, 67</sub> = 4972,  $P = 0.002$ ) and among tissue types (LR, Deviance<sub>10, 59</sub> = 2961,  $P = 0.002$ , Table S2 in the Supporting Information). Post hoc pairwise comparisons among species and seawater resolved holm free stepdown adjusted  $P$ -values <0.05 (observed holm free stepdown adjusted test statistic >572) for all combinations except between *Mastocarpus stellatus* and *Sargassum muticum* ( $P = 0.104$ , observed holm free stepdown adjusted test statistic = 362). Variation in community composition among species and seawater was also noticeable from the nMDS plot with partial or no overlap in the species centroid and 95% confidence regions (Fig. 3a).

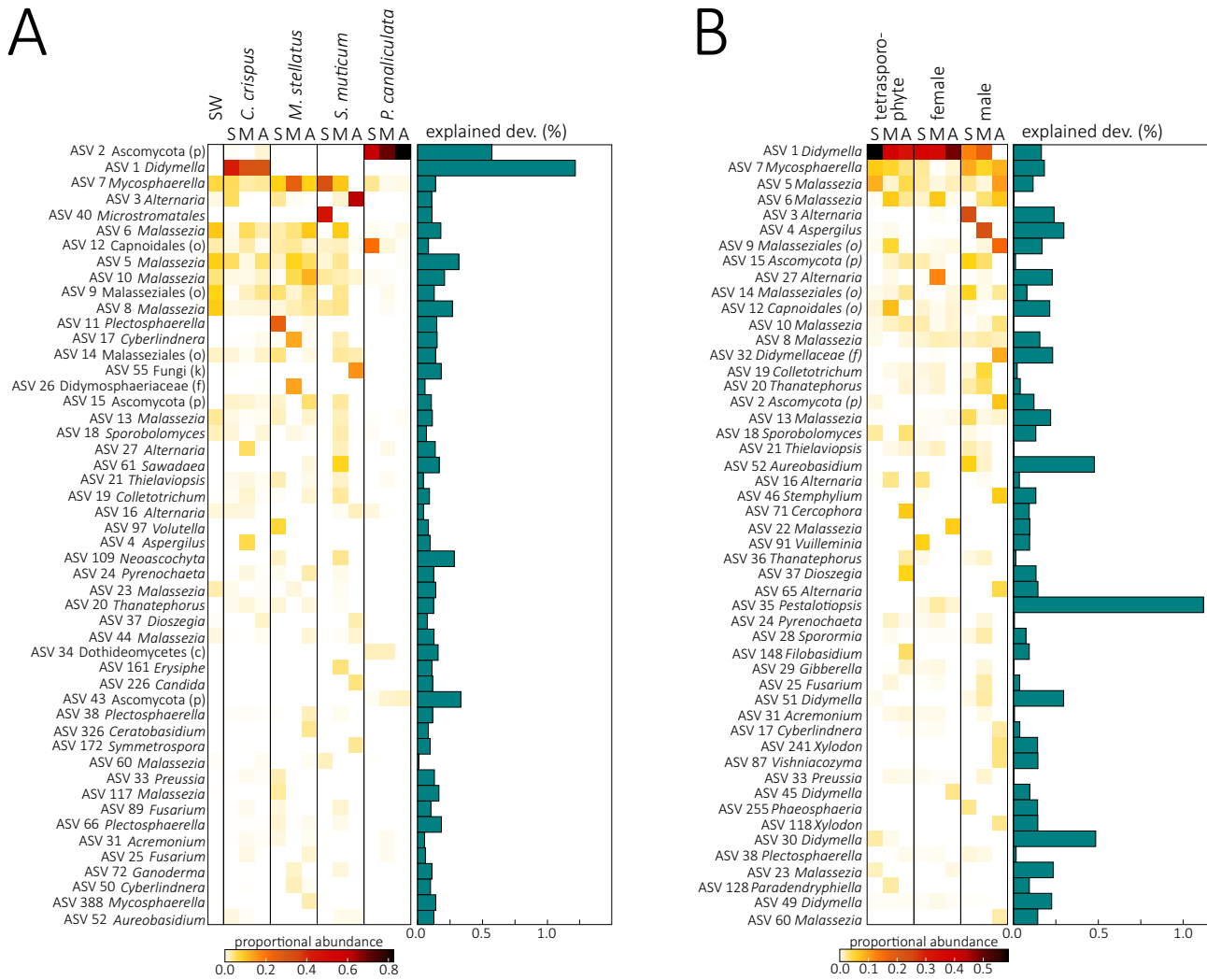


Fig. 2. Heatmaps displaying the relative abundance (group averages from proportional abundances) of the 50 most abundant ASVs and the deviance explained by each ASV in the model in percentages (a) by species and (b) and life cycle stages of *Chondrus crispus*.

Asymptotic richness varied among species (Wald test,  $\chi^2_{3,64} = 17.181$ ,  $P < 0.001$ ) and also among tissue types (Wald test,  $\chi^2_{8,56} = 39.233$ ,  $P < 0.001$ ). ASV richness was highest in *Chondrus crispus* and lowest in *Sargassum muticum*. Post hoc pairwise comparisons resolved differences for some combinations (Fig. 3b, Table S3 in the Supporting Information). Evenness (measured by logit PIE) varied among species (Wald test,  $\chi^2_{3,64} = 23.13$ ,  $P < 0.001$ ), with higher values for *C. crispus* and *Mastocarpus stellatus*, but no significant effect of tissue type (Fig. 3c, Table S3).

