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RESEARCH ARTICLE

18S rDNA gene metabarcoding of microeukaryotes and epi-endophytes in the holobiome of seven species of large brown algae

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Abstract

Brown algae (Phaeophyceae) are habitat-forming species in coastal ecosystems and include kelp forests and seaweed beds that support a wide diversity of marine life. Host-associated microbial communities are an integral part of phaeophyte biology, and whereas the bacterial microbial partners have received considerable attention, the microbial eukaryotes associated with brown algae have hardly been studied. Here, we used broadly targeted "pan-eukaryotic" primers (metabarcoding) to investigate brown algal-associated eukaryotes (the eukaryome). Using this approach, we aimed to investigate the eukaryome of seven large brown algae that are important and common species in coastal ecosystems. We also aimed to assess whether these macroalgae harbor novel eukaryotic diversity and to ascribe putative functional roles to the host-associated eukaryome based on taxonomic affiliation and phylogenetic placement. We detected a significant diversity of microeukaryotic and algal lineages associated with the brown algal species investigated. The operational taxonomic units (OTUs) were taxonomically assigned to 10 of the eukaryotic major supergroups, including taxonomic groups known to be associated with seaweeds as epibionts, endobionts, parasites, and commensals. Additionally, we revealed previously unrecorded sequence types, including novel phaeophyte OTUs, particularly in the *Fucus* spp. samples, that may represent fucoid genomic variants, sequencing artifacts, or undescribed epi-/endophytes. Our results provide baseline data and technical insights that will be useful for more comprehensive seaweed eukaryome studies investigating the evidently lineage-rich and functionally diverse symbionts of brown algae.

KEYWORDS
endophyte, epiphyte, eukaryome, fungi, holobiont, microeukaryote, Phaeophyceae, protist, seaweed, symbiome

Abbreviations: ASV, amplicon sequence variant; ASW, artificial sterile seawater; HTS, high-throughput sequencing; MAST, marine stramenopiles; OTU, operational taxonomic unit.

INTRODUCTION

The nutrient-rich surfaces of brown algae and other seaweeds harbor a wide diversity of microbial epibionts and endobionts, comprising eukaryotes (Armstrong et al., 2000; Bernard, Rousvoal, et al., 2019; Bernard, Strittmatter, et al., 2019; Gachon et al., 2009; Zuccaro et al., 2008), prokaryotes (Capistrant-Fossa et al., 2021; Egan et al., 2013; Goecke et al., 2013; Hollants et al., 2013; Singh & Reddy, 2015), and viruses (Lachnit et al., 2016; McKeown et al., 2017; McKeown et al., 2018; Müller et al., 1998), i.e., the symbiome. These, together with the host, are collectively referred to as the seaweed holobiont and can be considered a localized ecosystem living on and in a host (Egan et al., 2013; Margulis & Fester, 1991; Skillings, 2016; van der Loos et al., 2019). It is increasingly recognized that host-associated microbial communities (microbiomes) are an integral part of host biology, exerting diverse and strong influences on their hosts (del Campo et al., 2019; van der Loos et al., 2019). Although the term microbiome usually refers mostly or exclusively to bacteria, it is important to consider the whole microbial symbiome, including microbial eukaryotes (microeukaryotes) and larger epi-endophytic symbionts, to enable a comprehensive understanding of holobiont functioning (Bass et al., 2019; van der Loos et al., 2019). To date, bacterial symbionts of brown algae have received the most study (e.g., Capistrant-Fossa et al., 2021; Egan et al., 2013; Florez et al., 2017; Hollants et al., 2013; Singh & Reddy, 2015) and have been shown to represent complex and highly dynamic relationships, ranging from interactions necessary for algal development to those that have detrimental effects on their hosts (de Mesquita et al., 2018; Egan et al., 2013, 2014; Lage & Graça, 2016; Malik et al., 2020; Wahl et al., 2012; Zhang et al., 2020).

Microeukaryotes in the brown algal symbiome are far less known. Only a small number of studies have investigated these associations, mostly using traditional culturing/cell isolation methods or targeted molecular approaches (although Bringloe et al., 2021 recently used a metagenomic sequencing approach to study the epi-endobionts of the brown alga *Alaria*). Previous studies demonstrate that a broad taxonomic diversity of microeukaryotes is associated with brown algae, including surface-dwelling heterotrophic diatoms, dinoflagellates, and ciliates (Armstrong et al., 2000), naked amoebae (Rogerson, 1991), epiphytic and endophytic diatoms (Baardseth, 1969; Lam et al., 2008; Totti et al., 2009), and algal epi-endophytes (Bernard et al., 2017; Bernard, Rousvoal, et al., 2019; Bringloe et al., 2021; Rinkel et al., 2012) in addition to parasitic or saprotrophic labyrinthulids (Raghukumar, 2002; Raghukumar & Damare, 2011), oomycetes (Gachon et al., 2009, 2017; Strittmatter et al., 2013), phytomyxids (Goecke et al., 2012; Murúa et al., 2017; Neuhauser et al., 2014), and fungi (Küpper & Müller, 1999; Tourneroche et al., 2020; Vallet et al., 2018; Zuccaro et al., 2003, 2008). The nature of these microeukaryote–host relationships is mostly unknown, although some symbionts can have detrimental effects on their macroalgal hosts, for example, phytomyxids (Goecke et al., 2012; Murúa et al., 2017; Neuhauser et al., 2014), oomycetes (Gachon et al., 2009, 2017; Strittmatter et al., 2013), chytridiomycete fungi (Küpper et al., 2006; Küpper & Müller, 1999), and phaeophycean parasites and pathogens (Bernard et al., 2017; Bernard, Rousvoal, et al., 2019; Bernard, Strittmatter, et al., 2019; Bringloe et al., 2021; Heesch et al., 2008). Other microeukaryotes are suspected to have a beneficial effect on their hosts, for example, fungal mutualists (Garbary & MacDonald, 1995; Toxopeus et al., 2011; Zuccaro et al., 2008) and endophytes that might protect seaweeds against pathogenic protists (Vallet et al., 2018).

Adding to the complexity of the microeukaryotic biodiversity, the brown algae with which they are associated are often overgrown with a wide variety of smaller red, green, and brown seaweeds ranging from epiphytes through epi-endophytes to endophytes. These epi-/endophytes can have negative effects, such as imposing physical and physiological stress on their host, or positive ecosystem effects, such as increasing available habitats and food for both macroscopic and microscopic life (Potin, 2012). As macroalgal symbiomes comprise multicellular algae as epiphytes (Bjærke & Fredriksen, 2010; Fredriksen et al., 2005) and potentially pathogenic endophytes (Amsler et al., 2009; Bernard, Rousvoal, et al., 2019; Bernard, Strittmatter, et al., 2019; Bringloe et al., 2021; Ellertsdóttir & Peters, 1997; Heesch et al., 2008; Murúa et al., 2018) in addition to microeukaryotes, the almost complete lack of knowledge of these eukaryotes in a holobiont context is a fundamental knowledge gap which needs to be filled in order to improve our understanding of brown algal holobionts. Consequently, there is a need for studies that apply comprehensive high-throughput sequencing (HTS) techniques to explore seaweed-associated microbiomes (Bringloe et al., 2021; van der Loos et al., 2019).

The aim of this study was to use 18S rDNA gene (18S) high throughput metabarcoding to gain a preliminary insight into the eukaryome of a selection of brown macroalgal species that are key components of Northeast Atlantic Ocean rocky shore ecosystems (Fucales: *Fucus vesiculosus*, *F. serratus*, *Himanthalia elongata*, and *Ascophyllum nodosum*; Laminariales: *Laminaria digitata* and *Saccharina latissima*; and Tilopteridales: *Saccorhiza polyschides*). To avoid excluding certain (micro)eukaryotic symbionts, such as other stramenopiles that potentially could be very closely related to their brown algal hosts, we used a broadly targeted HTS approach without attempting to reduce host 18S rDNA gene amplification. We also aimed to assign putative functional roles to the host-associated

microeukaryotes (e.g., putative parasites) and to assess whether brown algae harbor a potentially novel diversity of (micro)eukaryotes.

METHODS

Sample collection

Five individuals each of *Ascophyllum nodosum*, *Fucus vesiculosus*, *Laminaria digitata*, and *Saccharina latissima* were sampled from the Oslofjord (59°40′26.9″ N, 10°35′13.2″ E), while five individuals each of *F. vesiculosus*, *F. serratus, Himanthalia elongata*, *Saccorhiza polyschides*, and *L. digitata* were collected at Newton's Cove (Dorset, UK: 50°36′12.5″ N, 2°26′49.3″ W). All samples were collected in October 2015. In addition, five samples of *F. vesiculosus* sampled in May 2013 from the same location in the Oslofjord were included in our data set (these samples were collected and processed identically to the 2015 samples, described in detail below). Samples were collected by free diving and shore-based collection and were kept cool in separate containers of local seawater from the isolation site while being transported to molecular laboratories where they were immediately subjected to subsampling of tissues for molecular analyses.

