Fungal Ecology 25 (2017) 1–13

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Coastal marine habitats harbor novel early-diverging fungal diversity

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ARTICLE INFO

Article history:
Received 12 September 2016
Received in revised form
20 October 2016
Accepted 27 October 2016
Available online 23 November 2016

Corresponding Editor: Felix Bärlocher

Keywords:
Marine fungi
Ion torrent
LSU rDNA
Chytridiomycota
Neocallimastigomycota
Diversity
Coastal

ABSTRACT

Despite nearly a century of study, the diversity of marine fungi remains poorly understood. Historical surveys utilizing microscopy or culture-dependent methods suggest that marine fungi are relatively species-poor, predominantly Dikarya, and localized to coastal habitats. However, the use of high-throughput sequencing technologies to characterize microbial communities has challenged traditional concepts of fungal diversity by revealing novel phylotypes from both terrestrial and aquatic habitats. Here, I used ion semiconductor sequencing (Ion Torrent) of the ribosomal large subunit (LSU/28S) to explore fungal diversity from water and sediment samples collected from four habitats in coastal North Carolina. The dominant taxa observed were Ascomycota and Chytridiomycota, though all fungal phyla were represented. Diversity was highest in sand flats and wetland sediments, though benthic sediments harbored the highest proportion of novel sequences. Most sequences assigned to early-diverging fungal groups could not be assigned beyond phylum with statistical support, suggesting they belong to unknown lineages.

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1. Introduction

Fungi are among the most diverse groups in Eukarya with estimates of total global diversity projecting upwards of 5.1 million species (O'Brien et al., 2005; Blackwell, 2011; Taylor et al., 2014). However, with only ~100,000 circumscribed taxa (Kirk et al., 2008), the overwhelming majority of which belong to the Ascomycota and Basidiomycota (~96,000 species), our current understanding of fungal diversity remains incomplete. As a consequence, efforts to reconstruct evolutionary relationships within and among major fungal lineages that lie outside of the crown groups have been stymied by limited taxon sampling. Further, the potential ecological roles that these poorly known taxa may play in different environments, and how important they might be in ecosystem functioning, largely remain a mystery.

Marine fungi, which represent less than 1% of described fungal species (Kis-Papo, 2005; Richards et al., 2012), are particularly poorly characterized, despite a century of study (Jones, 2011). Historically, marine fungi were either isolated from or observed on substrata such as vegetation, macroalgae, and driftwood, reported as parasites of animal, plant, and algal hosts, or cultured from water, sediments, and sea foam (Kohlmeyer and Kohlmeyer, 1979). The taxa recovered from these marine surveys were predominantly Dikarya and localized to coastal habitats, where organic matter was readily available. The relative paucity of marine taxa from other fungal lineages (especially the zoosporic groups) or taxa from surface waters led Kohlmeyer and Kohlmeyer (1979) to conclude that marine fungi were relatively species-poor and that the open oceans were largely a 'fungal desert'. These observations, coupled with phylogenetic studies showing that many marine ascomycetes are secondarily derived from terrestrial groups (Spatafora et al., 1998; Schoch et al., 2007; Suetrong et al., 2009) rather than descended from an ancient obligately marine lineage, in many ways cemented the view that the marine realm, though a vast reservoir of microbial diversity (Sogin et al., 2006), was home to only a few fungi outside of the Ascomycota. To wit, when discussing habitats that might harbor as-yet undiscovered fungi, Hawksworth and Rossman (1997) mention marine environments only briefly, and with regard to endophytes of marine plants.

Over the past two decades, culture-independent methods, including environmental cloning and, increasingly, next-generation sequencing, have begun to reveal substantial fungal diversity from previously un- and under-sampled habitats across the globe, including soils (Penton et al., 2013; Tedersoo et al., 2014), freshwater lakes (Lefevre et al., 2008; Monchy et al., 2011; Ishida et al.,

