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Alternate life history phases of a common seaweed have distinct microbial surface communities

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Abstract

Macroalgal life histories are complex, often involving the alternation of distinct free-living life history phases that differ in morphology, longevity and ploidy. The surfaces of marine macroalgae support diverse microbial biofilms, yet the degree of microbial variation between alternate phases is unknown. We quantified bacterial (16S rRNA gene) and microeukaryote (18S rRNA gene) communities on the surface of the common intertidal seaweed, *Mastocarpus* spp., which alternates between gametophyte (foliose, haploid) and sporophyte (encrusting, diploid) life history phases. A large portion (97%) of bacterial taxa on the surface *Mastocarpus* was also present in samples from the environment, indicating that macroalgal surface communities are largely assembled from the surrounding seawater. Still, changes in the relative abundance of bacterial taxa result in significantly different communities on alternate *Mastocarpus* life history phases, rocky substrate and seawater at all intertidal elevations. For microeukaryote assemblages, only high intertidal samples had significant differences between life history phases although sporophytes were not different from the rocky substrate at this elevation; gametophytes and sporophytes did not differ in microeukaryote communities in the mid and low zones. By sequencing three host genes, we identified three cryptic species of *Mastocarpus* in our data set, which co-occur in the mid-to-low intertidal zone. In these samples, *M. alaskensis* sporophytes harboured distinct bacterial communities compared to *M. agardhii* and *M. intermedius* sporophytes, which were not distinguishable. Conversely, microeukaryote communities did not differ among species.

KEYWORDS
bacteria, macroalgae, marine microbes, *Mastocarpus*, microbial ecology

1 | INTRODUCTION

Multicellular organisms support complex ecosystems of microbial symbionts that facilitate many aspects of host biology including growth, development and immune function (Fraune & Bosch, 2010; McFall-Ngai et al., 2013; Zilber-Rosenberg & Rosenberg, 2008). Understanding how these host-associated communities are assembled and structured is a fundamental goal of microbial ecology. Factors including host phylogeny, geography and diet have all been shown to influence the structure of microbial communities (Jones, Sanchez, & Fierer, 2013; Ley, Lozupone, Hamady, Knight, & Gordon, 2008; Mikaelyan et al., 2015; Moeller et al., 2013), highlighting the combined importance of host evolutionary history and environmental interactions in shaping the microbiome.

Within a single species, microbial communities are dynamic during the growth and development of their hosts (Bengtsson, Sjotun,

Lanzen, & Ovreas, 2012; Koenig et al., 2011) and can shift dramatically as hosts pass through discrete stages in their development (Harrison, Urruty, & Forister, 2016; Kueneman et al., 2016; Wang, Gilbreath, Kukutla, Yan, & Xu, 2011). For example, egg, tadpole and adult stages of the amphibian life cycle have distinct microbial communities, which may reflect a shifting defensive function at each developmental stage (Kueneman et al., 2016; Prest Tiffany, Kimball Abigail, Kueneman Jordan, & McKenzie Valerie, 2018). The structuring of microbial communities with host ontogeny highlights the importance of considering host development when quantifying microbial communities, particularly in species that have morphologically or functionally distinct stages.

Marine macroalgae have exceptionally complex life histories (reviewed by Dewreede & Klinger, 1988) often involving the alternation of distinct free-living life history phases with dramatically different morphologies. It is well documented that the surfaces of marine macroalgae harbour diverse communities of microbial symbionts that are integral for proper development, disease resistance and defence (reviewed by Egan et al., 2013). Yet, previous research has focused almost entirely on the microbiota of a single life phase. Comparing microbial communities between macroalgal life history phases offers a distinct perspective on the sources of intraspecific variation in host-associated microbial communities, and the roles played by the host versus the environment in structuring the microbiota. This is because in contrast to the example of amphibian development in which the same individual transitions through relatively brief larval and tadpole stages, alternate macroalgal life history phases (sporophyte and gametophyte) are discrete free-living entities separated from each other by the release of either spores or gametes—one phase does not "grow" into the other. The discrete nature of alternate macroalgal generations provides little opportunity for the direct transfer of microbes between phases. However, these alternate phases can coexist in sympatry for several years, meaning that despite their differences, they are subject to the same source pools of environmental microbiota.

We focus on species within the red algal genus *Mastocarpus* (Rhodophyta: Florideophyceae; Figure 1). *Mastocarpus* species alternate between a flat encrusting sporophyte diploid phase and a branched upright gametophyte haploid phase (Guiry, West, Kim, & Masuda, 1984; Slocum, 1980; Zupan & West, 1988), which co-occur in sympatry along rocky intertidal coastlines. *Mastocarpus* is an intriguing system for this research because the morphological divergence between life phases is so extreme that they were originally described as distinct species; the upright gametophyte (formerly in the genus *Gigartina*) was believed to lack a sporophytic life history phase, whereas the crustose sporophyte phase was placed in a different genus (*Petrocelis*) for which no gametophytes had ever been observed. Culturing experiments (West, 1972) and subsequent genetic analyses (Bird, Sosa, & Mackay, 1994) confirmed that these distinct morphologies are in fact alternate life history phases of the same organism. Within the genus *Mastocarpus* at least 11 species occur along the Pacific coast of North America, with at least six of these species present at our study site on the central coast of British Columbia (Lindstrom, 2008; Lindstrom, Hughey, & Martone, 2011). Many *Mastocarpus* species are morphologically cryptic and occur in sympatry (Lindstrom et al., 2011).

We sampled sympatric individuals of each *Mastocarpus* life history phase to test for differences in their microbial communities while controlling for environmental factors. Samples from the surrounding environment (rocky substrate and seawater) were used to parse out the influence of environmental microbes in shaping seaweed microbial communities. Genetic testing revealed three cryptic

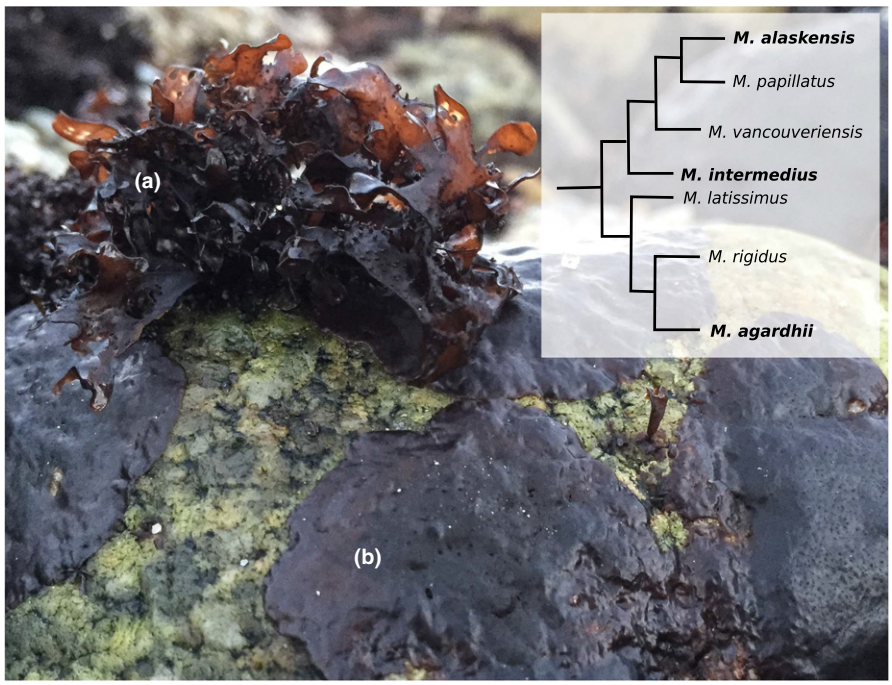

FIGURE 1 *Mastocarpus* exhibits alternation of heteromorphic generations: (a) The upright haploid gametophyte phase and (b) the crustose diploid sporophyte phase co-occur on rocky intertidal beaches. A phylogram of select Northeast Pacific *Mastocarpus* species is inset based on the three-gene consensus tree (ITS, COI and *rbc*L) from Lindstrom et al. (2011); the three cryptic species present in this study are indicated in bold font. Photograph by B. Clarkston