All samples were handled under laminar flow hoods to limit their exposure to airborne contaminants. The brown algae were rinsed in sterile artificial seawater (ASW) to remove loosely attached organisms (but not true epibionts) and debris from the surface. This was done in three consecutive steps by vortexing the samples in sterile 50-mL tubes with ASW for 5–10 s. The rinsed individuals were placed in sterile Petri dishes, and one subsample of the algal thallus (squares of approximately 1–2 cm^2) per individual was excised and placed in a separate 2-mL tube. The tissue samples were excised from the middle part of the thallus (i.e., not the apex, stipe, or holdfast). Every specimen appeared healthy; we did not specifically target potentially infected tissues.

The samples were freeze-dried (Freeze drying systems, FreeZone® 2.5 bench top) under sterile conditions by perforating the sample tube lids and placing the sample tubes inside sterile culture flasks with 0.2-μm filter caps (Thermo Scientific™ Nunclon™). After freeze-drying, samples were weighed, placed in new 2-mL sample tubes, and stored at −80°C until DNA extraction.

Molecular methods

The freeze-dried samples were mechanically disrupted by adding two sterile tungsten carbide beads to each sample tube and tissue-lyzed at 20 Hz for 2 min or longer, until the tissues were completely pulverized. Thereafter, a modified lysis buffer (Snirc et al., 2010) was added to the tubes proportional to the sample weight. This lysis buffer contained antioxidant compounds such as PVP (polyvinylpyrrolidone) and BSA (bovine serum albumin) that bind polyphenols and have high salt concentrations, which decrease the levels of co-extracted polysaccharides. The samples were homogenized and divided into two or more tubes so that the samples used for DNA isolation did not exceed 20 mg dry-weight tissue. DNA was extracted following the protocol by Snirc et al. (2010).

PCR amplification of the V4 region of the 18S rDNA gene was performed with "broadly-targeted" eukaryotic primers; the forward primer V4_1f (5′-CCAGCASCYGC GGTAATWCC-3′) and reverse primer TAReukREV3 (Bass et al., 2016; Stoeck et al., 2007; 5′-ACTTTCGT TCTTGATYRA-3′), producing a ~400 bp fragment. PCR reactions (25 μL) contained 1x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 0.4 μM of each primer, 0.5 $mg \cdot mL^{-1}$ BSA (Promega), and 1 μL genomic DNA. The PCR program had an initial denaturation step at 98°C for 2 min, 15 cycles of 30 s at 98°C, 30 s at 53°C, and 45 s at 72°C, then 20 similar cycles except that the annealing temperature was 48°C, and a final elongation step at 72°C for 10 min. DNA was titrated into several dilutions to take into account potential inhibiting substances from the brown algae. Each brown algal individual sample was amplified separately in triplicate reactions for each sample. PCR products were purified and eluted (20 μL) with ChargeSwitch PCR Clean-Up kit (ThermoFisher Scientific) and quantified with the dsDNA BR Assay Kit and Qubit 2.0 Fluorometer (ThermoFisher Scientific) before they were pooled equimolarly, according to species and geographic location. For example, the purified PCR products of five *Fucus vesiculosus* individuals from Norway were pooled into one sample before sequencing library preparation. Negative control PCRs were included in all batches of PCR reactions, and the absence of visible products confirmed in each case.

Sequencing libraries were prepared using the TruSeq Nano DNA Library Preparation Kit at The Natural History Museum, London, UK, following the manufacturer's protocol. High-throughput sequencing was conducted on a MiSeq v3 flow cell using 2 × 300 bp paired-end reads. Base calling was performed by Illumina Real-Time Analysis v1.18.54 and was demultiplexed and converted to FastQ files with Illumina Bcl2fastq v1.8.4, coupled with removal of adaptor sequences.

The complete sequencing data set is available at the European Nucleotide Archive under the study accession number PRJEB45285 (http://www.ebi.ac.uk/ena/data/view/PRJEB45285; Sample ERS649 5425-ERS6495434).

To obtain full-length 18S rDNA gene sequences of the brown algal host species, three DNA extracts

from each brown algal species were subjected to PCR amplification using the eukaryotic 18S primers NSF83 (5′-GAAACTGCGAATGGCTCATT-3′; Hendriks et al., 1989) and 1528R (5′-TCCTTCTGCAGGTTCAC CTAC-3′; Medlin et al., 1988; Orr et al., 2018) utilizing Illustra™ PuReTaq Ready-To-Go™ PCR beads (GE Healthcare). PCR reactions contained 1 Illustra PuReTaq Ready-To-Go bead, 1 μL of DNA template, and 0.5 μM of each primer, filled to 25 μL with water. PCR settings were as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, elongation at 68°C for 1.5 min; final elongation step at 68°C for 15 min. The PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen), according to the manufacturer's protocol. The clones were grown overnight in Luria-Bertani (LB) media amended with 50 μg · mL^{-1} ampicillin. From each brown algal species, 20–30 bacterial colonies/cloned fragments were subjected to PCR reactions with the vector primers T7 and M13R and using approximately 0.5 μL of the bacterial suspension as a template. PCR settings were as follows: initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min, elongation at 72°C for 2 min, final elongation at 72°C for 10 min. The PCR products were cleaned using Illustra™ ExoProStar 1-Step (GE Healthcare) and then Sanger sequenced (GATC Eurofins Genomics). The 18S rDNA gene clone sequences from the brown algal hosts are available at NCBI GenBank under the Accession numbers OQ883584–OQ883643.

Bioinformatics

Cutadapt v3.5 (Martin, 2011) was used to clean the Illumina data by removing primers, adapters, and sequences with low quality, using default settings. DADA2 v1.26 (Callahan et al., 2016) was used for additional quality control, trimming, and denoising. Reads were trimmed with the filterAndTrim() function with the following settings: The forward reads were trimmed at 280 bp, the reverse reads were trimmed at 250 bp, the maximum number of expected errors (MaxEE) was set to 2, and reads with ambiguous bases after trimming were discarded (maxN = 0). The error rates were estimated independently for the trimmed forward and reverse reads with learnErrors(), and reads were denoised with the dada() function, again independently for the forward and reverse reads. The denoised pair of forward and reverse reads were then merged with mergePairs(). Chimeras were removed with the function isBimeraDenovo in DADA2. After denoising and removal of chimeric sequences dereplication, sorting by abundance and discarding of singletons was done with VSEARCH v2.13.4 (Rognes et al., 2016). Finally, the sequences were clustered with VSEARCH into operational taxonomic units (OTUs) using a 97% sequence similarity threshold.

Operational taxonomic units were taxonomically assigned using BLASTn against the PR2 v. 4.12.0 (Guillou et al., 2012) and GenBank (Sayers et al., 2019) databases. For each OTU, we retrieved the 100 best matches that were sorted by the highest bit score and lowest e-value. If hits were identical in e-value and bit score, the match with the longest alignment score and highest percentage identity was kept. The PR2 taxonomy was used for taxonomical assignment for the majority of the following analyses.

Post-VSEARCH trimming and diversity analyses

Species (OTU) composition analyses and further trimming of the data set were done using R v. 4.2.3 (R Core Team, 2013). As we expected, the data set was dominated by brown algal reads, and we therefore did not use a stringent threshold to remove nonbrown algal OTUs with low abundances. However, all OTUs with ≤2 reads were discarded, and the remaining OTUs were subjected to thorough inspections following these criteria: to ensure that all OTUs kept in the data set were valid, we performed careful inspections of Blastn hits against two reference sequence databases (PR^2 and GenBank), sequence alignments, and phylogenetic placement of the OTUs. Operational taxonomic units assigned to land plants and *Homo sapiens* were also removed from the data set. Based on these criteria, 16 OTUs were removed from the data set (Table S1 in the Supporting Information). Further, the 44 OTUs (15.7% of the total number of OTUs) that matched reference sequences of Metazoa were removed from the data set, as shown in Figure S1 and Table S2 in the Supporting Information. The trimmed data set, containing only nonmetazoan eukaryotes, was used for further analyses. The taxonomy of OTUs was visualized using the ggplot2 package v. 3.4.1. (Wickham, 2016).

Phylogenetic analysis

Based on the taxonomic assignment of OTUs as described above, individual phylogenies of the most abundant/diverse groups of microeukaryotes and those with known or potential symbiotic associations with brown algae were constructed, that is, fungi; oomycetes; labyrinthulids; Cercozoa and Endomyxa (Rhizaria); diatoms; brown, green and red algae; ciliates; other alveolates; and centroheliozoans. Each of these alignments also contained the most closely related (lowest e-value) taxonomically characterized sequence from GenBank (or that sequence plus an environmentally derived sequence if the latter was more closely related

to the query sequence). Sequence alignments were carried out using MAFFT v7.300b with the G-ins-i option (Katoh & Standley, 2013).