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http://dx.doi.org/10.1016/j.funeco.2016.10.006
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2015), and glacial snowpack (Brown et al., 2015). Taxa recovered in these studies can and do belong to well-characterized fungal lineages, but many others represent entirely novel clades that have previously eluded detection. Though there are undescribed taxa across the fungal tree—recently termed the "dark matter fungi" (DMF) by Grossart et al. (2016)—they are especially common among the zoosporic fungi (Blastocladiomycota, Chytridiomycota, Cryptomycota, Neocallimastigomycota, and the genus *Olpidium*) and former zygomycotan (Entomophthoromycota, Kickxellomycotina, Mortierellomycotina, Mucoromycotina, Zoopagomycotina) lineages. The nameless, faceless members of these early-diverging groups are often microscopic and may have very specific nutritional requirements [e.g., obligate endoparasites in the Cryptomycota, putative symbionts in the Chytridiomycota (Newell, 1981; Nyvall et al., 1999; Picard et al., 2013)] making them difficult to isolate into culture. Most notably, the recently described phylum Cryptomycota was established using phylotypes recovered almost exclusively from environmental surveys (Jones et al., 2011a). Taxa in this group have subsequently been shown to be not only ubiquitous in their distribution (Livermore and Mattes, 2013; Matsunaga et al., 2014; Lazarus and James, 2015), but also diverse—and often abundant—relative to other microbial eukaryotes (Taib et al., 2013; Capo et al., 2015; Debroas et al., 2015).

In addition to revealing new taxa among better characterized terrestrial and freshwater habitats, culture-independent methods have increasingly reported novel clades from marine environments, many of which are allied to the early-diverging branches of the fungal tree (Bass and Richards, 2011; Richards et al., 2012, 2015). Recent culture-independent studies describing fungi from marine environments have investigated deep-sea and benthic sediments (Nagano et al., 2010; Edgcomb et al., 2011; Nagahama et al., 2011; Thaler et al., 2012; Richards et al., 2015; Pachiadaki et al., 2016; Tisthammer et al., 2016), hydrothermal vents (Burgaud et al., 2015), oxygen-deficient environments (Stoeck et al., 2006; Stock et al., 2009; Jebaraj et al., 2012; Wang et al., 2014b), and global surface waters (Wang et al., 2014a; de Vargas et al., 2015; Richards et al., 2015; Stern et al., 2015; Tisthammer et al., 2016). Comparatively fewer studies have focused on marine fungi in coastal habitats (Arfi et al., 2012; Jeffries et al., 2016), which have historically been the best studied.

In this study, I used ion semiconductor sequencing of the nuclear large subunit (LSU, 28S) to investigate the taxonomic richness and diversity of marine and estuarine fungi from four disparate habitats in coastal North Carolina over the course of a year. My primary objectives were: (i) to characterize the fungal communities in coastal habitats and compare community composition across sites; (ii) assess the difficulty in classifying putative marine taxa across fungal lineages; and (iii) elucidate potential ecological roles for marine fungi as suggested by spatio-temporal distribution of taxa in coastal sites.

2. Materials and methods

2.1. Study sites and sampling regime

A total of four sampling sites located in coastal Carteret County, North Carolina, USA were sampled quarter-annually between April 2011 and May 2012. For the first two sites, sediments were collected from persistent intertidal wetlands (Town Marsh; 34° 42′ 45.5832″ N × 76° 40′ 17.7492″ W) and intertidal sand flats (Bird Shoal; 34° 42′ 28.7928″ N × 76° 39′ 42.8796″ W)—part of the Rachel Carson site within the North Carolina National Estuarine Research Reserve (NCERR) (Fig. 1A). Town Marsh is a sandy island whose interior is dominated by supratidal grasslands and scrub-shrub vegetation such as southern redcedar (*Juniperus virginiana* var. *silicicola*), yaupon (*Ilex vomitoria*), loblolly pine (*Pinus taeda*), and Hercules' club (*Zanthoxylum clava-herculis*). The periphery of the island comprises intertidal persistent wetlands that support oyster beds and avian rookeries. Adjacent to Town Marsh, Bird Shoal primarily comprises intertidal sand- and mud-flats dominated by dwarf glasswort (*Salicornia bigelovii*) and smooth cordgrass (*Spartina alterniflora*). Town Marsh and Bird Shoal are subject to diurnal tides. Sediments from both sites were collected at low tide, using sterile 50 mL centrifuge tubes, up to a depth of 5 cm.

Piver's Island (34° 43′ 12.4782″ N × 76° 40′ 22.7388″ W), home to the National Oceanic and Atmospheric Administration (NOAA) Fisheries Lab and the Duke University Marine Lab, is situated in the lower Newport River estuary less than 1 km west of Bird Shoal and Town Marsh, and approximately 2 km from the Beaufort Inlet (Fig. 1A). This site experiences semi-diurnal tides of approximately 1 m (NOAA, 2012). A thorough description of the tidal and climatic variables at this site can be found in DeVries et al. (1994). To facilitate surveying surface water fungi, especially potential phytopathogens, plankton tows were performed from a platform under the Piver's Island bridge using a 0.5 m diameter 80 μm plankton net. The net was deployed for 15 min and a total of 200 mL of surface water was collected in sterile 50 mL centrifuge tubes.