species among our *Mastocarpus* samples, providing an opportunity to test the hypothesis that phylogenetically divergent, yet morphologically indistinguishable, cryptic species harbour distinct microbial communities. We contrast the degree to which microbial communities differ between life history phases with interspecific differences among cryptic host species.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We collected sporophyte (crustose; n = 19) and gametophyte (upright; n = 15) life history phases of *Mastocarpus* spp. in March 2015 along three permanent intertidal transects located in a boulder field just south of West Beach, Calvert Island, British Columbia (51.6509°, -128.1484°). These horizontal transects, corresponding to the low, mid and high intertidal zones, are permanently fixed at an average height of 1.3, 1.9 and 2.5 m, respectively, above chart datum. We first rinsed each specimen with sterile seawater to remove transient environmental microbes and then sampled surface microbial communities using a Puritan® sterile swab for 10 seconds over an area approximately 2–3 cm^2, with the area of surface sampled kept consistent for gametophytes and sporophytes. Each microbial sample was stored in an individual sterile cryovial (VWR) on ice for transport back to the laboratory and then transferred to −80°C for storage. After collecting the microbial sample, a small section of each macroalgal specimen (~2 × 2 cm) was placed in a 20-ml scintillation vial containing silica beads for long-term storage of host tissue for DNA barcoding. The remainder of each macroalgal specimen was dried as a herbarium voucher.

Microbial samples from the surrounding environment are important because they constitute the main source populations for seaweed-associated microbial communities. We sampled microbial communities from the rocky substrate (n = 29) at each transect using sterile swabs as described above. Seawater samples (n = 27) were collected at the water's edge, adjacent to the low zone transect, using sterile 500-ml plastic containers; microbes were filtered from seawater in the laboratory using a Cole-Parmer MasterFlex L/S peristaltic pump with a 0.22-μm Durapore® membrane filter (Merck Millipore Ltd) the same day as collection. Filters from each seawater sample were stored at −80°C in individual Whirl-Pak® bags.

2.2 | Molecular methods

We extracted DNA from swabs and water filters using the MoBio PowerSoil®-htp 96 Well DNA Extraction Kit (Carlsbad, CA) following the manufacturer's recommended protocol. To amplify bacterial DNA we targeted the V4 region of 16S rRNA gene using primers modified from Caporaso et al. (2012): *515f*: 5′–GTGYCAGCMGCCGCGGTAA–3′, and *806r*: 5′–GGACTACNVGGGTWTCTAAT–3′. Forward primers were tagged with unique 12-bp barcodes to facilitate sample pooling. Each PCR reaction contained 10 μl of 5-Prime Master Mix, 1 μl of each primer (final concentration = 0.2 μM each), 0.5 μl of peptide nucleic acid (PNA) chloroplast blocking primer (Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013; 0.2 μM final concentration, purchased from PNA Bio Inc., Thousand Oaks CA), 2 μl of DNA and PCR-grade water to a final volume of 25 μl. PCR was carried out with an initial denaturation step at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, PNA clamping at 75°C for 60 s, primer annealing at 50°C for 60 s and extension at 72°C for 90 s, with a final extension step at 72°C for 10 min. All PCR products were quantified using Quant-iT PicoGreen® dsDNA Assay Kit (Life Technologies), and equal amounts (25 ng) of each sample were pooled and then purified using the MoBio UltraClean® PCR clean-up kit. Pooled library quantification and paired-end Illumina MiSeq sequencing (2 × 300 bp) were carried out at the Integrated Microbiome Resource (IMR) facility in the Centre for Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Canada).

To amplify microeukaryotic DNA, we submitted DNA samples for library preparation and sequencing to the IMR facility at Dalhousie University. Library preparation was carried out using the protocol described by Comeau, Li, Tremblay, Carmack, and Lovejoy (2011), Comeau, Douglas, and Langille (2017) to amplify the V4 region of the 18S rRNA gene with primers E572F: 5′-CYGCGGTAATTCCAGCTC-3′ and E1009R: 5′-AYGGTATCTRATCRTCTTYG-3′. Paired-end sequencing was carried out using the Illumina MiSeq (2 × 300 bp) platform.

2.3 | Host taxonomy

Total genomic DNA was extracted from each macroalgal host sample following the methods of Saunders (2008) with modifications from Saunders and McDevit (2012) to include the use of red algal extraction buffer and the extraction buffer additions. For each sample, we targeted a ~664-base pair fragment at the 5′ end of the cytochrome c oxidase subunit 1 DNA barcode region (COI) using the M13 linked primers *LF3* and *Rx* and associated protocol from Saunders and Moore (2013). PCR products were sequenced using a 3730xl DNA Analyzer (Applied Biosystems, California, USA) and the resulting sequence fragments were edited and aligned using Geneious 7.1.7 (Kearse et al., 2012). Taxonomic assignment was based on similarity searches to taxa accessioned in the Barcode of Life Data system (BOLD; Ratnasingham & Hebert, 2007).

Sequencing the COI region identified the presence of three *Mastocarpus* species within our samples (*M. alaskensis* S.C. Lindstrom, Hughey & Martone, *M. intermedius* S.C. Lindstrom, Hughey & Martone and *M. californianus* S.C. Lindstrom, Hughey & Martone). *Mastocarpus alaskensis* (gametophyte and sporophyte) was the only species present in the high intertidal zone. Sporophyte, but not gametophyte, *M. alaskensis* was also present in the mid/low intertidal zone. Both life history phases of the remaining two species were present in the mid/low intertidal zones (Supporting information Data S1).

Previous taxonomic research on *Mastocarpus* has found some discordance between nuclear, mitochondrial and plastid markers in some individuals (Lindstrom et al., 2011), possibly indicating chimerism

(Santelices et al. 1999). For example, Lindstrom et al. (2011) found that some individuals identified as *M. californianus* based on a plastid gene had nuclear genotypes consistent with *M. agardhii* (Setchell & N.L. Gardner) S.C. Lindstrom, Hughey & Martone. To account for this discordance in our taxonomic designations, samples identified as either *M. californianus* or *M. intermedius* based on mitochondrial COI haplotypes were subject to additional DNA sequencing using the nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast-encoded large rubisco subunit locus (*rbc*L) following the methods described by Lindstrom (2008). This additional sequencing found that samples identified as *M. californianus* based on COI and *rbc*L had ITS genotypes matching *M. agardhii*. Similarly, samples identified as *M. intermedius* based on COI and the ITS regions had *rbc*L haplotypes matching *M. intermedius* or *M. latissimus* (Supporting information Data S2). For consistency, we use the nuclear marker (ITS) to name these species throughout, but see Supporting information Data S2 for complete results of host tissue sequencing. No additional sequencing was carried out for samples identified as *M. alaskensis* (based on COI) as this species generally has consistent taxonomic designation among nuclear, mitochondrial and plastid markers (Lindstrom et al., 2011).