Phylogenetic trees were built with MrBayes v.3.2.6 (Ronquist et al., 2012). Two separate MC3 runs with randomly generated starting trees were carried out for four million generations each with one cold and three heated chains: 500,000 generations were discarded as "burnin". The evolutionary model applied a GTR substitution matrix, with a four-category autocorrelated gamma correction. All parameters were estimated from the data. The trees were sampled every 1000 generations and the first million generations discarded as burn-in. All phylogenetic analyses were carried out on the Cipres server (Miller et al., 2010). Heatmaps were made for all phylogenetic trees to display the proportional read abundance ($\log_{10}$) for each OTU in the different samples.

RESULTS

Composition of the brown algal eukaryome

A total of 236 eukaryotic OTUs were recovered. Their read abundance in each library is shown in Table S3 in the Supporting Information. Each library represented five brown algal samples pooled according to species and geographic location hereafter referred to as a sample. The sequence data set was dominated by brown algal reads (97.6% of all sequences, Table S4 in the Supporting Information). Brown algae also displayed the highest OTU richness (25.4% of the total OTUs; Figure 1a,b, and they were accountable for 25.0%–78.6% of the OTUs per sample; Table S4). The richness recovered from each sample ranged from 14 to 76 OTUs, and their taxonomic profiles are shown in Figure 1a and Table S3. Nevertheless, there was a broad taxonomic diversity of microeukaryotes in the brown algal samples with OTUs taxonomically assigned to 10 of the eukaryotic major supergroups (Figure 1a,b).

Brown, green, and red algae

We detected a total of 60 brown algal OTUs (four brown algal OTUs were removed because of spurious alignment, Table S1). Considerable levels of microdiversity in the V4 OTUs within species were seen in the phaeophyte clade (Figure 2). Operational taxonomic units corresponding to the host species were inferred based on their phylogenetic proximity to, and high proportions of reads associated with, reference sequences from that host, either from GenBank or full-length 18S rDNA gene sequences generated in this study. However, of the brown macroalgae included in this study, only 18S rDNA gene sequences of *Saccorhiza polyschides* were available in GenBank; 18S rDNA gene sequences were lacking for all other hosts in GenBank, Silva, and PR2. Inferred host OTUs and 18S rDNA clone sequences are indicated by labeled vertical lines in Figure 2. A heatmap, displaying the proportional read abundance ($\log_{10}$) of each OTU in the different samples, shows that many host-derived OTUs were present in samples from multiple host species, although generally represented with lower read abundance in the "nonhost" samples (see, e.g., OTU 003 in the inferred *Himanthalia elongata* host clade and OTU 004 in the inferred *S. polyschides* clade, Figure 2).

Some brown algal OTUs did not cluster with host-derived V4 amplicons or 18S clone sequences. The 35 OTUs in the Uncharacterized clade in Figure 2 were clearly distinct from any characterized or environmental sequences in reference sequence databases. This clade encompassed OTUs that were mainly detected in the *Fucus* spp. samples, but two of them were also detected in lower abundance in other brown algal samples (Figure 2). Additionally, six OTUs in the *Ascophyllum nodosum* clade (OTUs 022, 065, 043, 281, 001, and 104) may derive from that species but grouped separately from the sequences encompassing the cloned 18S sequences generated from that host.

Some of our OTUs grouped strongly with known epi-endophytic lineages, such as the two OTUs (008 and 072) clustering with GenBank sequences from *Pylaiella*, *Halothrix*, and *Myrionema strangulans*. OTUs 008 and 072 were observed in all, or in the majority of, the different brown algal hosts (Figure 2), and some were also detected in 18S clone sequences from *Saccorhiza polyschides*.

We also detected OTUs from green and red algae (Figure 2). The majority of the six green algal OTUs (Figure 2) were very similar (>99% identity) to characterized and sequenced Ulvales taxa such as *Ulvella* (formerly *Acrochaete*) *leptochaete* and *Umbraulva japonica*, which are known endophytes of macroalgae (Gunnarsson & Nielsen, 2016; Nielsen et al., 2014; Rinkel et al., 2012). Similarly, the five red algal OTUs (Figure 2) displayed >98% similarity to taxa that are common epiphytes on seaweeds and a variety of other substrata, including *Ceramium* sp. and *Cryptopleura ramosa*.

Wider phylogenetic diversity of eukaryotic OTUs

Phylogenetic analyses were conducted for taxonomic groups displaying either high taxonomic diversity and/or taxonomic groups previously documented to be in a symbiotic relationship (*sensu lato*) with brown algae. The resulting phylogenies (Figures 3–6; Figures S2 and S3 in the Supporting Information) include OTUs and the closest sequence matches in NCBI GenBank

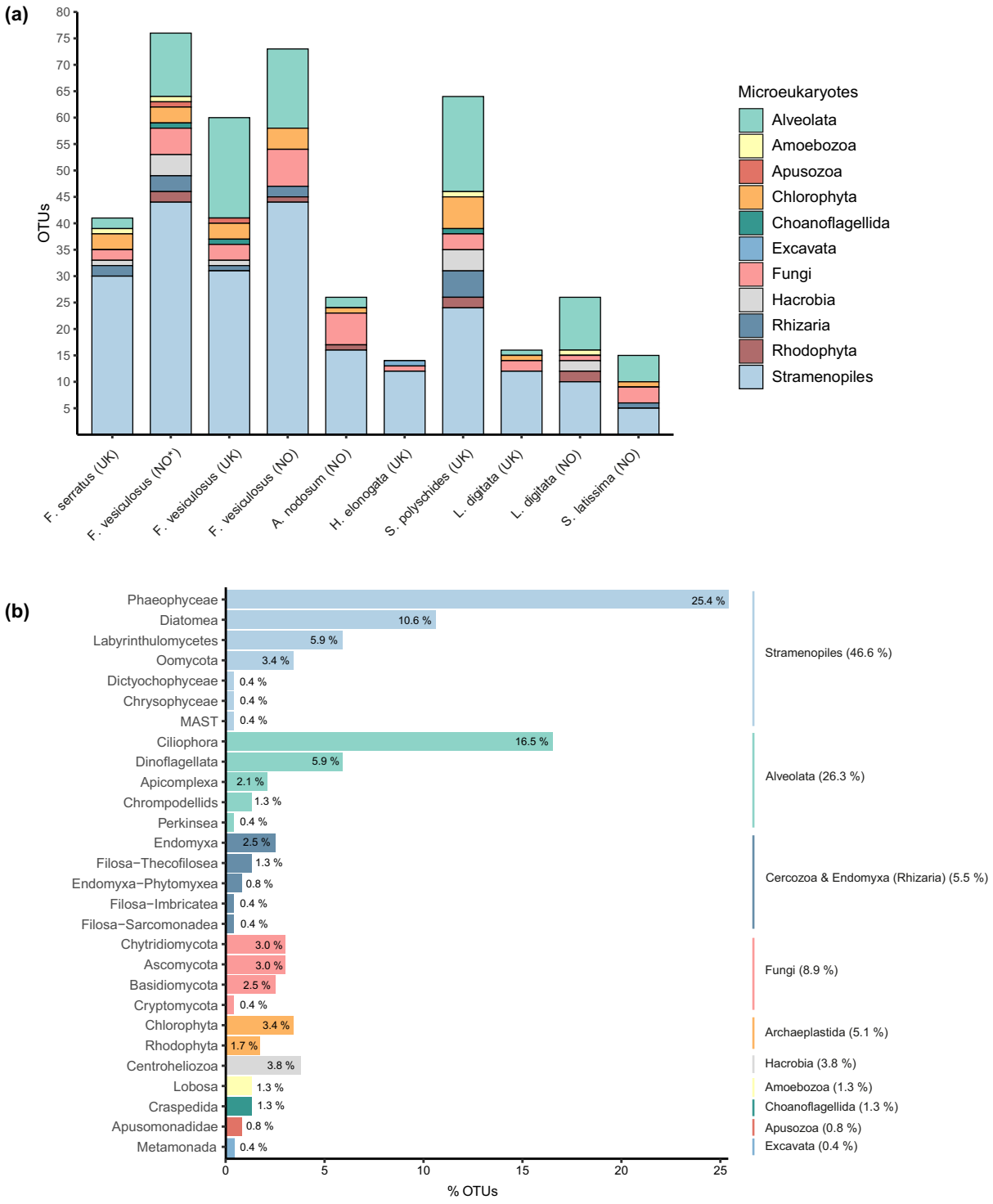


FIGURE 1 Abundance and diversity of eukaryotes in brown algal holobionts. (a) Abundance of operational taxonomic units (OTUs) taxonomically assigned to eukaryotic major Kingdoms or "supergroup" in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parenthesis, NO = Norway and UK = The United Kingdom, together with the total number of OTUs per library. The asterisk (NO*) represents *F. vesiculosus* sampled in Norway, May 2013. All other samples were collected in October 2015. (b) Percentage representation of OTUs assigned to the different taxonomic groups in all the brown algal samples combined. MAST = Marine Stramenopiles. To see the percentage of reads and percentage of OTUs for all the taxonomic groups per brown algal sample, see Table S3. [Color figure can be viewed at wileyonlinelibrary.com]