Finally, marine sediments were collected from the shallow waters (~9 m) at Station A-1 (34° 37′ 7.0422″ N × 76° 32′ 43.1160″ W) in Cape Lookout Bight, located at the southern tip of the Outer Banks (Martens and Klump, 1980) (Fig 1B). This small marine basin is rich in organic detritus originating from barrier islands upstream, with sediments containing 3–5% organic C (Martens and Klump, 1984). Sampling was performed seasonally over the course of a year (July and October 2011, February and May 2012). Sediments were collected using a piston core deployed from the research vessel *Susan Hudson*; collected sediment cores measuring 100–120 cm in total length were divided into 2 cm strata. Due to high activity of sulfate-reducing and methanogenic bacteria in the spring and summer months, respectively (Alperin et al., 1994), and limited penetration of dissolved oxygen from overlying water in the winter (Martens and Klump, 1984), surface sediments in the bight quickly become anoxic. Therefore, only the upper 2 cm of the core was included in this study. The upper core sediments were subsampled with sterilized, ethanol-rinsed spatulas and placed into sterile 15 mL centrifuge tubes.

All samples taken from Town Marsh, Bird Shoal, Piver's Island, and Cape Lookout Bight were sealed with parafilm, transported to Duke University on ice, and stored at −80 °C until the extraction of genomic DNA.

2.2. DNA extraction and sequence data generation

Collected sediments (Town Marsh, Bird Shoal, and Cape Lookout Bight) were thawed at room temperature and homogenized by hand. Large pieces of plant matter and other detritus were removed manually, if present. For the plankton tow site (Piver's Island), tissue from thawed samples was collected through centrifugation (4000×*g* for 15 min at 4 °C) in volumes of 100 mL, and dried at 30 °C in a Vacufuge® concentrator (Eppendorf, Hamburg, Germany) for 15–30 min. Following mixing and/or drying steps, approximately 1 g of sediment or mixed planktonic tissue was used for total genomic DNA extraction using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's protocol. Extracted DNA was eluted in 100 μL of Solution C6 (10 mM Tris) that had been heated to 55 °C.

Amplicon libraries were generated using nuclear LSU primers LR0R [5′- ACCCGCTGAACTTAAGC-3′ (Moncalvo et al., 2000);] and EDF360R (5′-TACTTGTICGCTATCGGTCTC-3′; designed here for this study to accommodate the 400 bp read length of Ion Torrent), with