**Fungal ASV variation between *Chondrus crispus* life cycle stages.** Amplicon sequence variant composition varied among *Chondrus crispus* life cycle stages (LR, Deviance<sub>2,37</sub> = 2088,  $P = 0.012$ ), among tissues (LR, Deviance<sub>2,35</sub> = 1765,  $P = 0.002$ ), and also the interaction between life cycle stages and tissue type (LR, Deviance<sub>4,31</sub> = 902,  $P = 0.006$ ; Table S2). Post hoc

pairwise comparisons among life cycle stages indicated significant differences for each combination (observed holm free stepdown adjusted test statistic  $\geq 667$ ,  $P \leq 0.034$ ). The fungal communities associated with female gametophytes appeared compositionally more dispersed, while the fungi associated with tetrasporophytes and male gametophytes were less variable within the life cycle stage (Fig. 4a).

Asymptotic ASV richness differed among tissue types (Wald test,  $\chi^2_{2,37} = 8.26$ ,  $P < 0.001$ ), but not among life cycle stages (Wald test,  $\chi^2_{2,35} = 4.91$ ,  $P = 0.086$ ). However, the interaction between tissue type and life cycle stage was significant (Wald test,  $\chi^2_{4,31} = 43.37$ ,  $P < 0.001$ ) and post hoc comparisons showed that richness in apical tissue differed among life cycle stages, being higher for female apices compared to tetrasporophyte and male apices (Fig. 4b, Table S3). Further, tetrasporophytes and females

TABLE 1. Summary of the fungal isolates obtained in this study.