2.4 | Microbial sequence data

We processed raw Illumina reads separately for the 16S rRNA gene (bacteria and archaea) and 18S rRNA gene (microeukaryote) data sets. Sequence data were first demultiplexed using the Split Libraries function from the Quantitative Insights into Microbial Ecology (QIIME v.1.9) analysis pipeline (Caporaso, Kuczynski et al., 2010), trimmed to a uniform length of 250 bp using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and processed into operational taxonomic units (OTUs) using the minimum entropy decomposition method (MEDs; Eren et al., 2015) as implemented in the Oligotyping microbial analysis software package (Eren et al., 2013). MEDs partitions the data into phylogenetically homogeneous units (MED-nodes) for downstream bacterial diversity analyses. This is accomplished using Shannon entropy to separate biologically meaningful patterns of nucleotide diversity from sequencing noise. We set the minimum substantive abundance parameter (-M) at 250 reads and used default settings for all other parameters. In practice, the MED-nodes identified in this study are analogous to ≥99% OTUs.

Taxonomic assignment of OTUs from the 16S rRNA gene was carried out using UCLUST V1.2.22q (Edgar, 2010) as implemented in the Assign Taxonomy function of QIIME v.1.9 and the SILVA 128 database (Quast et al., 2013; Yilmaz et al., 2014). We removed OTUs that annotated to either mitochondria or chloroplast sequences, as these are likely due to host contamination. We also removed any OTU with fewer than 100 sequences and OTUs only present in a single sample (regardless of the number of reads). The remaining bacterial OTUs (n = 1,044) were aligned with PYNAST v.1.2.2 (Caporaso, Bittinger et al., 2010) using the GreenGenes 13_8 alignment as a template, and a tree was constructed using FastTree (Price, Dehal, & Arkin, 2010) as implemented in QIIME v.1.9.

Taxonomic assignment of OTUs from the 18S rRNA gene were carried out as for bacteria using UCLUST v 1.2.22q (Edgar, 2010) to match sequences to the SILVA 128 ribosomal RNA database (Quast et al., 2013; Yilmaz et al., 2014). As with bacteria, we removed OTUs with fewer than 100 sequences, OTUs that were only detected in a single sample (regardless of read depth), OTUs with unassigned taxonomy and any OTU with taxonomic assignment that matched macroalgal host species. The remaining microeukaryote OTUs (n = 817) were aligned with PYNAST v.1.2.2 (Caporaso, Bittinger et al., 2010) using the Silva v.123 core alignment as a template. A tree of microeukaryote OTUs was constructed using FastTree (Price et al., 2010) as implemented in QIIME v.1.9.

2.5 | Microbial diversity among life history phases and environmental samples

We used the Chao1 index (Chao, 1984) to estimate the richness of microbial taxa for each sample; this was carried out using the bias-corrected version of Chao1 after rarefying to 1000 sequences/sample as implemented in QIIME v.1.9. We tested for differences in microbial richness (Chao1 index) between samples using a linear model in which substrate type (four levels: *Mastocarpus* sporophyte, *Mastocarpus* gametophyte, rocky substrate and seawater) and tidal height (three levels: low, mid and high transects) were coded as fixed factors. This analysis was carried out separately for the bacterial and microeukaryote data sets in R v.3.2.3, with a posteriori contrasts assessed using the lsmeans package (Lenth, 2016).

For analyses of microbial community composition we constructed dissimilarity matrices based on weighted and unweighted UniFrac distance (Lozupone & Knight, 2005) and Bray–Curtis distance (rarefied to 1000 sequences/sample) as implemented in QIIME v.1.9. Distance matrices were visualized using principal coordinates plots in PRIMER E v. 6 (Clarke & Gorley, 2006) and UPGMA trees.

Statistical tests for differences in microbial community structure among substrate types (four levels: *Mastocarpus* sporophyte, *Mastocarpus* gametophyte, rocky substrate and seawater) were carried out using a permutational multivariate analysis of variance (PERMANOVA) with tidal height (low, mid, high transects) included as a fixed factor. This analysis was carried out for each distance matrix (weighted and unweighted UniFrac distances and Bray–Curtis distance) as implemented in PRIMER v. 6 (Clarke & Gorley, 2006) with 9999 permutations using a Type III sum of squares. Sums of squares were used to calculate R^2 values for the main factors in each test.

2.6 | OTU-specific differences between life history phases

We sought to identify specific microbial taxa associated with each life history phase by testing for differences in the prevalence and abundance of OTUs between gametophytes and sporophytes. We restricted these analyses to only those OTUs that were significantly enriched on *Mastocarpus* compared to the environment. In doing so, we avoid mistaking bacteria that are common on all surfaces (e.g., those abundant on

rocky substrate) as being specific to *Mastocarpus*. We identified the subset of OTUs that are enriched on *Mastocarpus* using the Sloan neutral model (Sloan et al., 2006) with R scripts and methods described by Venkataraman et al. (2015). For this analysis macroalgal samples were coded as the target and samples from the environment (rocky substrate and seawater) as the source. We identified 514 of 1,044 bacterial OTUs (49%) that were significantly enriched (called overrepresented in the neutral model) on macroalgal surfaces relative to the environment, and 341 of 817 microeukaryote OTUs (42%) were enriched on macroalgae.

Using the subset of enriched OTUs, we identified OTUs with high prevalence on each life history phase by calculating the common core of microbial taxa present on *Mastocarpus* overall and on sporophytes and gametophytes individually. For these analyses the common core was defined as OTUs present in ≥90% of samples in each group. To be considered present in a sample, an OTU had to be represented by at least 2 reads.

We then used a differential abundance algorithm (DESeq2; Love et al. 2014) to test for bacterial and microeukaryote OTUs that significantly differed in abundance between alternate life history phases. This analysis was implemented using the Phyloseq package (McMurdie and Holmes 2013) in R. We controlled the false discovery rate using the Benjamini–Hochberg procedure for multiple comparisons (FDR = 0.1, the program default). As with the identification of core taxa, differential abundance analyses were carried out using only OTUs that were overrepresented on macroalgal hosts based on results of the Sloan neutral model.

2.7 | Microbial differences among host species

The genetic identification of the three *Mastocarpus* species in our data provided an opportunity to test for interspecific microbial differences between cryptic hosts. Statistical tests for microbial differences among cryptic host species were restricted to samples from the mid/low intertidal zones because only a single host species (*M. alaskensis*) was present in the high zone, whereas all three species co-occur in the mid/low zones. We tested for differences in microbial community composition among host species using a PERMANOVA implemented in PRIMER (Clarke & Gorley, 2006). Life history phase was included in the model as a fixed factor so that we could tease apart the relative effect of life history and host species on bacterial community structure. We tested for differences in microbial OTU richness (Chao1 index) among host species using a linear model as previously described. These statistical tests were carried out separately for the bacterial and microeukaryote data.

3 | RESULTS

We collected microbial samples from *Mastocarpus* sporophyte (crust; n = 19) and gametophyte (upright; n = 15) phases, from rocky substrates (n = 29) and from seawater (n = 27). Sequencing the V4 region of the 16S rRNA gene produced an average coverage of 18,228 quality-filtered reads per sample. Minimum entropy decomposition and subsequent filtering of these data resulted in a total of 1,044 bacterial OTUs for downstream analyses. A large proportion of OTUs were shared among samples; of the 1044 bacterial OTUs (16S rRNA gene), 97% of these were shared between *Mastocarpus* and rocky substrate and 86% were shared between *Mastocarpus* and seawater (Supporting information Data S3).