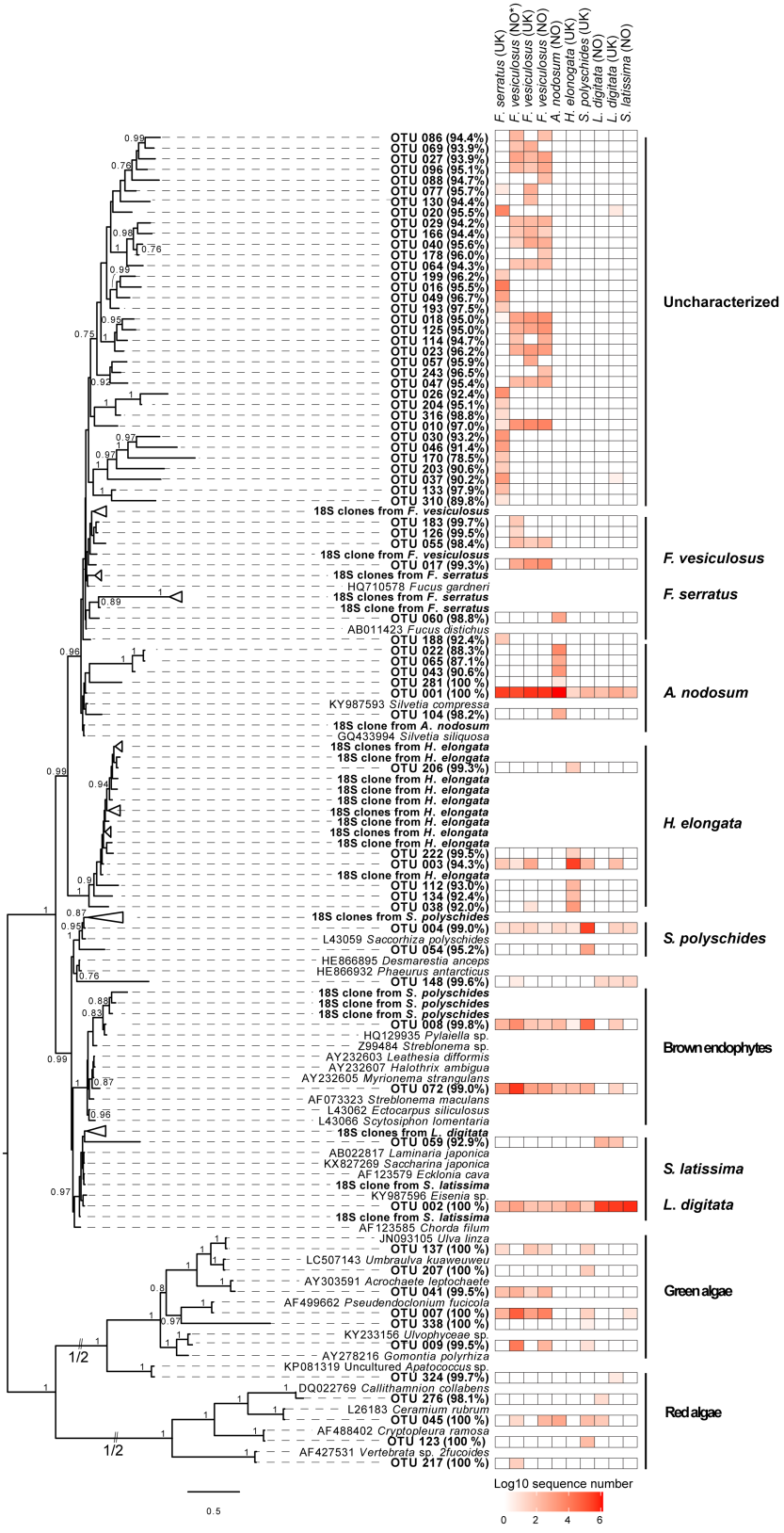
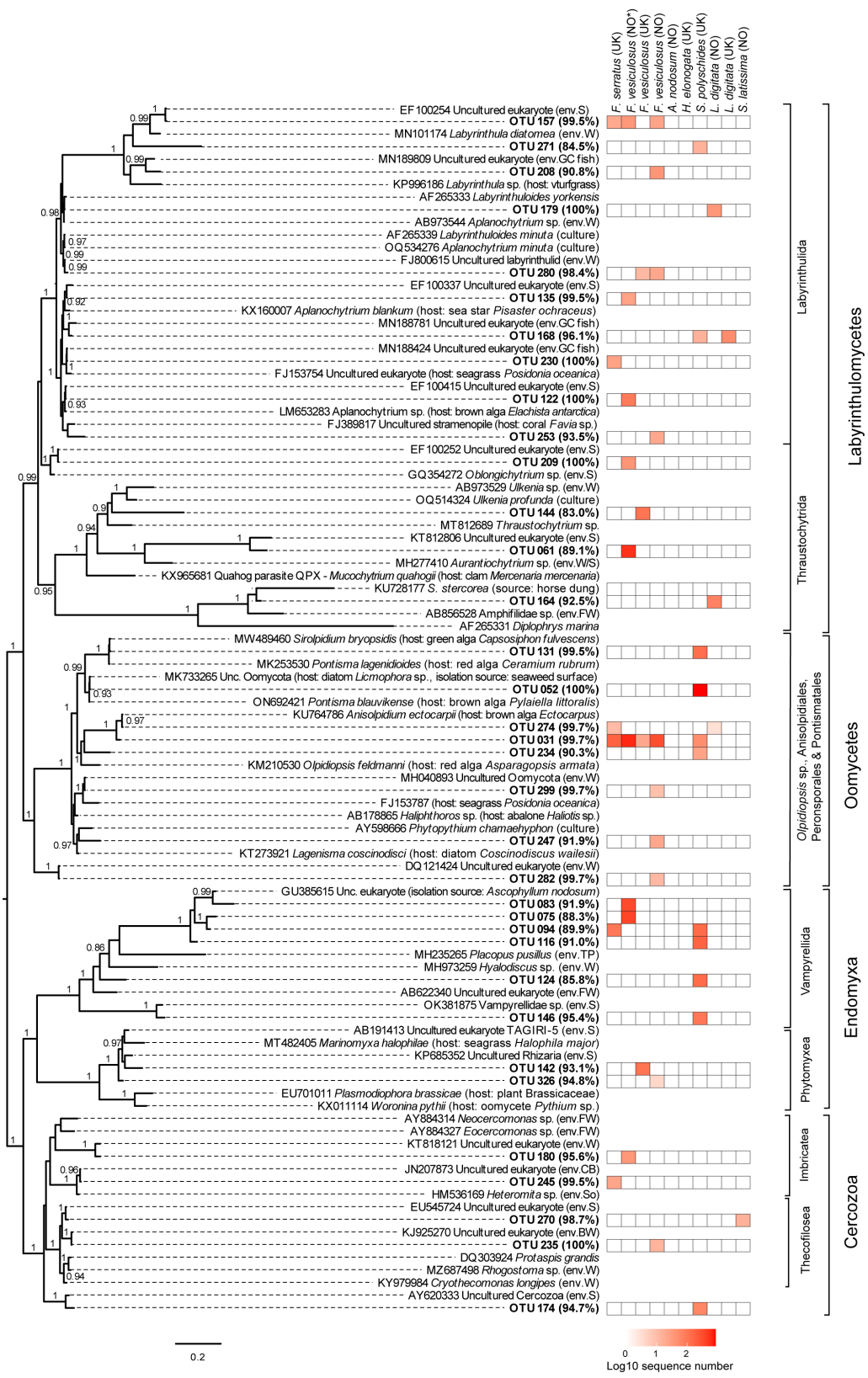


FIGURE 2 Brown, green, and red algal Bayesian phylogeny with heatmap representing proportional read abundance (log_{10}) of operational taxonomic units (OTUs) per sample. OTUs from this study are shown in bold. The percentage identity to the most similar reference sequence is shown in parentheses after each OTU. The longer 18S rDNA gene sequences generated by cloning and Sanger sequencing in this study include the species name of the brown alga they were amplified from and are shown in bold. The heatmap illustrates the log_{10} read abundance for each OTU in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parentheses: NO = Norway and UK = The United Kingdom. All samples were collected in October 2015 except *F. vesiculosus* (NO*), which was sampled from Norway in May 2013. The scale bar represents 0.5 substitutions per site. Several of the taxonomic names for the red algal reference sequences included in the phylogeny are out of date: *Ceramium* spp. is an aggregate of species (*Ceramium rubrum* in this figure) that are members of the order Ceramiales together with *Callithamnion* spp.; *Polysiphonia sensu lato* (*Polysiphonia fucoides* is now *Vertebrata fucoides*) and *Cryptopleura* spp. [Color figure can be viewed at wileyonlinelibrary.com]

as of August 30, 2022. Each phylogeny is accompanied by heat maps showing the proportional read abundance (log_{10}) of each OTU in the different samples.