Algal host	LCS <sup>1</sup>	Tissue	ASV	$\Delta nt^2$	UNITE taxonomy <sup>3</sup> Species	Best BLAST hit <sup>4</sup>		
						Species	%	Accession
<i>Himantalia elongata</i>		Apex	7451		<i>Westerdykella multisporea</i>	<i>Westerdykella dispersa</i>	96.3	NR_111187
<i>Chondrus crispus</i>	⊕	Mid	0016		<i>Alternaria metachromatica</i>	<i>Alternaria conjuncta</i>	99.5	MH861940
	⊕	Stipe	1772	>10	<i>Metschnikowia reukaufii</i>	<i>Metschnikowia zobellii</i>	99.4	NR_138246
	♀	Stipe	0170		<i>Acremonium exuviarum</i>	<i>Emericellopsis stolckiae</i>	98.4	MH871826
	♂	Apex	0128		<i>Stemphylium vesicarium</i>	<i>Paradendryphiella arenariae</i>	98.6	MH857747
	♂	Stipe	3868		<i>Rhodotorula mucilaginoso</i>	<i>Rhodotorula mucilaginoso</i>	99.5	KY104874
<i>Mastocarpus stellatus</i>		Mid	0057		<i>Didymella glomerata</i>	<i>Epicoccum huancayense</i>	98.1	MH861244
		Apex	0670		<i>Didymella exigua</i>	<i>Epicoccum nigrum</i>	97.6	NR_165920
		Apex	8526	>10	<i>Mucor laxorrhizus</i>	<i>Mucor laxorrhizus</i>	100	NR_103642
<i>Pelvetia canaliculata</i>		Stipe	0012		<i>Cladosporium cladosporioides</i>	<i>Cladosporium angustisporum</i>	100	MH863862
		Stipe	0170		<i>Acremonium exuviarum</i>	<i>Emericellopsis stolckiae</i>	98.4	MH871826
		Stipe	4182	>10	<i>Chlamydocillium</i> sp.	<i>Chlamydocillium cyanophilum</i>	99.8	NR_153914
		Stipe	0016		<i>Alternaria metachromatica</i>	<i>Alternaria conjuncta</i>	99.8	MH861940
		Mid	0016		<i>Alternaria metachromatica</i>	<i>Alternaria conjuncta</i>	99.8	MH861940
		Stipe	0016		<i>Alternaria metachromatica</i>	<i>Alternaria conjuncta</i>	99.8	MH861940
		Stipe	0125		<i>Didymella exigua</i>	<i>Epicoccum phragmospora</i>	98.3	NR_165920
		Mid	0125		<i>Didymella exigua</i>	<i>Epicoccum phragmospora</i>	98.3	NR_165920
		Apex	0128	1	<i>Stemphylium vesicarium</i>	<i>Paradendryphiella arenariae</i>	99.6	MH857747
		Mid	0007		<i>Mycosphaerella tassiana</i>	<i>Cladosporium phlei</i>	100	NR_120013
<i>Sargassum muticum</i>		Apex	0012		<i>Cladosporium cladosporioides</i>	<i>Cladosporium xylophilum</i>	100	MH863875
		Stipe	0316	1	<i>Aspergillus awamori</i>	<i>Aspergillus foetidus</i>	100	NR_163668
		Mid	1777	>10	<i>Acrostalagmus luteoalbus</i>	<i>Sodiomyces tronii</i>	90.2	NR_155790
		Mid	4548	>10	<i>Tolyposcladium</i> sp.	<i>Tolyposcladium cylindrosporium</i>	99.6	NR_167967
		Stipe	9372	>10	<i>Fusarium proliferatum</i>	<i>Fusarium concentricum</i>	99.4	MH862659
		Stipe	0057		<i>Didymella glomerata</i>	<i>Epicoccum huancayense</i>	98.1	MH861244
		Apex	0125		<i>Didymella exigua</i>	<i>Epicoccum phragmospora</i>	98.3	NR_165920
		Apex	0621	3	<i>Paraphaeosphaeria pilleata</i>	<i>Paraphaeosphaeria sardoa</i>	98.1	NR_145167
		Mid	5985	>10	<i>Daldinia raimundi</i>	<i>Daldinia martinii</i>	100	MH862913
		Stipe	5985	>10	<i>Daldinia raimundi</i>	<i>Daldinia martinii</i>	100	MH862913
	Apex	5985	>10	<i>Daldinia raimundi</i>	<i>Daldinia martinii</i>	100	MH862913	

<sup>1</sup>Life cycle stages (LCS) identified for *C. crispus*: ⊕ = tetrasporophyte, ♀ = female, ♂ = male.

<sup>2</sup>Number of nucleotides differing between isolate and most related ASV when not identical.

<sup>3</sup>Taxonomic classification using the UNITE reference database, with >50 bootstrap support values.

<sup>4</sup>BLAST parameters were set to include only type specimens. For BLAST hits with 100% similarity, multiple 100% may exist and are not displayed.

were clearly dominated by ASV1 (*Didymella*), in contrast to males, where this ASV was completely absent in the apices (Fig. 2b). For evenness, the main effect of the life cycle stage was detected (Wald test,  $\chi^2_{2,35} = 9.74$ ,  $P = 0.008$ ) with a marginally significant difference between female and male gametophytes in which females exhibited greater evenness (Tukey method,  $z$ -ratio = 2.893,  $P = 0.049$ ; Fig. 4c).