Sequencing the V4 region of the 18S rRNA gene produced an average coverage of 19,664 quality-filtered reads per sample. Minimum entropy decomposition and subsequent filtering of these data resulted in a total of 817 microeukaryote OTUs. Of these 817 microeukaryote OTUs, 84% were shared between *Mastocarpus* and rocky substrate, and 63% were shared between *Mastocarpus* and seawater (Supporting information Data S3).

3.1 | Microbial diversity among life history phases and environmental samples

Bacterial community composition significantly differs among substrate types (*Mastocarpus* sporophyte, *Mastocarpus* gametophyte, rocky substrate and seawater; Figure 2, Table 1). Analyses run with different distance metrics give similar results; thus, only unweighted UniFrac analyses are presented. Tidal height and the interaction term (tidal height x substrate type) were also significant in this model. In the light of the significant interaction term, pairwise comparisons for differences among substrate types were carried out separately at each tidal height. This analysis revealed that bacterial communities on all substrate types were significantly different at each tidal height (Table 2).

Bacterial OTU richness (Chao1 index) is significantly different among substrate types (Figure 3, Table 1). Tidal height also had a significant effect on OTU richness, with no significant interaction between tidal height and substrate type. Pairwise comparisons of substrate types (across all tidal heights) show that *Mastocarpus* sporophytes have significantly lower bacterial OTU richness compared to gametophytes, rocky substrate and seawater.

Microeukaryote community composition also significantly differed among substrate types (Figure 2, Table 1). As with the bacterial data, tidal height and the interaction term (tidal height × substrate type) were also significant. However, in pairwise comparisons (Table 2), we did not see differences between life history phases except in the high intertidal zone; in the high zone microeukaryote communities were significantly different between sporophytes and gametophytes, but sporophytes were not different from rocky substrate. Sporophytes and gametophytes did not differ in community composition in the mid and low zones, although they did differ from rocky substrate at these elevations.

Microeukaryote OTU richness (Chao1 index) was greatest in seawater compared to all other samples and did not differ between to life history phases (Figure 3, Table 1).

3.2 | OTU-specific differences between life history phases

To assess the taxa driving community differences between life history phases, we tested whether there are microbial OTUs indicative

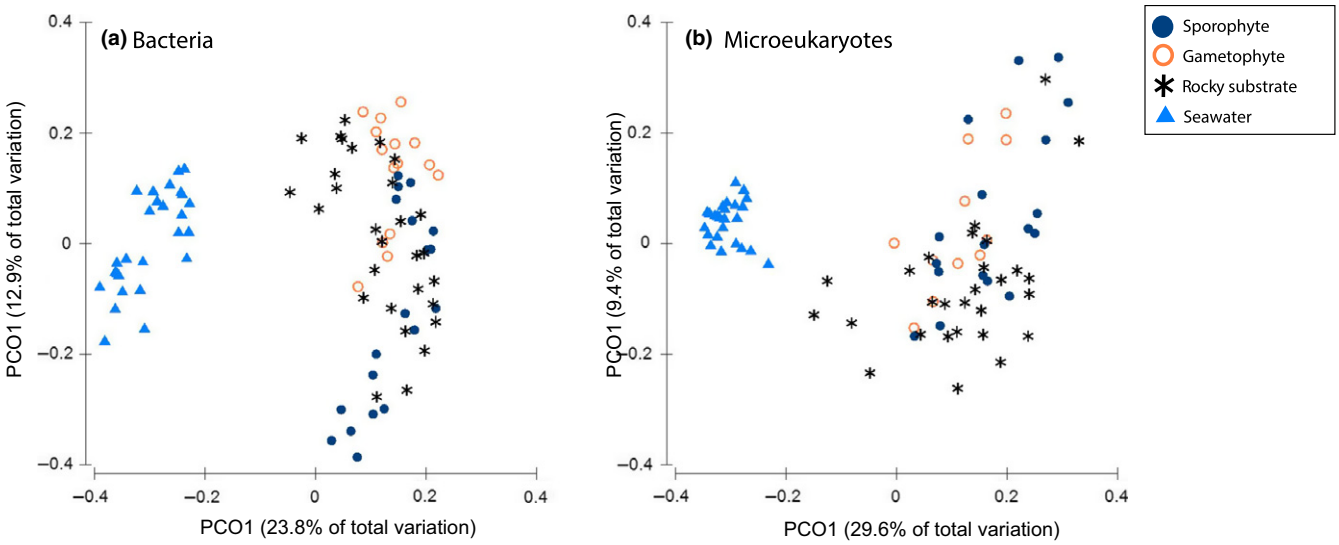

FIGURE 2 Principal coordinates analysis of unweighted UniFrac distance comparing microbial communities on each substrate type

TABLE 1 Statistical comparisons of microbial communities among substrate types (*Mastocarpus* sporophytes, *Mastocarpus* gametophytes, rocky substrate and seawater)

	ANOVA (Chao1 index)				PERMANOVA (unweighted UniFrac)			
	df	SS	*F*-value	*p*-value	*df*	SS (R^2)	Pseudo-F	*p*-value
(A) Bacteria								
Substrate type	3	194,610	11.2	<0.0001	3	3.9 (0.25)	13.0	0.0001
Tidal height	2	148,418	12.8	<0.0001	2	1.3 (0.08)	6.4	0.0001
Interaction	4	17,000	0.7	0.6	4	1.1 (0.07)	2.7	0.0001
Residuals	80	463,951			80	8.1		
(B) Microeukaryotes								
Substrate type	3	70,562	10.8	<0.0001	3	3.2 (0.24)	11.0	0.0001
Tidal height	2	616	0.1	0.9	2	1.0 (0.07)	4.8	0.0001
Interaction	4	16,584	1.9	0.1	4	1.0 (0.07)	2.2	0.0005
Residuals	72	156,908			72	7.2		

ANOVA was used to compare OTU richness (Chao1 index) among groups, PERMANOVA was used to compare community structure (unweighted UniFrac distance). Intertidal height (low, mid, high) was included as fixed factor in this model. (A) Tests for differences in richness and composition in bacterial (16S) OTUs; (B) tests for differences in richness and composition in microeukaryote (18S) OTUs. See Table 2 for pairwise comparisons among substrate types.

of *Mastocarpus* overall and of each life history phase (i.e., common core microbiome). Given the high proportion of taxa shared with the environment, we first used the Sloan neutral model (Sloan et al., 2006; Venkataraman et al., 2015) to identify and remove OTUs from this analysis that are likely acquired passively from the environment and therefore may be transient on macroalgal surfaces. The neutral model identified 514 bacterial OTUs (49% of total) and 341 microeukaryote OTUs (42% of total) that were significantly enriched on macroalgal surfaces relative to the environment.