The heatmaps indicated that for some taxonomic groups, the majority of OTUs were only detected in one brown algal sample, such as Cercozoa (Figure 3), non-ciliate alveolates (Apicomplexa, Perkinsea, and Dinoflagellata; Figure S2), and Centroheliozoa (Figure S3). For other taxonomic groups, such as Labyrinthulomycetes and Oomycetes (Figure 3), Fungi



(Figure 4), ciliates (Figure 5), and diatoms (Figure 6), the heatmaps showed that several of the OTUs (>20%) were detected in multiple samples.

Several OTUs clustered with reference sequences reflecting protist-host associations reported in previous studies, such as the labyrinthulids and oomycetes clustering with *Aplanochytrium* (Labyrinthulida) and Anisolpidiales associated with various hosts (Figure 3), and the endomyxan vampyrellids (OTUs 075, 094, 083, and 116; Figure 3) and the

FIGURE 3 Cercozoa, Endomyxa, oomycete, and labyrinthulid Bayesian phylogeny with heatmap representing proportional read abundance (log_{10}) of operational taxonomic units (OTUs) per sample. Operational taxonomic units from this study are shown in bold. The percentage identity to the most similar reference sequence is shown in parentheses after each OTU. Host or type of environment of the reference sequences retrieved from previous studies is listed in parentheses after each GenBank accession number. The scale bar represents 0.2 substitutions per site. Abbreviations used for descriptions of environment: env.S = environmental sample, marine sediment; env.W = environmental sample, marine water; env.FW = environmental sample, fresh water; env.BW = environmental sample, brackish water; env.So = environmental sample, soil; env.GC = environmental sample gut content; env.CB = cyanobacterial mat; env.TP = environmental sample, tidal pool. The heatmap illustrates the log_{10} read abundance for each OTU in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parentheses: NO = Norway and UK = The United Kingdom. All samples were collected in October 2015 except *F. vesiculosus* (NO*) which was sampled from Norway in May 2013. [Color figure can be viewed at wileyonlinelibrary.com]

diatoms (OTUs 014 and 182; Figure 6) clustering with reference sequences previously detected in samples of *Ascophyllum nodosum*.

Potential novel diversity in the brown algal eukaryome

We determined that ~30% of the OTUs had a lower percentage identity (<95%) to any known close relatives in reference sequence databases (Table S3). The majority of OTUs taxonomically and phylogenetically placed within Fungi, ciliates, and diatoms displayed a high percentage identity (>95%) to known reference sequences (Figures 4–6, respectively). Conversely, within Cercozoa, Endomyxa, and labyrinthulomycetes (Figure 3), and Centroheliozoa (Figure S3), ≥50% of the OTUs had relatively low similarity to known reference sequences (<95%). Some of the low-similarity OTUs were represented by specific clades in the phylogenetic trees, such as the well-supported labyrinthulid clade within Thraustochytrida that encompassed three OTUs with very low percentage identity (ranging between 83.0% and 92.5%; Figure 3). Similarly, within Endomyxa, the eight vampyrellid, and phytomyxean OTUs had relatively low similarity to reference sequences (from 85.8% to 94.8%; Figure 3).

DISCUSSION

Broad diversity of microeukaryotes associated with brown algae

The wide taxonomic diversity we detected suggests that microeukaryotes and eukaryotic epi-/endophytes are an integral part of brown algal holobionts. Our findings show that metabarcoding using general eukaryotic primers can be a valuable approach for investigating the diversity of eukaryotes as part of brown algal holobiomes, including lineages closely related to the host that might be excluded by more targeted primers or host amplification-blocking approaches. Although the sequence data were dominated by brown algal reads, we were able to identify a broad diversity of microeukaryotes representing most of the main branches in the eukaryotic tree of life (Figure 1a,b).

Epi–/endophytic brown, green, and red algae

Some of the brown algal OTUs detected in our study, such as the ones clustering with the epiphytic *Pylaiella* and *Halothrix* and other brown algal epi-/endophytes (Figure 2), represented known epi-/endophytic lineages (e.g., Fredriksen et al., 2005; Lee, 2001; Longtin et al., 2009). These OTUs were detected in all the brown algal host species included in this study except *Saccharina latissima* and were also detected in several 18S rDNA clones generated from the *Saccorhiza polyschides* samples, indicating a broad distribution and host range. The brown algal epi-/endophytes formed a maximally supported clade (Figure 2), including 18S rDNA clones from *Saccorhiza polyschides*, which we infer to represent potentially novel epi-/endophytes present in those samples.

Although we performed careful inspections of the sequence alignment and removed all spurious OTUs, the diversity of brown algal OTUs that apparently derived from the host organisms was much higher than expected (i.e., the OTUs we inferred to represent the different hosts). This may partly be caused by artefactual sequence variants amplified to a discernible extent by the depth of Illumina sequencing, and/or sequence differences between multiple copies of the 18S rDNA gene/rDNA array in the host genome (intragenomic polymorphism). Intragenomic polymorphism has been described in, for example, picoeukaryotes (Zhu et al., 2005), radiolarians (Decelle et al., 2014), and fungi (Ganley & Kobayashi, 2007), but the levels of polymorphism are not known for these brown algal species. Therefore, we have been conservative in ascribing sequence variants indicated in Figure 2 to represent the host alga, e.g., we have ascribed all V4 OTUs clustering with 18S rDNA clone sequences generated from the hosts in the clades bracketed as *Ascophyllum nodosum* and *Himanthalia elongata* in Figure 2 as being host-derived, but there remains the possibility that they represented different organisms.

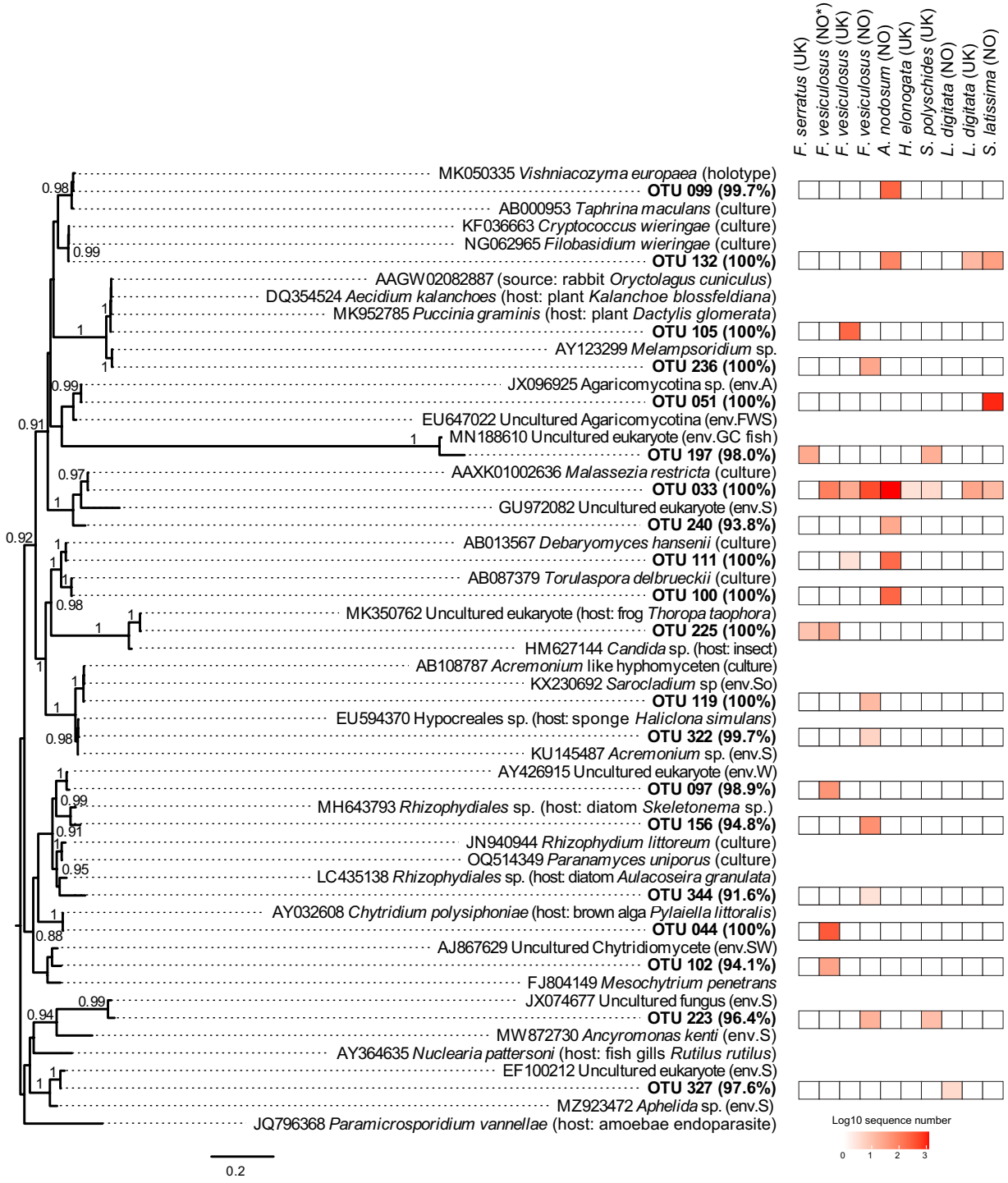


FIGURE 4 Fungal Bayesian phylogeny with heatmap representing proportional read abundance (log_{10}) of operational taxonomic units (OTUs) per sample. Operational taxonomic units from this study are shown in bold. The percentage identity to the most similar reference sequence is shown in parentheses after each OTU. Host or type of environment of the reference sequences retrieved from previous studies is listed in parentheses after each GenBank accession number. The scale bar represents 0.2 substitutions per site. Abbreviations used for descriptions of environment: env.S = environmental sample, marine sediment; env.W = environmental sample, marine water; env.So = environmental sample, soil; env.A = environmental sample, air; env.FWS = environmental sample, fresh water sediment; env. SW = environmental sample, melted snow water. The heatmap illustrates the log_{10} read abundance for each OTU in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parentheses: NO = Norway and UK = The United Kingdom. All samples were collected in October 2015 except *F. vesiculosus* (NO*), which was sampled from Norway in May 2013. [Color figure can be viewed at wileyonlinelibrary.com]