## DISCUSSION

Using simple cultivation conditions, we successfully recovered 18 cultures with identical sequence matches to ASVs from amplicon sequencing, nine of which were abundant in the host thalli (e.g., ASV7, ASV12, and ASV57; classified to *Mycosphaerella* sp., *Cladosporium* sp., and *Didymella* sp., respectively). Combining the culture-dependent and -independent approaches, our data suggest a high degree of a novelty among algicolous fungi; some of these novel taxa can be isolated with ease. This opens the door to further understanding the ecological and evolutionary interactions between fungal endophytes and their macroalgal hosts. Unlike

previous work that suggested brown algae harbored greater fungal diversity (Raghukumar 2017), the red alga *Chondrus crispus* had the greatest asymptotic richness. Moreover, as found previously in different species of *Sargassum* (Wainwright et al. 2019) as well as in the seagrass *Zostera marina* (e.g., Ettinger and Eisen 2019), fungal diversity varied among tissue types. Depending on the taxon, we found the greatest fungal diversity in the stipes (e.g., *C. crispus*) or the apices (e.g., *Mastocarpus stellatus*). Finally, fungal communities differed among life cycle stages in *C. crispus*.

*Cultivation and detection of specific fungal taxa.* In line with other studies that have recently explored marine host-associated fungi (e.g., Amend et al. 2012, Wainwright et al. 2017, 2019, Bonthond et al. 2018), we found a high degree of taxa that could not be classified suggesting that they are novel species or have no available reference sequences. Our study shows that with simple culturing media and conditions, many algicolous fungi detected with amplicon sequencing can also be recovered in culture.

One of the most noticeable ASVs in our dataset is ASV2, the dominant taxon associated with *Pelvetia*