Only two of these 514 bacterial OTUs were present in ≥90% of all *Mastocarpus* samples (Figure 4), both belonging to *Litorimonas* (Alphaproteobacteria; Hyphomonadaceae) and are 99% similar to *Litorimonas cladophorae*, isolated from the green alga *Cladophora* (Nedashkovskaya, Kukhlevskiy, Zhukova, Kim, & Rhee, 2013). These two "core" OTUs were also prevalent in environmental samples, but were an order of magnitude more abundant on macroalgae,

From the same subset of enriched bacterial OTUs (n = 514) we identified 14 and 10 core OTUs that were present on ≥90% of sporophytes and gametophytes, respectively (Figure 4, Supporting information Data S4). Six of 14 core OTUs on sporophytes were assigned to *Portibacter* (Bacteroidetes; Saprospiraceae) and appear to be specific to *Mastocarpus* sporophytes as they are at very low abundance in the environment and on *Mastocarpus* gametophytes (less than 1% of total sequences; Figure 4). Outside of *Portibacter*, many of the core OTUs belong to clades commonly found on macroalgae, such as Flavobacteria (*Dokdonia*), Gammaproteobacteria (*Granulosicoccus*) on sporophytes and Alphaproteobacteria (*Robiginitomaculum* and *Litorimonas*), Gammaproteobacteria (*Vibrio*, *Granulosicoccus*, *Colwellia*) and Flavobacteria (*Maribacter*) on gametophytes.

TABLE 2 Pairwise comparisons of microbial community structure among substrate type at each tidal height (based on PERMANOVA of unweighted UniFrac distance)

		Bacteria		Microeukaryotes	
Tidal height	**Pairwise comparisons**	***t***	***p*-value**	***t***	***p*-value**
High zone	**Gametophyte vs. sporophyte**	2.6	**0.0009**	1.7	**0.0055**
	Gametophyte vs. rocky substrate	2.1	0.0023	1.5	0.0115
	Sporophyte vs. rocky substrate	2.4	0.0005	1	0.5
Mid zone	**Gametophyte vs. sporophyte**	2.2	**0.0193**	1	**0.3**
	Gametophyte vs. rocky substrate	2	0.0004	1.6	0.0027
	Sporophyte vs. rocky substrate	2	0.004	1.6	0.008
Low zone	**Gametophyte vs. sporophyte**	1.8	**0.0011**	1.2	**0.2**
	Gametophyte vs. rocky substrate	2	0.0002	1.4	0.016
	Sporophyte vs. rocky substrate	1.8	0.0001	1.9	0.0008
	Gametophyte vs. seawater	3.6	0.0001	3.9	0.0001
	Sporophyte vs. seawater	3.7	0.0001	5.2	0.0001
	Rocky substrate vs. seawater	3.9	0.0001	4.4	0.0001

Microbial differences between life history phases of *Mastocarpus* (sporophyte and gametophyte) are highlighted with bold font.

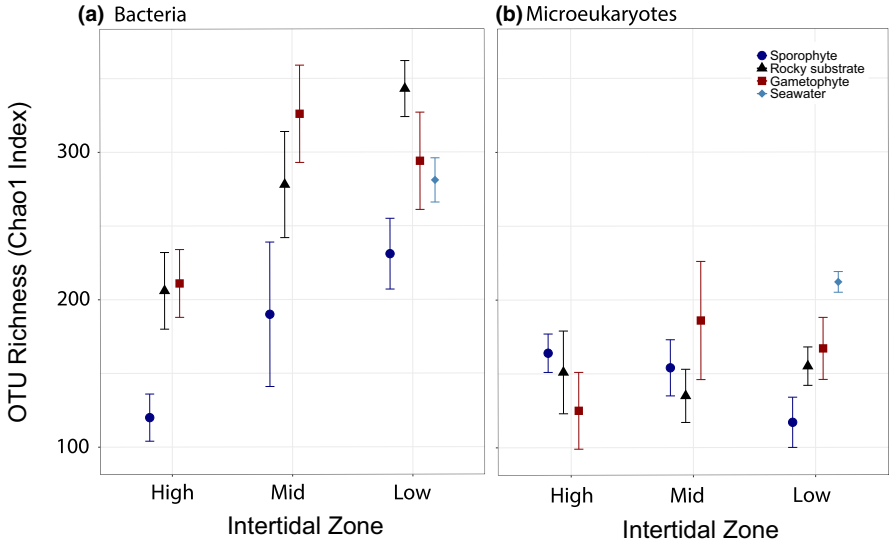

FIGURE 3 OTU richness (Chao1 index) across intertidal zones and substrate types: (a) Bacterial richness is lower on sporophytes and in the high intertidal zone; (b) the richness of microeukaryote OTUs does not significantly differ across tidal heights or substrate types, with the exception of seawater having significantly greater average richness. Data presented are mean OTU richness ± standard error

From the microeukaryote data, we find a small core of six diatom OTUs present on ≥90% of *Mastocarpus* samples, and in fact, all OTUs with greater than 50% prevalence on *Mastocarpus* are diatoms (Supporting information Data S5). In contrast to bacteria, core OTUs on sporophytes and gametophytes are a subset of the *Mastocarpus* core rather than being unique to specific life history phases.

Differential abundance analysis carried out using the DESeq2 package on OTUs overrepresented on macroalgal hosts identified 128 bacterial OTUs (25%) that significantly differed between gametophytes and sporophytes (Figure 5, Supporting information Data S4). These differentially abundant bacterial OTUs were split approximately between life history phases; 66 OTUs were enriched on gametophytes and 63 OTUs were enriched on sporophytes. The majority of differentially abundant bacterial OTUs were from the Bacteroidetes (Saprospiraceae: 29%; and Flavobacteriaceae: 13%), Alphaproteobacteria (22%) and Gammaproteobacteria (20%) (Figure 5). More Flavobacteriaceae were enriched on sporophytes, whereas more Gammaproteobacteria were enriched on gametophytes. However, in many cases, bacterial genera contained OTUs that were variably enriched on sporophytes or gametophytes, and again, these enriched taxa typically fell within clades commonly found on other macroalgae.

We identified 40 overrepresented microeukaryote OTUs with differential abundances between gametophytes and sporophytes, with the majority of these enriched on sporophytes (Figure 5, Supporting information File 5). Two striking patterns emerged from the microeukaryote data: Most of the microeukaryote OTUs enriched on sporophytes were diatoms (29 OTUs), and no diatom OTUs were enriched on gametophytes (Figure 5). Conversely, the five of six microeukaryote OTUs enriched on gametophytes were metazoans (animals), including two bivalves (Mytiloida), two copepods (Harpacticoida) and one gastropod (Caenogastropoda); no metazoan OTUs were