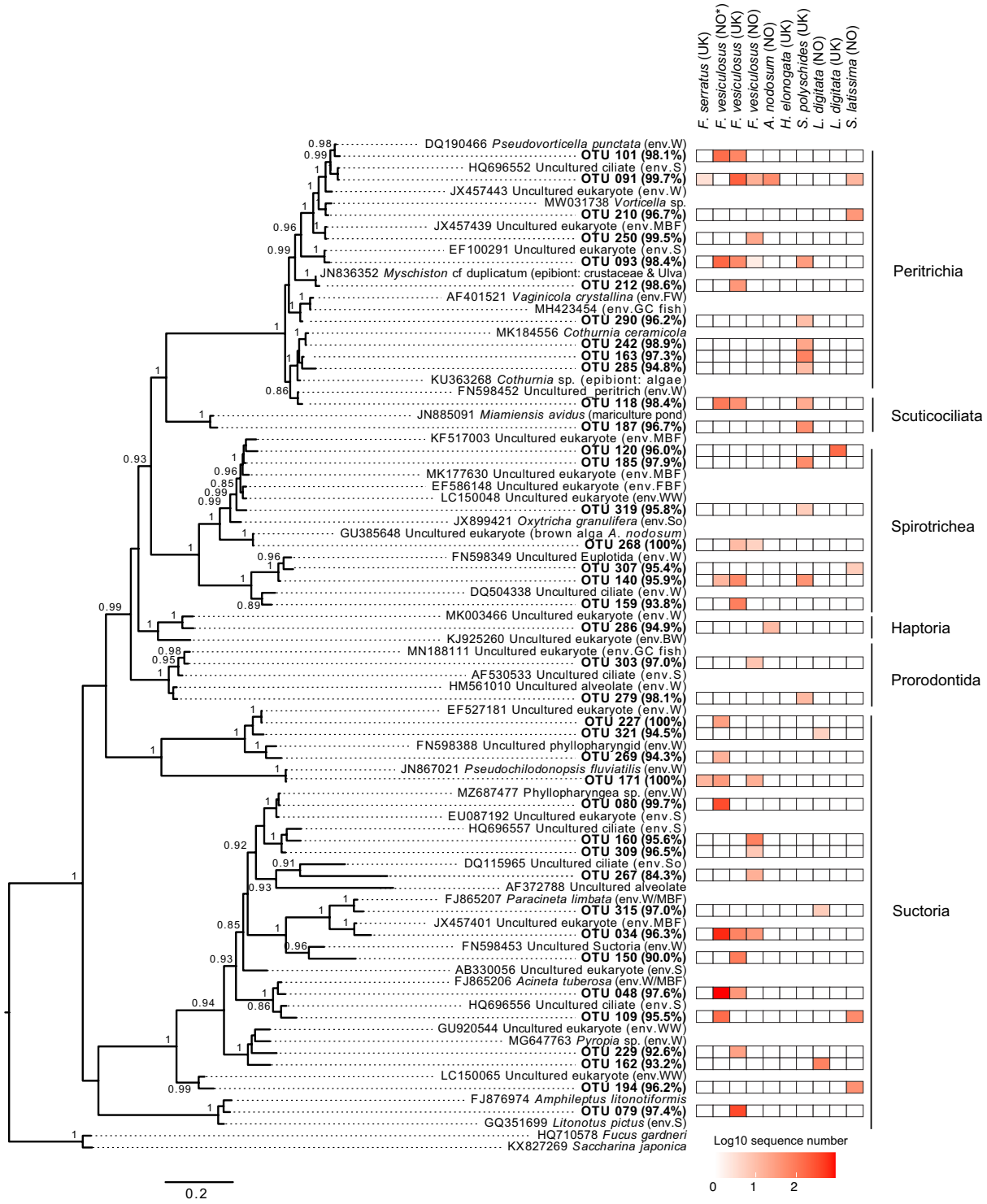


FIGURE 5 Ciliate Bayesian phylogeny with heatmap representing proportional read abundance (log_{10}) of operational taxonomic units (OTUs) per sample. Operational taxonomic units from this study are shown in bold. The percentage identity to the most similar reference sequence is shown in parentheses after each OTU. Host or type of environment of the reference sequences retrieved from previous studies is listed in parentheses after each GenBank accession number. The scale bar represents 0.2 substitutions per site. Abbreviations used for descriptions of environment: env.S = environmental sample, marine sediment; env.W = environmental sample, marine water; env.FW = environmental sample, fresh water; env.WW = environmental sample, wastewater; env.BW = environmental sample, brackish water; env.MBF = environmental sample, marine biofilm; env.FBF = environmental sample, freshwater biofilm; env.So = environmental sample, soil. The heatmap illustrates the log_{10} read abundance for each OTU in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parentheses: NO = Norway and UK = The United Kingdom. All samples were collected in October 2015 except *F. vesiculosus* (NO*), which was sampled from Norway in May 2013. [Color figure can be viewed at wileyonlinelibrary.com]