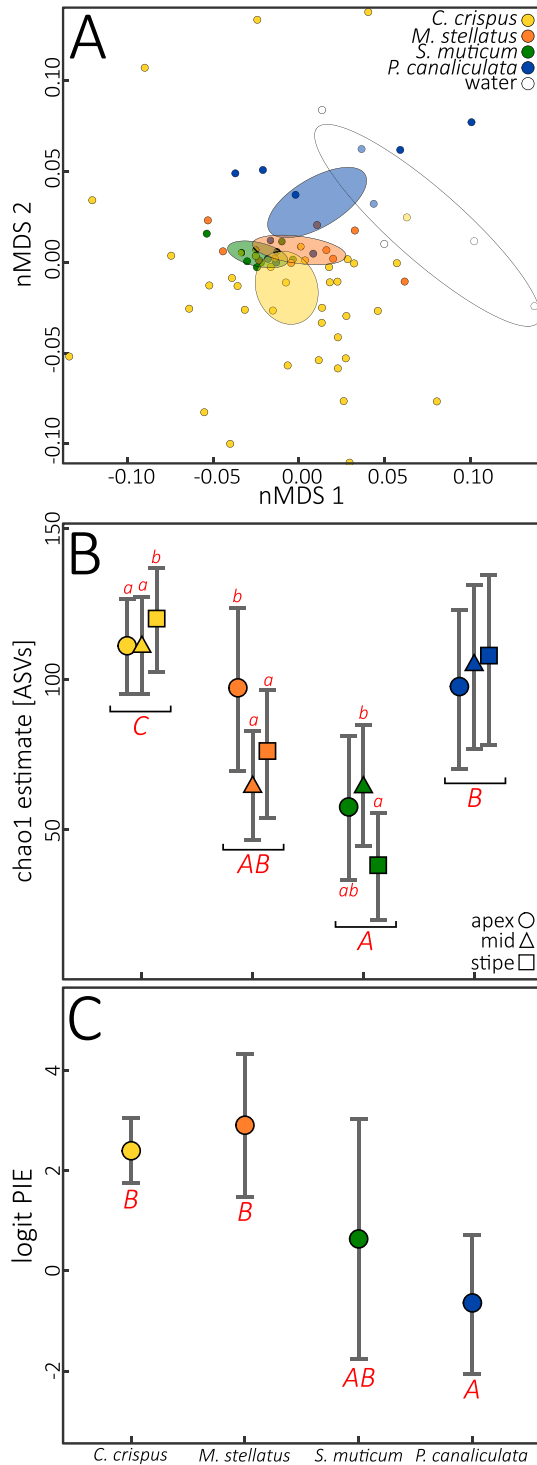


FIG. 3. Composition and diversity are displayed by species. (a) An nMDS plot based on ASV composition using the rescaled residuals from multivariate GLMs accounting for sequencing depth. The estimated group centroid 95% confidence intervals are indicated as ellipses. (b) Asymptotic richness (Chao1 parameter) is estimated by species and tissue type. (c) Evenness (logit PIE) is estimated by species. Significant differences among species are indicated with red capital letters and differences among tissue types within species with small letters.

*canaliculata*, but for which we could not classify beyond the phylum level (Ascomycota). An additional BLAST search revealed the best hit to an isolate from *Ascophyllum nodosum* classified to the genus *Moheitospora*, yielding only 93.6% similarity (Acc. No. MH397636). As *Pelvetia* spp. are known to host the ascomycete *Stigmidium ascophylli* as an endosymbiont (Kohlmeyer and Kohlmeyer 1979, Stanley 1992), one possibility is that the detected ASV2 represents this fungus. At present, there are no ITS rDNA sequences available for *S. ascophylli* in GenBank or UNITE. However, as the genus *Stigmidium* is classified under the Mycosphaerellaceae, it would be expected that the ASV, if indeed it represents the fungus studied by Kohlmeyer and Kohlmeyer (1979), would at least be classified to the family level.

Similarly, there are no ITS rDNA sequences available for *Lautitia danica*, the pathogen of *Chondrus crispus* (Schatz 1984). It is uncertain if our dataset contains ASVs from this species. We found that *C. crispus* was dominated by ASV1 which was classified to the genus *Didymella*—a genus including four marine species (Jones et al. 2012). The best hit recovered from a BLAST search for ASV1 against ex-type sequences only yielded a 94.40% similarity match (Acc. No. EF192138) with a fungus isolated from *Fucus vesiculosus* identified as *Dichothrix fucicola* (Table 1). Therefore, the current taxonomic identification of this ASV should be interpreted with caution and may well belong to a species that is not described or to a species, such as *L. danica*, for which ITS rDNA sequences are not available.

*Host-specific fungi.* In addition to characterizing novel diversity, we first tested the hypothesis that fungi will differ among macroalgal hosts. Macroalgal hosts varied in community composition and in terms of diversity. This pattern was, however, more evident in certain macroalgal species. For example, in *Chondrus crispus* (dominated by ASV1) and *Pelvetia canaliculata* (dominated by ASV2), the most abundant ASVs were absent or rare in the other macroalgae and the seawater. While these two ASVs explained most of the observed deviance, this was less than 2%, suggesting the detected difference among hosts is not driven solely by highly abundant ASVs. Instead, differences are driven by multiple fungal taxa which vary rather subtly among macroalgal hosts.

For terrestrial plants, host phylogeny has been shown to explain differences in fungal communities at the local scale (Gilbert et al. 2007). Our results reflect that composition varies among macroalgal hosts from the same locality, which suggests that host phylogeny may be of similar importance to algal-fungal communities. This is also in line with Wainwright et al. (2017) who found fungal communities were differentiated by macroalgal hosts on mesophotic reefs. While sampled from the same