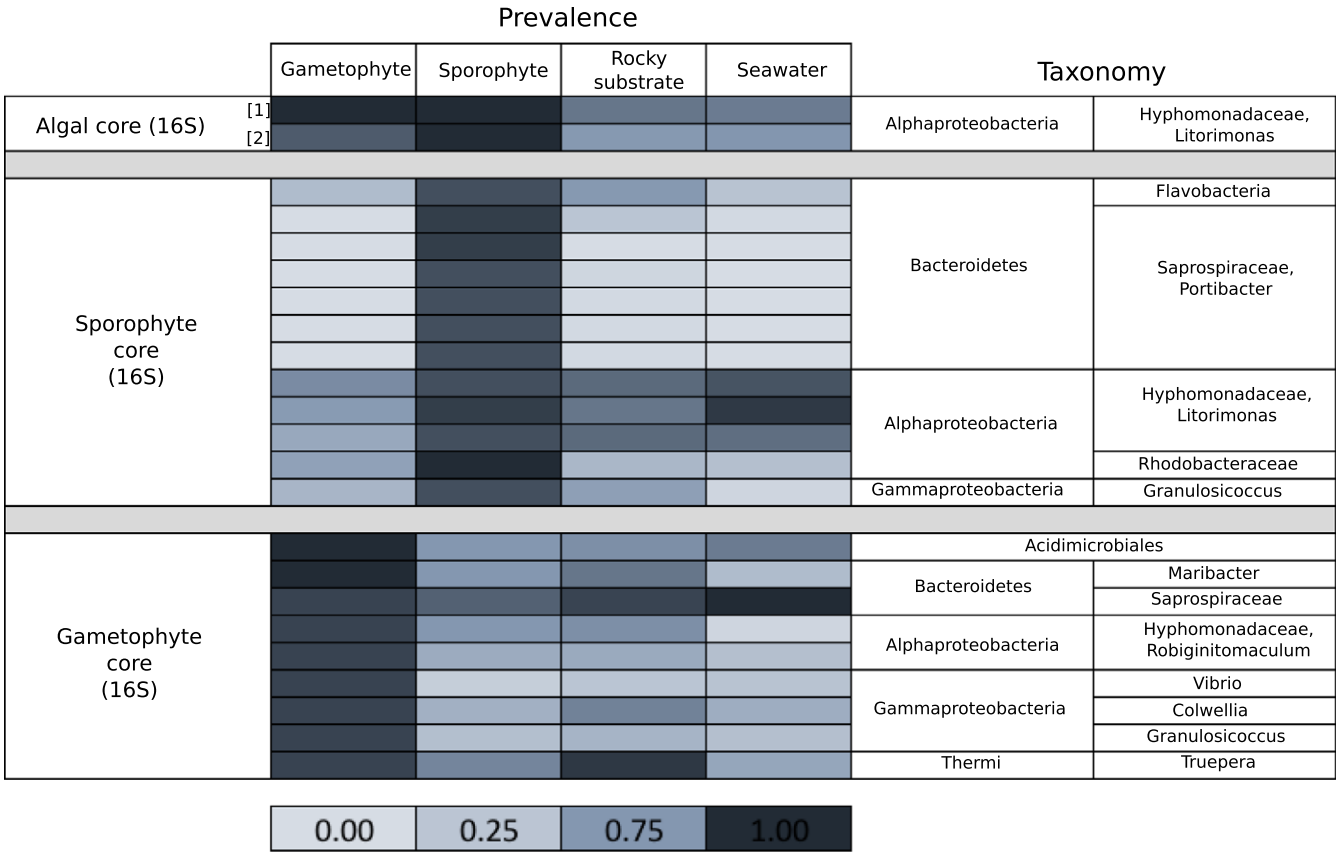

FIGURE 4 Core bacterial OTUs identified in ≥90% individuals from (1) all *Mastocarpus* samples, (2) encrusting sporophytes and (3) upright gametophytes, respectively. The groups from which core OTUs were identified are given along the *y*-axis, and the prevalence of each OTU is presented for gametophyte, sporophyte, rock and seawater samples (*x*-axis). [1] This OTU was also a member of both the sporophyte and gametophyte cores, but is only presented here. [2] This OTU was also a member of the sporophyte core, but is only presented here

enriched on sporophytes. We hypothesize that these OTUs are from animal larvae that were using *Mastocarpus* gametophytes as habitat.

3.3 | Microbial differences among host species

By sequencing genetic markers for the host, we identified three species of *Mastocarpus* in our data (Supporting information Data S1 and S2). COI sequence similarity was very high within species (99.9% or greater), and there was no COI sequence divergence between sporophyte and gametophyte individuals of the same species. Between-species divergence was much greater: Mean pairwise COI sequence similarity between *M. intermedius* and *M. agardhii* was 90% and between *M. alaskensis* and *M. agardhii* was 88%, and similarity was lowest between *M. alaskensis* and *M. intermedius* (mean = 85%; Supporting information Data S6). This mitochondrial (COI) similarity among species contrasts with the three-gene consensus phylogeny reconstructed from nuclear, chloroplast and mitochondrial markers showing *M. alaskensis* and *M. intermedius* as the most closely related pair (Lindstrom et al., 2011). For this reason, sequencing of the COI region was only used to infer taxonomy and not to reconstruct evolutionary relationships among host samples.

Mastocarpus alaskensis was the only species identified in the high intertidal, whereas both phases of *M. intermedius* and *M.* agardhii as well as sporophytes of *M. alaskensis* occurred at mid/low tidal heights. The overlapping distribution of sporophyte samples from all three species in the mid/low intertidal enabled us to test for differences in host-associated microbial communities among these cryptic species in a common environment. Samples from the mid/low intertidal had significantly different bacterial communities among species (PERMANOVA: $df = 2$, pseudo-$F = 2.6$, $p < 0.0002$, $R^2 = 0.19$). Pairwise comparisons revealed that the bacterial communities on *M. alaskensis* sporophytes were significantly different from those on *M. agardhii* and *M. intermedius*, but *M. agardhii* and *M. intermedius* were not different from each other (Figure 6, Supporting information Data S7 and S8). The relative proportion of variation explained by host species and life history phase are nearly identical in this model, but we are cautious about drawing strong conclusions from these species comparison due to the small samples sizes of each species and the fact that no gametophytes of *M. alaskensis* were sampled in the mid/low zone, leading to an unbalanced design. Given this imbalance, we carried out an additional PERMANOVA that was restricted to only the sporophyte samples from the mid/low intertidal zone to test for differences among host species; this analysis also found significant differences in bacterial community structure among species (Supporting information Data S7).

Microeukaryote communities did not differ across host species (PERMANOVA: $df = 2$, pseudo-$F = 1.0$, $p = 0.5$, $R^2 = 0.1$; Figure 6,