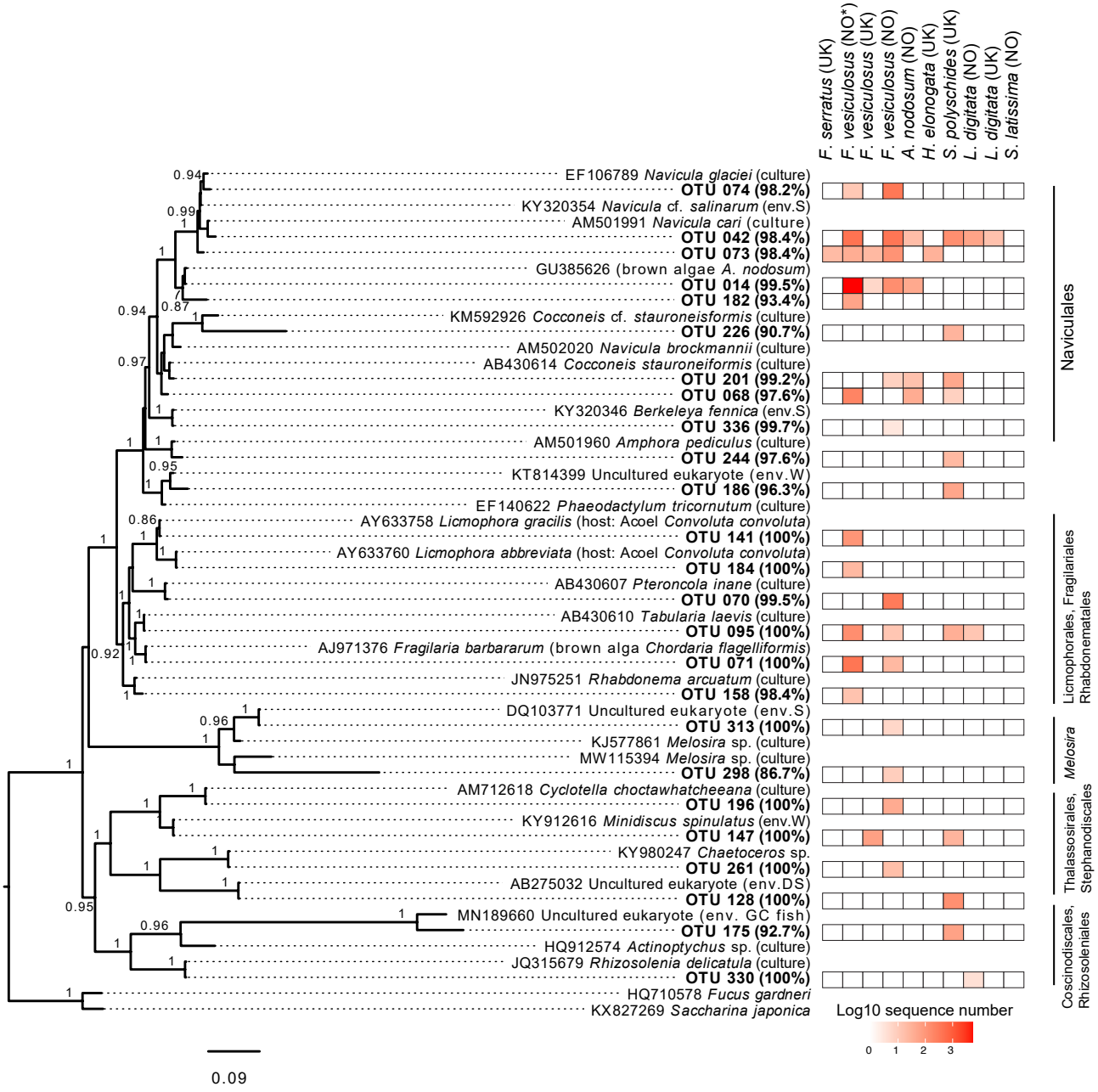


FIGURE 6 Diatom Bayesian phylogeny with heatmap representing proportional read abundance (log_{10}) of operational taxonomic units (OTUs) per sample. Operational taxonomic units from this study are shown in bold. The percentage identity to the most similar reference sequence is shown in parentheses after each OTU. Host or type of environment of the reference sequences retrieved from previous studies is listed in parentheses after each GenBank accession number. The scale bar represents 0.09 substitutions per site. Abbreviations used for descriptions of environment: env.S = environmental sample, marine sediment; env.W = environmental sample, marine water; GC = gut content; env.DS = environmental sample, deep sea sediment. The heatmap illustrates the log_{10} read abundance for each OTU in the brown algal samples of *Fucus serratus*, *F. vesiculosus*, *Ascophyllum nodosum*, *Himanthalia elongata*, *Saccorhiza polyschides*, *Laminaria digitata*, and *Saccharina latissima*. The sampling location for each brown alga is shown in parentheses: NO = Norway and UK = The United Kingdom. All samples were collected in October 2015 except *F. vesiculosus* (NO*), which was sampled from Norway in May 2013. [Color figure can be viewed at wileyonlinelibrary.com]

It is clear that 18S rDNA gene sequence differences between closely related species (e.g., between species of the genera *Fucus* and *Silvetia* and among *Saccharina*, *Laminaria*, *Ecklonia*, and *Chorda*) are relatively small (Figure 2). A long-read metabarcoding approach targeting longer rDNA array regions (e.g., Jamy et al., 2019) could be employed in future studies to enable better phylogenetic characterization of novel host-associated sequence types and their relationships to characterized taxa.

The OTUs that are clearly distinct from host reference sequences (e.g., the "Uncharacterized" clade

and OTUs 022, 065, 043, 281, 001, and 104 bracketed with *Ascophyllum nodosum* and OTUs 206, 222, 003, 112, 134, and 038 bracketed with *Himanthalia elongata* in Figure 2) are worthy of further investigation to determine whether they derive from the related host genomes or (partly) represent other organisms. Microscopic life stages (zoospores, gametophytes, gametes, and juvenile sporophytes) of brown algae are known to grow epi-/endophytically on macroscopic sporophytes of other seaweeds (e.g., Fox & Swanson, 2007; Garbary et al., 1999a, 1999b; Hubbard et al., 2004; Lane & Saunders, 2005; Schoenrock et al., 2020). If spores of different species of Fucales or the gametophytes of Laminariales included in our study grew on/in the brown algal host samples (or were associated with the epi-endophytic red or green algae associated with these), this would have been captured in our sequence data, which can also explain why host-derived OTUs were detected in lower abundance in the other brown algal samples (Figure 2).

It is possible that the *Fucus*-associated "Uncharacterized" clade (at least partly) represents currently unknown fucoid diversity (e.g., vastly morphologically reduced fucoids, or microscopic life stages) or that these OTUs may represent unusually divergent 18S rDNA gene variants present in the host genomes. Either way, high throughput single-cell sequencing approaches could be used to address both this question and whether the sequence variants, if derived from the fucoid hosts, are associated with particular algal cell types.

Microeukaryotes in the seaweed holobiont

Several of the microeukaryotic groups associated with brown algae also include taxa known to live as epi-endophytes. These associations can be facultative (i.e., the epi-endophytes can grow on a variety of biotic and/or abiotic substrata) or obligate (dependent on one or more specific hosts). Like many biological categorizations, the boundaries between epiphytes and endophytes in real life can be blurred. The nature of the association can be affected by both biotic and abiotic factors and can range from mutualism to parasitism (Bringloe et al., 2021; Correa, 1994; Eggert et al., 2010; Potin, 2012).

Diatoms are regularly observed in the biofilm on seaweed surfaces, where they live as epiphytes and often occur in large numbers (Costa et al., 2016; Lage & Graça, 2016; Tiffany, 2011; Totti et al., 2009). Although many diatoms associated with brown algae are epiphytes, there are also studies that have demonstrated that certain diatom species, such as the ones belonging to the genera *Navicula* and *Cocconeis* can live as endophytes, intercellularly in tissues of both brown algae and red algae (Baardseth, 1969; Baardseth & Taasen, 1973; Hasle, 1968; Klochkova et al., 2014). Recent studies have suggested that some diatom taxa might have adapted to an endophytic life style to avoid the antifouling mechanisms of their hosts (Mayombo et al., 2019, 2020). Several of the OTUs from our study, which were taxonomically and phylogenetically assigned to different species of the orders Naviculales, Cocconeidales, and Fragilariales (Figure 6), were present in multiple samples/hosts independent of geographic location. Our molecular data in combination with previous observational studies (Baardseth, 1969; Baardseth & Taasen, 1973; Hasle, 1968; Klochkova et al., 2014; Mayombo et al., 2019, 2020; Totti et al., 2009) support the hypothesis that some diatom species such as *Navicula* sp. and *Cocconeis* sp. might have a more intimate, potentially endophytic, association with seaweeds.

Ciliates represent another ubiquitous group of microeukaryotes living as epiphytes in the biofilm on seaweeds (Armstrong et al., 2000; Gismervik, 2004), and this was also the dominating group of alveolates in our study (Figure 1b). Many ciliates are predators/grazers feeding on bacteria and microeukaryotes (Armstrong et al., 2000; Lynn, 2010), whereas other ciliates can be involved in symbiotic interactions (Bjorbækmo et al., 2019; Lynn, 2010). Most of the ciliates (~75%) detected in our study were only seen in single samples (Figure 5), which might indicate that these ciliates were random visitors without any specific interaction with brown algae. Approximately 25% of the ciliate OTUs, however, appeared in several samples from both Norway and the UK and were highly similar to reference sequences from different marine environmental samples. This suggests that these ciliates are generalist biofilm grazers on different organisms and substrata. However, one of these OTUs (OTU 268, Spirotrichea, Figure 5) was identical to a reference sequence observed in samples of *Ascophyllum nodosum* used as live bait wrapping (Haska et al., 2011), which could indicate that some of these ciliates have a closer association with brown algae. It is certainly possible that many of the microeukaryotes that are frequently detected from environmental samples might also be host-associated (del Campo et al., 2019).

Less abundant, but potentially important symbionts associated with brown algae

Some of the less abundant microeukaryotes we detected such as Fungi (Figure 4) and Cercozoa, Endomyxa, oomycetes, and labyrinthulomycetes (Figure 3) were taxonomically and phylogenetically placed with known algal symbionts.

Fungi associated with brown algae can have a wide variety of effects on their hosts, from detrimental to beneficial (Suryanarayanan, 2012, and references

therein). Two of the Ascomycota OTUs clustered with taxa known to be parasites of brown algae such as *Hypocreales* and *Acremonium* (Vicente et al., 2021; Wong Chin et al., 2022; Figure 4). Several of the basidiomycete OTUs clustered with reference sequences related to *Cryptococcus* and *Cystofilobasidium* that have previously been found associated with various marine invertebrates, algae, and seaweeds, and as parasites on other fungi (Figure 4; Duarte et al., 2013; Lo Giudice et al., 2019; Ogaki et al., 2019; Vaca et al., 2012). In addition, within Chytridiomycota, there were several OTUs that clustered with *Rhizophydium* sp., which comprise parasites with broad host ranges known to infect both macroalgae and protists (Frenken et al., 2017; Gromov et al., 1999). One OTU (OTU 044; Figure 4) also displayed high similarity to *Chytridium polysiphoniae*, a parasite of brown algae (Küpper et al., 2006; Müller et al., 1999).

The cercozoan and endomyxan diversity (Figure 3) included vampyrellid amoebae, with four OTUs forming a distinct clade with GU385680, which was previously detected on *Ascophyllum nodosum* used as live bait wrapping (Haska et al., 2011). Vampyrellids exhibit a wide diversity of feeding strategies and often feed omnivorously, but it is noteworthy that this clade comprises only lineages associated with brown algae, which suggests a potentially specific association. Phytomyxids, which are biotrophic parasites of angiosperms, oomycetes, and stramenopile algae (Neuhauser et al., 2014), were represented by two OTUs which may represent organisms interacting with the brown algae themselves or algal/oomycete epiphytes of the hosts we sampled. The other cercozoans detected (Figure 3) are more likely to be commensals, feeding on microorganisms growing on the algal surfaces.