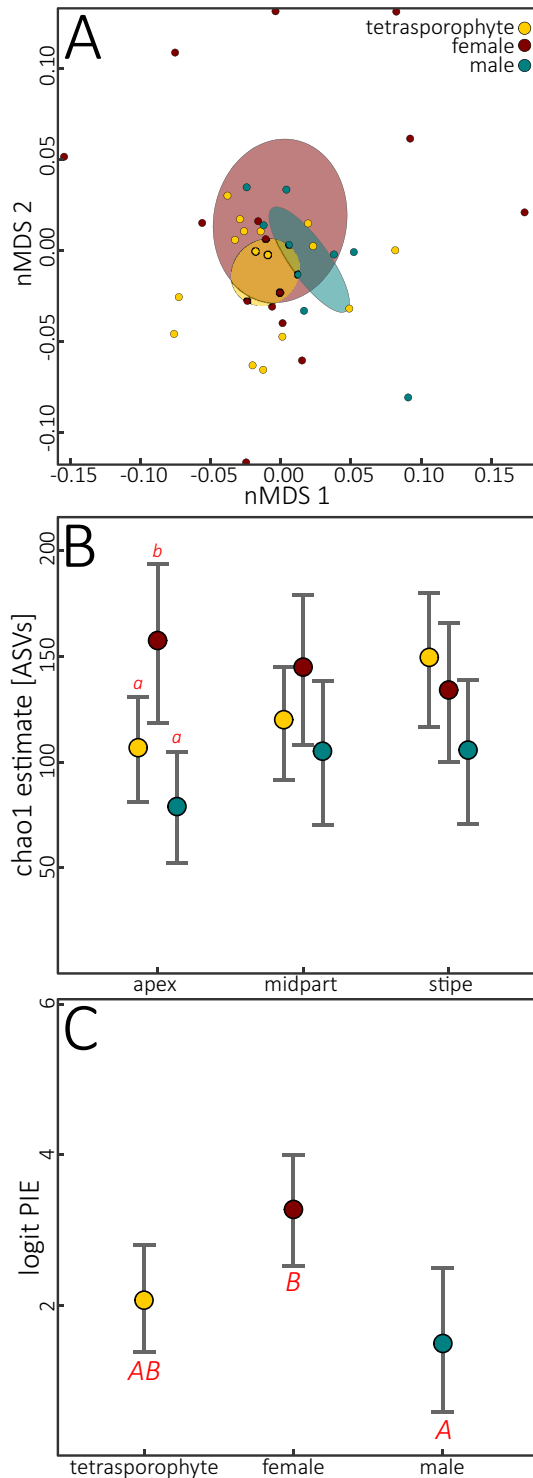


FIG. 4. Composition and diversity are displayed by *Chondrus crispus* life cycle stages. (a) An nMDS plot based on ASV composition using the rescaled residuals from multivariate GLMs accounting for sequencing depth. The estimated group centroid 95% confidence intervals are indicated as ellipses. (b) Asymptotic richness (Chao1 parameter) is estimated by LCS and tissue type. (c) Evenness (logit PIE) is estimated by LCS. Significant differences among LCS are indicated with red capital letters and differences among tissue types within LCS with small letters.

locality, *Pelvetia canaliculata* occurs at the higher end of the tidal gradient and its distinct fungal ASV composition may thus also be explained by the local variation driven by the tidal gradient. For example, if some fungal taxa indeed aid their algal hosts with desiccation resistance (Garbary and London 1995), then we might expect further differentiation in fungal endophyte communities between macroalgae based on their tidal distributions. Future work investigating patterns of fungal community composition along intertidal gradients of exposure, seasonality, and among taxonomic groups could give further insight into these patterns as well as the potential functional role some of these endophytes may fulfill in the macroalgal holobiont.

*Mastocarpus stellatus* and *Sargassum muticum* shared the most abundant ASV (ASV7, classified to *Mycosphaerella*) that was also abundant in the seawater, although not the dominant fungal ASV in the seawater samples. Similarly, ASV12 (not classified beyond the order Capnodiales) was abundant in both the seawater and *Mastocarpus stellatus* and at somewhat lower abundance in *Chondrus crispus* and *S. muticum*. Five Malasseziales ASVs (four of which classified to the genus *Malassezia*) were especially abundant in the seawater, but also detected in *C. crispus*, *M. stellatus*, and *S. muticum*. Whether these taxa are abundant in the seawater or live in closer association with macroalgae remains difficult to answer. Chen and Parfrey (2018) showed that *Nereocystis luetkeana* and *Mastocarpus* spp. strongly influence bacterial composition and richness in the surrounding seawater. However, *Malassezia* and its order are well known as yeasts growing on human skin (Findley et al. 2013). They are also commonly detected on a variety of marine substrates in amplicon-based sequencing studies and as such, considered as environmentally highly widespread fungi (Amend 2014). Given that these ASVs were practically absent in *Pelvetia canaliculata*, the high intertidal species, they may well originate from the seawater. Given that we used ASVs in this study, opposed to an OTU-clustering approach, it should be kept in mind that the *Malassezia* ASVs may represent different copies from single species or subvariants.