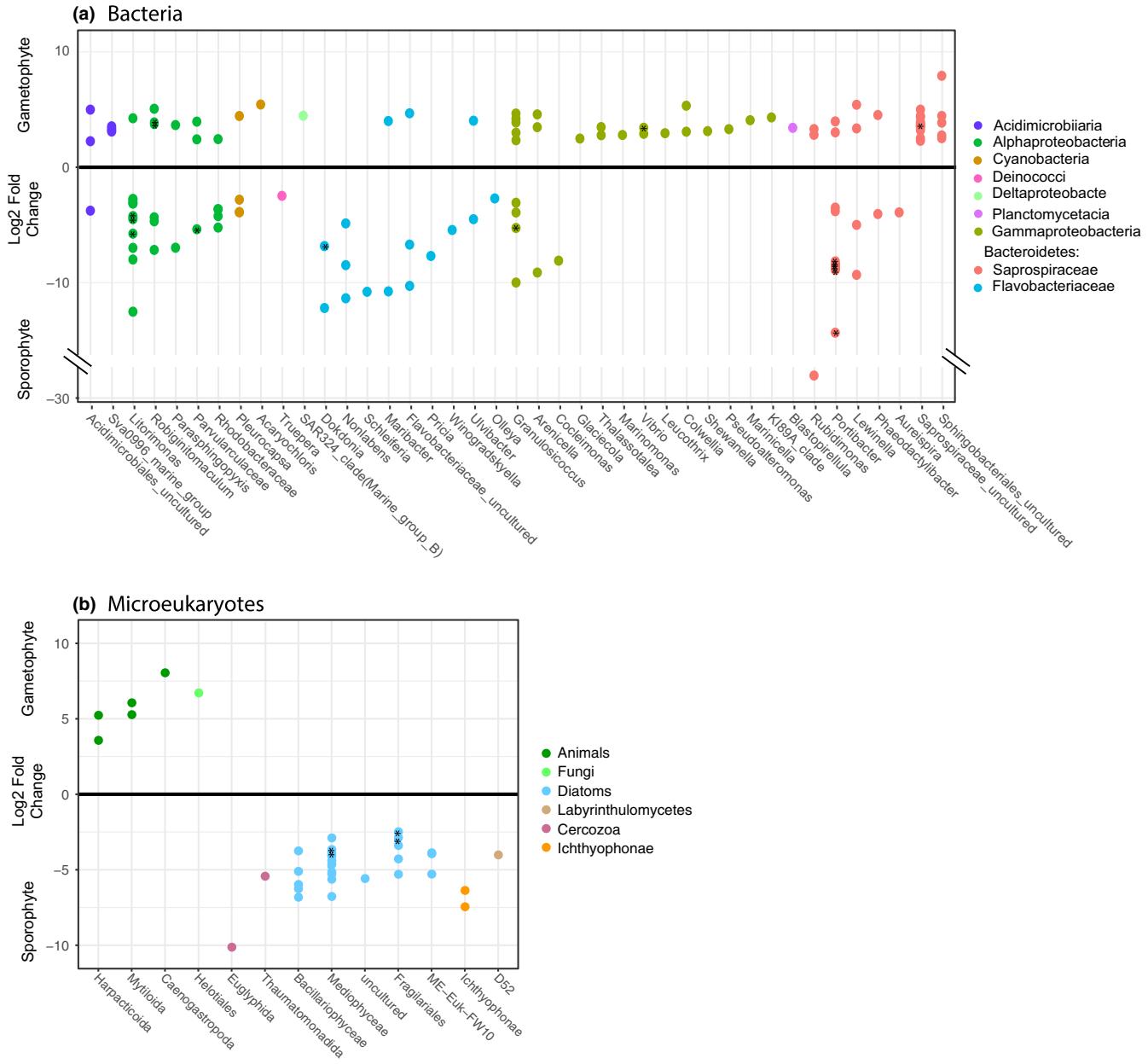

FIGURE 5 Differential abundance analysis (DESeq2) comparing sporophyte and gametophyte samples. Each circle represents an OTU with significantly different relative abundance, after FDR correction. OTUs present in either the sporophyte or gametophyte core are indicated with asterisks

Supporting information Data S7 and S8). Richness of OTUs (Chao1 index) did not differ among host species for bacteria (ANOVA: $df = 2$, $F = 3.5$, $p = 0.06$) or microeukaryotes (ANOVA: $df = 2$, $F = 0.5$, $p = 0.66$).

4 | DISCUSSION

Alternate life history phases (sporophyte and gametophyte) of the *Mastocarpus* spp. have statistically distinct bacterial surface communities. Each life history phase supported a small common core bacterial community (Figure 4), and differential abundance analysis pointed to dozens of OTUs that were enriched on either the sporophyte or gametophyte (Figure 5). Overall, we detected bacterial taxa that were widely reported on other macroalgae, including Saprospiraceae (*Portibacter*), Gammaproteobacteria (e.g., *Granulosicoccus*) and Hyphomonadaceae (*Litorimonas*), most of which were common on both life history phases (Figure 4), suggesting differentiation between life history phases occurs at a fine taxonomic scale for bacteria (i.e., at the OTU level).

In contrast to bacteria, microeukaryote community differentiation between life history phases was only observed in the high intertidal zone, where a single species (*M. alaskensis*) was present. The small common core of microeukaryotes consisted exclusively of diatoms,

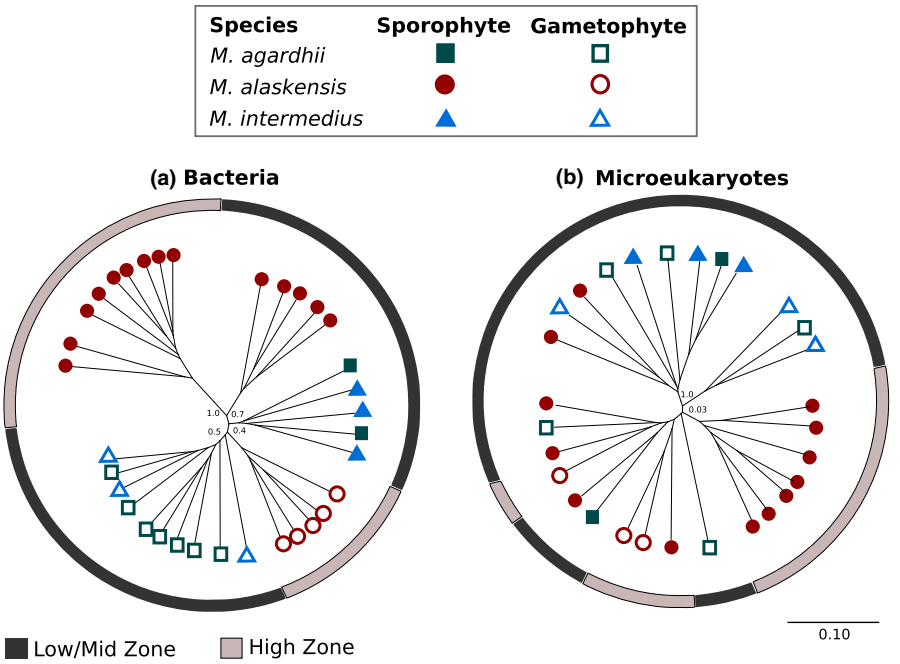

FIGURE 6 An UPGMA tree based on unweighted UniFrac distance of bacterial communities (left panel) and microeukaryote communities (right panel). Jackknife support values based on 100 replicate trees are provided for major clades

and we did not see distinct taxa comprising a core for each life history phase. Gametophytes in particular did not appear to host a unique and specific community; only six OTUs (out of 817) were statistically enriched on gametophytes. Five of these OTUs were animals common in the intertidal and one was a fungus present at very low abundance (~250 total reads) and on only 20% of gametophyte samples. In contrast, 34 OTUs were enriched on sporophytes and were predominately diatoms (Figure 4).

The biological mechanism driving microbial community structure between sporophyte and gametophyte life history phases is unknown, but we hypothesize that this pattern can be attributed to differences in host morphology, chemistry and age. The most obvious difference between *Mastocarpus* life history phases is their morphology. The complex foliose branching pattern of the gametophyte phase provides much greater structural complexity than the crustose sporophyte, and this host complexity has previously been shown to increase the settlement of larval marine invertebrates (Smith, Johnston, & Clark, 2014; Stevens & Kittaka, 1998). Indeed, we observed an enrichment of metazoan OTUs on the *Mastocarpus* gametophytes that match common intertidal invertebrates including bivalves, gastropods and arthropods. These likely represent invertebrate larvae taking refuge on the branched gametophytic thallus.

Morphological differences between macroalgae are also predicted to alter their response to water flow, which may in turn affect their microbiota. Whereas turbulent water motion over the macroalgal thallus is essential for the uptake of nutrients and removal of metabolic products (Hurd et al. 1996; Hurd and Stevens 1997), excessive drag forces from extreme water flow in the intertidal zone risks dislodging upright macroalgae (Carrington 1990, Denny 1994, Denny and Gaylord, 2002, Martone, Kost, & Boller, 2012). To mitigate the risk of dislodgement, foliose macroalgae, including *Mastocarpus*, reconfigure their shape to minimize drag (Martone et al., 2012), and in so doing locally reduce flow through their branches (Hurd and Stevens 1997), which reduces the dislodgement risk of large epiphytic symbionts (Anderson & Martone, 2014) and likely reduces gas, nutrient and microbial exchange with the surrounding water. On the other hand, crustose macroalgae, like the *Mastocarpus* sporophyte, have limited influence on local flow dynamics because of their flat and simple morphology.