Labyrinthulomycetes (Figure 3), which are heterotrophic Stramenopiles, are abundant and diverse in a wide range of marine and freshwater habitats, where they play important roles as saprotrophs/decomposers (Nakai & Naganuma, 2015; Pan et al., 2017). Some labyrinthulomycetes are also known as important symbionts (parasites, mutualists, or commensals) of marine organisms, including brown algal seaweeds (Gleason et al., 2013; Mystikou et al., 2014; Raghukumar, 2002). Although most thraustochytrids are free-living, a few species have been associated with diseases in marine metazoans, including the quahog parasite QPX (*Mucochytrium quahogii*), which parasitizes clams (Mo et al., 2002; Stokes et al., 2002). Several of the OTUs in our study clustered in three highly supported clades within Labyrinthulida (Figure 3) and had high similarity to *Aplanochytrium* labyrinthulids, which have been observed in previous studies to live associated with various hosts such as corals (Siboni et al., 2010), the pseudoparenchymatous brown alga *Elachista* sp. (Mystikou et al., 2014), seagrass (Medina-Pons et al., 2009), and sea stars (FioRito et al., 2016).

Certain oomycetes are common parasites of brown and red algae (Badis et al., 2018, 2020; Gachon et al., 2010; Strittmatter et al., 2013), and *Olpidiopsis* species have been shown to have cosmopolitan occurrence and broad host ranges (Badis et al., 2020; Sekimoto et al., 2009). In this study, some of the OTUs clustering within the clade labeled "*Olpidiopsis* sp., Anisolpidiales, and Pontismatales" were highly similar to reference sequences of *Anisolpidium rosenvingei* and *A. ectocarpii* (Figure 3), which are parasites of filamentous brown algae (Gachon et al., 2017). Others displayed high similarity to *Pontisma lagenidioides*, *Ectrogella*, and *Sirolpidium bryopsidis*, which parasitize the diatom *Licmophora* sp., the red alga *Ceramium virgatum*, and the green alga *Capsosiphon fulvescens* (Buaya et al., 2019, 2021; Garvetto et al., 2019). Further, we detected two OTUs with low similarity to available reference sequences (Figure 3): OTU 247 with 91.9% identity to JN635125 (*Phytophthora* sp.) and OTU 234 with 90.3% identity to *Lagenisma coscinodisci* (KT273921), of which the latter is an oomycete parasitizing diatoms (Garvetto et al., 2019; Thines et al., 2015). This highlights one of the intrinsic problems when inferring symbiont–host relationships relying on molecular metabarcoding data alone; it is challenging to infer whether these oomycete OTUs are symbionts (parasites) of the brown algal hosts or if they infect other taxa associated with the host (e.g., diatoms). This question is not specific to oomycetes, however, and arguably applies to most biodiversity described in this study.

Challenges and recommendations for future holobiome studies

Although our approach successfully captured a wide diversity of eukaryotes associated with brown algae, it is well known that "general" or "universal" primers do not amplify consistently across the diversity of eukaryotes, since several taxonomic groups, for example within Rhizaria and Excavata, have sequence mismatches against general primers and consequently fail to amplify (Bass & del Campo, 2020; Vaulot et al., 2021). Therefore, in future holobiome studies of brown algae, it would be optimal to combine general eukaryotic metabarcoding with PCR-free approaches such as metagenomic/transcriptomic shotgun sequencing (Bass & del Campo, 2020; Bringloe et al., 2021; Tully et al., 2018), which would also enable genomic assemblies (e.g., of viruses and bacteria) and functional gene searches, for example by E-probes (Espindola et al., 2018). In a recent study, deep whole-genome sequencing was successfully used to investigate the brown alga *Alaria*, whereby the researchers observed several epi-endophytic brown algae, including a likely novel parasitic brown algal endophyte (Bringloe et al., 2021). Combining metagenomic/transcriptomic sequencing

and targeted metabarcoding will ensure a holistic approach representing all available genomic information in a sample and is a promising approach to capture "everything" associated with brown algae (or other eukaryotic hosts); including DNA and RNA viruses, bacteria and archaea, eukaryotic protists, fungi, and epi/endophytes. This approach would provide a) insight into the whole symbiome (via metagenomics: broad sequencing of relatively few samples) and b) robust testing of hypotheses requiring large sample sizes (via metabarcoding: narrow and deep sequencing of many samples). Further PCR amplicon analyses of brown algal symbiome would nonetheless be very valuable, especially when using long-range amplification and sequencing, which is becoming more established for both bacterial and eukaryote metabarcoding (e.g., Jamy et al., 2019), providing greater phylogenetic resolution for placing novel lineages with respect to host and other taxa.

One of the main challenges when using molecular methods alone, however, is to differentiate "host-associated" from "random co-occurrence," which is the case for several of the lineages we detected in our study. To determine the localization of microbes in the holobiont and the functional roles of the microbial partners, and to gain insight into the nature of the microbial-host associations (e.g., their impact on host tissues), molecular methods should be integrated with microscopy-based approaches such as (Fluorescent) In Situ Hybridisation or (F)ISH, histology, fluorescence confocal microscopy, and Transmission Electron Microscopy (TEM), in addition to isolation and culture-based experiments.

Another challenge is the lack of reference sequences in public databases. The low percentage identity to known reference sequences displayed by OTUs phylogenetically placed within Cercozoa, Labyrinthulomycetes, and Centroheliozoa hints at a large unknown diversity in these lineages as opposed to marine protist groups like diatoms and ciliates that have been better studied.

In conclusion, to understand how microeukaryotes and epi-endophytes interact with and affect their hosts and how environmental change and aquaculture affect the brown algal holobionts (i.e., effects on the microeukaryome composition), we first need to know the identity of these organisms. Our results provide important baseline data from which to study those interactions and changes. The potentially novel eukaryotic diversity we have observed and that the vast majority of macroalgae in marine habitats remain unexplored for their eukaryotic symbiome demonstrates that brown algae and other seaweeds are potentially rich sources for a large and hidden diversity of novel microeukaryotes and epi-endophytes.

AUTHOR CONTRIBUTIONS

Marit F. Markussen Bjorbækmo: Conceptualization (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (equal); project administration (equal); resources (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Juliet Brodie:** Investigation (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (lead). **Anders K. Krabberød:** Formal analysis (equal); resources (equal); supervision (equal); visualization (equal); writing – review and editing (equal). **Ramiro Logares:** Formal analysis (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Janina Fuss:** Funding acquisition (equal); investigation (equal); writing – review and editing (equal). **Stein Fredriksen:** Supervision (supporting); writing – review and editing (equal). **Anders Wold-Dobbe:** Investigation (equal); writing – review and editing (equal). **Kamran Shalchian-Tabrizi:** Conceptualization (equal); resources (equal); supervision (supporting); writing – review and editing (equal). **David Bass:** Conceptualization (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (equal); project administration (equal); resources (equal); supervision (lead); visualization (equal); writing – original draft (equal); writing – review and editing (lead).

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DATA AVAILABILITY STATEMENT

The complete sequencing data set is available at the European Nucleotide Archive under the study accession number PRJEB45285 (http://www.ebi.ac.uk/ena/data/view/PRJEB45285). Amplicon V4 sequence data are in Samples ERS6495425-ERS6495434, full-length 18S clone sequences from the brown algal hosts are available at NCBI GenBank under the Accession numbers OQ883584–OQ883643. All used R packages as well as other software are cited in "Methods" section.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Diversity of metazoan OTUs detected in brown algal holobionts.

Figure S2. "Non-ciliate" Alveolates phylogeny with heatmap representing proportional read abundance (log10) of OTUs per sample.

Figure S3. Centroheliozoa phylogeny with heatmap representing proportional read abundance (log10) of OTUs per sample.

Table S1. OTUs removed from the OTU-table.

Table S2. Overview of all metazoan OTUs, their read abundance in the different samples, the taxonomic classification of each OTU, closest reference sequence in Protist Ribosomal Database (PR2). Accession# = PR2 accession number.

Table S3. Overview of all microeukaryotic OTUs, their read abundance in the different samples, the taxonomic classification of each OTU, and the percentage identity (%id) to closest reference. sequence in GenBank (NCBI) and the Protist Ribosomal Database (PR2). acc# = accession number. Abbreviations: NCBI/PR2_bit = bitscore, NCBI/PR2_len = length of the High-scoring Segment Pairs (HPS), NCBI/PR2_eval = e-value.

Table S4. Percentage of total number of OTUs and percentage of total number of reads are shown in the first two columns. Percentage of of OTUs per taxonomic group in each sample is shown outside brackets, while percentage of reads per taxonomic group in each sample is shown inside brackets.

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