*Fungal association with specific macroalgal tissues.* Previous studies have observed that fungal (Wainwright et al. 2019) and bacterial communities (Staufenberger et al. 2008, Serebryakova et al. 2018, Bonthond et al. 2020) differ among macroalgal thallus tissue. We also found differences in fungal community composition depending on tissue type in *Chondrus crispus*, *Mastocarpus stellatus*, and *Sargassum muticum*. The patterns of greatest asymptotic ASV richness were greatest in *C. crispus* stipes as compared to *M. stellatus* apices (Fig. 4). In both red macroalgal species, the apices are the newest tissue, whereas the stipe is the oldest part of the thallus. Both Correa et al. (1987) and Mathieson and Prince

(1973) speculated that algal or fungal endophytes gain entry through reproductive structures upon spore release. Yet, the *C. crispus* and *M. stellatus* thalli sampled in our study were at peak reproduction with few empty reproductive structures. Fungi may gain entry into the thallus below the apices as the thallus is growing and the cuticle is disrupted (Correa et al. 1987), but this does not explain the greater diversity of fungi in *C. crispus* stipes. However, Correa and McLachlan (1991) noted that *C. crispus* tetrasporophytes were not only more likely to be infected by a pathogenic green algal endophyte, but that infected tetrasporophytes were more susceptible to bacteria and fungi. As the infection by *Ulvella operculata* (as *Acrochaete operculata*) progresses, the algal filaments become concentrated in the older parts of the thallus near the lower part of the mid-thallus and stipe. It is possible that the fungal endophytic diversity, which was overall greater in *C. crispus* more generally, may increase as the frond grows. The rugose structure of papillate female gametophytes in *M. stellatus* may also harbor greater fungal diversity as the papillae may be easier for fungal spores to colonize. Future studies should explore the differences between these two common taxa in the Gigartinales.

While other studies have seen differences in fungal communities depending on tissue type in the genus *Sargassum*, to the best of our knowledge, no studies have investigated the fungal endophytes in *S. muticum*. The success of invasive species is often thought to be the result of enemy release (Colautti et al. 2004). Mitchell and Power (2003) observed that up to 84% fewer fungi were found to infect plant species in their non-native ranges. In our study, *S. muticum* had the lowest asymptotic richness compared to the native species, including other fucoids (Fig. 4). Serebryakova et al. (2018) found differences in the microbial communities associated with *S. muticum* depending on the season, thallus location (basal vs. apical), and location in a study along the coast of Portugal. We found differences in asymptotic richness between the mid-thallus and stipe in *S. muticum*. Future studies should investigate the fungal communities in the native range in the northwest Pacific as well as throughout the invasion range as *S. muticum*. Its circumglobal distribution is the result of different invasion pathways (Le Cam et al. 2020) which may enable tests of the enemy release hypothesis.

It is curious that *Pelvetia canaliculata* showed no significant differences between its apices, mid-thallus, or stipe. *P. canaliculata* has the same fungus—*Stigmidium ascophylli*—as the fucoid *Ascophyllum nodosum* (Selosse and Le Tacon 1998). As *P. canaliculata* occupies an extremely high intertidal zone, *S. ascophylli* has been postulated to protect the alga against desiccation (Garbary and London 1995). Perhaps the zone at which this species is found necessitates fungal symbionts throughout its entire thallus. Future work should ascertain to what species

the dominant ASV2 in *P. canaliculata* belongs to, as we were unable to assign this ASV to a lower taxonomic level or isolate it.