Beyond their morphology, chemical differences between *Mastocarpus* life history phases may play a role in structuring their surface microbiota. In particular, polysaccharide (carrageenan) chemistry differs between the gametophyte and sporophyte life history phases of other red algal species within the Gigartinales including *Chondrus crispus* (McCandless, Craigie, & Walter, 1973) and *Gigartina atropurpurea* (Falshaw, Bixler, & Johndro, 2003). We hypothesize that differences in the composition of compounds available for bacterial metabolism may also influence microbial colonization.

Finally, the age of host tissue may also play a role in structuring microbial communities (Bengtsson et al., 2012; Lemay et al., 2018). *Mastocarpus* gametophytes are relatively short-lived, likely persisting for a few years, whereas the sporophytes are long-lived perennials. Paine and Vadas (1969) found that ephemeral and annual marine macroalgae have greater caloric value than perennials, and hypothesized that the rapid growth and maturation in shorter lived species may promote the accumulation and excretion of energy-rich compounds. This hypothesis is consistent with previous research on *Mastocarpus* that the gametophyte phase is significantly more palatable to grazers than the long-lived sporophyte phase (Littler & Littler, 1980; Slocum, 1980). The long-lived sporophyte phase also provides more time for the assembly of microbial communities, potentially supporting a later successional stage microbial community relative to the shorter lived gametophyte.

Microbial communities on the surface of *Mastocarpus* are distinct from communities in the surrounding abiotic environment (seawater and rocky substrate). These differences are largely driven by changes in relative abundances of shared OTUs rather than the presence of distinct microbial taxa. For example, the microbiota on rocky substrate share ~97% of bacterial taxa and 84% of microeukaryotes with *Mastocarpus* samples. Yet, Cyanobacteria are much more abundant and Flavobacteria less abundant on rocky substrate compared to *Mastocarpus*, and within Saprospirae, *Portibacter* is common on *Mastocarpus* sporophytes whereas *Lewinella* is more common on rocky substrate (Supporting information Data S3 and S4).

The high degree of overlap and presence of only a very small core community support the hypothesis that macroalgal surfaces are colonized from the pool of environmental microbes present in the surrounding seawater and on nearby surfaces, and few microbial taxa are specifically associated with their macroalgal hosts (Burke, Steinberg, Rusch, Kjelleberg, & Thomas, 2011). This is reinforced by the relatively few taxa that are consistently associated with *Mastocarpus* (two OTUs for bacteria and six for microeukaryotes), or life history phase of *Mastocarpus* (10 and 14 of 1044 bacterial OTUs for gametophytes and sporophytes, respectively).

These results suggest several areas for future research. For example, the inclusion of multiple geographically distinct study sites with differing abiotic conditions would be useful for testing the degree to which macroalgal communities change as a result of changes in the background environmental microbiota. Similarly, future research should investigate whether the observed differences in microbial communities between host life history phases translate to functional differences, or whether functions remain constant despite turnover of microbial taxa, as is seen in *Ulva* (Burke et al., 2011) and other systems (Louca et al., 2018). Finally, we suggest that examining the ecological interactions between host-associated bacterial and microeukaryote communities is an import area for future research. Amplicon sequences from bacterial (16S rRNA gene) and microeukaryote (18S rRNA gene) communities are generally treated as independent data, yet the ecological interactions between these organisms could be important drivers of community structure (Bengtsson et al., 2017).

The presence of three *Mastocarpus* species in our data allowed us to test whether morphologically cryptic macroalgal hosts can have distinct microbial communities. We found that *M. alaskensis* harbours distinct bacterial communities compared to *M. agardhii* and *M. intermedius*, which do not differ from each other. To control for potentially confounding effects of the abiotic environment, we confined our microbial analysis of cryptic host species to co-occurring individuals in the mid/low intertidal zone. These samples occurred in sympatry along the same transect, yet had significant differences in their bacterial community structure. The limited sample sizes and the lack of co-occurrence between all species and life history phases at a single tide height preclude definitive conclusions.

Mastocarpus alaskensis and *M. intermedius* share a more recent common ancestor than either does with *M. agardhii* (Figure 1), yet *M. intermedius* and *M. agardhii* harbour indistinguishable bacterial communities, suggesting that the bacterial differences observed on *M. alaskensis* are not related to host phylogeny. Instead, *M. alaskensis* has several ecological differences compared to both *M. agardhii* and *M. intermedius* that we hypothesize may contribute to observed differences in their bacterial communities. *Mastocarpus agardhii* and *M. intermedius* are southern species that we sampled at or near their northern limits, whereas *M. alaskensis* has a range from northern California to the Aleutian Islands. Divergent selection at northern and southern latitudes could contribute to differences in microbial symbiosis. In addition, *M. alaskensis* is also notable for being the only sampled species that has adapted to tolerate the high intertidal zone. The distribution of marine macroalgae within the intertidal zone is governed by their ability to cope with temperature and desiccation stress, with more tolerant species able to survive at higher elevations on the shore (Bell, 1995). It is possible that physiological changes required to facilitate adaptation to increased thermal stress may have an impact on microbial communities of *M. alaskensis*.

Irrespective of mechanism, the identification of microbial community structure among morphologically cryptic host species has received very little attention and warrants further research. Some recent examples include a study by Sun, Xiao, Cook, Feng, and Huang (2011) who identified three genetic clades of fig wasps that were all morphologically identified as the same species, *Eupristina verticillata*. These clades corresponded to differences in *Wolbachia* infection, leading to the conclusion that the clades were in fact cryptic host species with different bacterial associations. Similarly, McGovern and Hellberg (2003) found that morphologically cryptic bryozoans that vary in the palpability of their larvae to predators also vary in their complement of bacteria that may confer chemical defence. Further research into the structuring of bacterial communities among morphologically cryptic host species is an exciting avenue for future research that could be useful for better understanding the evolution of the microbiome during speciation events in their hosts.

Bacterial differences among cryptic hosts also suggest the potential utility of microbes as an additional source of data for resolving the taxonomic relationship of host species. In *Mastocarpus*, research by Lindstrom et al. (2011) provides molecular evidence based on nuclear, mitochondrial and plastid genes of 382 gametophytes that 11 species inhabit coastal regions between Alaska and California. Our data, which show significant differences in bacterial community structure among sporophyte life history phases sampled in sympatry at the same intertidal height, provide a novel line of evidence supporting the divergence of *M. alaskensis* from its congeners. These results also highlight the critical importance of confirming host taxonomy in ecological studies where cryptic species may be present. In the current study, failure to account for the presence of cryptic host species would have led to spurious patterns of microbial community diversity and ill-informed conclusions.

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DATA ACCESSIBILITY

Raw Illumina MiSeq reads and associated MiMARKS compliant metadata have been accessioned in the European Bioinformatics Institute (www.ebi.ac.uk; Accession no. PRJEB25010). Sanger sequences used for the identification of host species have been accessioned at NCBI (Accession nos. MH407244–MH407279).

AUTHOR CONTRIBUTIONS

MAL, PTM and LWP designed the study. MAL and KRH collected the samples. MAL, KRH and SCL performed laboratory procedures and analysed the data. All authors contributed to writing the manuscript.

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