*Fungal community differentiation among Chondrus crispus life cycle stages.* Unlike for pro- and eukaryotic communities in the isomorphic *Gracilaria vermiculophylla* (Bonthond et al. 2020) and in heteromorphic *Mastocarpus* spp. (Lemay et al. 2018), we found differences in the fungal communities among the isomorphic life cycle stages in *Chondrus crispus*. Not only does this support earlier work with filamentous algal endophytes (Correa and McLachlan 1991, 1992, Krueger-Hadfield 2011), it may also hint that host traits related to host-endophyte interaction may be involved in the evolutionary stability of haploid–diploid life cycles with morphologically similar stages (Hughes and Otto 1999). Although we do not know whether the fungi associated with female gametophytes, male gametophytes, or tetrasporophytes are mutualists or pathogens or under which conditions these relationships may be beneficial or detrimental, biotic interactions offer tantalizing clues to the niches that different “isomorphic” stages might inhabit (see discussion in Krueger-Hadfield 2020).

Future studies must further enumerate and cultivate the fungal taxa that are associated with *Chondrus crispus* in general and specifically by life cycle stage. We did not include non-reproductive thalli in our study, but Correa et al. (1987) found that non-reproductive thalli were less infected by algal endophytes as their thalli were not yet disrupted by reproductive structures. It would be interesting to compare vegetative with reproductive thalli to determine if there are differences between stages prior to the onset of sporogenesis or gametogenesis. Expanding our knowledge of fungal species associated with *C. crispus* will likely aid in our understanding of ploidy dominance that is characteristic of this species (see Krueger-Hadfield 2011, Krueger-Hadfield et al. 2013a). It may also enable us to explore the interaction of fungi with the elegant experimental *C. crispus*–*Ulvella operculata* pathosystem as fungal infections are thought to increase as a result of *U. operculata* advanced infections (Correa and McLachlan 1991, 1992, Bouarab et al. 2001).

*The gap in fungal taxonomy.* As has also been noted in previous studies (Amend 2014, Wainwright et al. 2017, Bonthond et al. 2018), the lack of available reference ITS rDNA sequences on fungal taxa from marine environments poses difficulties for the interpretation of amplicon-sequencing based fungal community data. To begin closing this gap, there is an urgent need for taxonomic efforts, including the collection and description of new species and sequencing of collected material and herbarium specimens. *Stigmidium ascophylli* and *Lautitia danica* are examples of well-described marine fungi for which there are no ITS rDNA sequence data available. While some of the ASVs are poorly classified

(e.g., ASV2, ASV15, and ASV43 for which we were only able to assign the phylum Ascomycota), and our cultivation efforts did not retrieve these isolates, only 8 out of 30 isolates yielded 100% similarity scores. It is likely that this strain or some of the strains with higher similarity hits represent undescribed species. In addition, with the development of more specific culturing strategies, poorly classifiable fungi may also become available.

**Conclusions.** Our study provided a window into the intertidal algal mycobiome. Distinct fungal communities were found at the species level, at different tidal heights, among thallus tissue types, and among the life cycle stages of *Chondrus crispus*. Relatively simple cultivation and sequencing approaches could be undertaken in intertidal macroalgae to further test hypotheses that were suggested in our study: (i) determining the role fungi play in the ecological patterns as emblematic as tidal zonation (Lüning 1990), (ii) the evolutionary stability of life cycle variation (Hughes and Otto 1999), or (iii) enemy release of fungal endophytes in macroalgal invasions. Developing culture collections and reference databases are critical next steps that will enable taxonomic descriptions of algaliculous fungi and the assessment of macroalgal–fungal symbioses.

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#### AUTHOR CONTRIBUTION

**G. Bonthond:** Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (equal); Visualization (lead); Writing – original draft (equal); Writing – review & editing (equal). **A. Barilo:** Data curation (equal); Investigation (equal); Methodology (equal); Writing – review & editing (equal). **R. J. Allen:** Data curation (equal); Investigation (equal); Methodology (equal); Writing – review & editing (equal). **M. Cunliffe:** Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Writing – review & editing (equal). **S. A. Krueger-Hadfield:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal); Writing – review & editing (equal).

#### CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

#### DATA AVAILABILITY STATEMENT

Merged and quality filtered forward and reverse Sanger sequences of the ITS region were deposited in GenBank under the accession numbers MZ081427–MZ081458. Raw high-throughput ITS2 amplicon sequencing reads were uploaded to the SRA (accession: PRJNA727013).

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Table S1.** Fungal isolates obtained in culture in this study with strain details.

**Table S2.** Statistical output of all multivariate models.

**Table S3.** Statistical output of all univariate models.