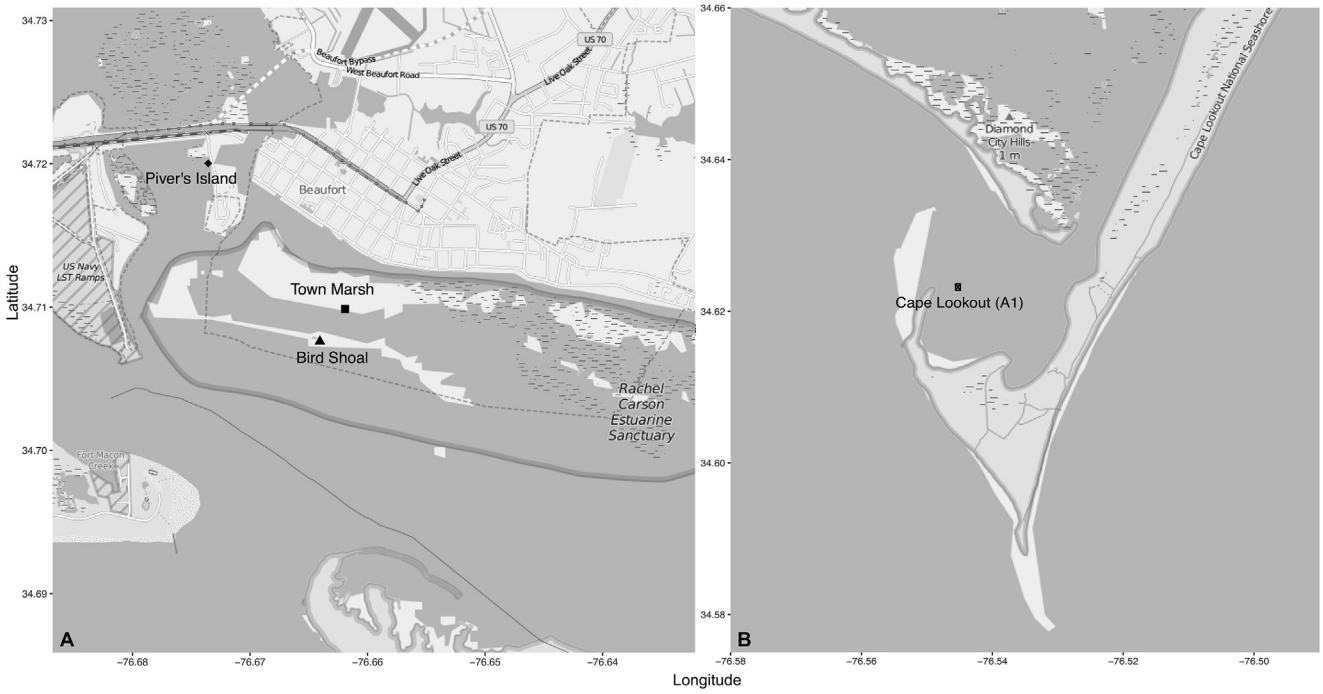

Fig. 1. Map of coastal North Carolina sampling sites. (A) Collection sites within Beaufort Inlet, near Beaufort, NC. ◆ – Piver's Island, plankton; ■ – Town Marsh, persistent wetland sediments; ▲ – Bird Shoal, intertidal sand flat sediments. (B) Collection site in Cape Lookout Bight, NC. ● – Station A-1 (Martens and Klump, 1980), shallow marine sediments.

Ion Torrent sequencing adaptors A (forward) and trP1 (reverse) (Life Technologies, Carlsbad, CA) and sample-specific DNA tags attached. For each sample, PCR reactions were performed in triplicate and pooled following purification to reduce bias. Conditions for each 25 μL reaction were: 20–100 ng template DNA per sample, 200 μM Invitrogen mixed dNTPs (Life Technologies, Carlsbad, CA), 10 μM forward (LR0R) and reverse (EDF360R) primers, 2.5 μL 10x Master *Taq* Buffer with 1.5 mM Mg^{2+} (5Prime, Hamburg, Germany), 5 μL 5x *Taq*Master PCR Enhancer (5Prime), 0.5 U *Taq* DNA Polymerase (5Prime), and 6 μL molecular biology grade water (Fisher Scientific). PCR reactions were carried out using a Veriti® thermal cycler (Applied Biosystems, Foster City, CA) with the following specifications: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C for 1.5 min, concluding with a final extension at 72 °C for 7 min. Negative controls containing only molecular biology grade water showed no amplification, indicating amplicon libraries were free from contamination. PCR replicates for each sample were pooled and then purified on a 0.8% high-melt agarose gel. Excised bands were cleaned using the illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Piscataway, NJ) according to manufacturer's protocol for maximum product recovery. After quantification using a Qubit® fluorometer (Life Technologies), samples were pooled in an equimolar solution and submitted to the Duke University Genome Sequencing and Analysis Core (Durham, NC). Following assessment for DNA concentration and size distribution on a BioAnalyzer 2100 (Agilent, Santa Clara, CA), amplicons were sequenced using the Ion Torrent PGM 400bp sequencing kit (Life Technologies) and one Ion 314™ chip. Raw sequence data have been submitted to the National Center for Biotechnology Information Sequence Read Archive under accession number SRP091681.

2.3. Sequence data processing

Sequence data were processed using the QIIME 1.9.1 framework (Caporaso et al., 2010). In the initial quality control filtering, reads were screened for the presence of the forward sequencing primer (LR0R) and a valid barcode, and discarded if they failed to meet the following criteria: average Phred quality score $\geq$ 25, no ambiguous bases, and homopolymer length $\leq$ 6. Reads were then screened for low-quality regions with 50 bp sliding window and removed if truncation at a low-quality region resulted in a sequence shorter than 200 bp. After quality filtering, reads shorter than 200 bp and longer than 400 bp were discarded. Using the USEARCH quality-filtering pipeline (Edgar, 2010) as implemented in QIIME, noisy sequences were filtered at 99% similarity before *de novo* and reference-based chimera checks were performed, the latter using the SILVA LSU 119 release as a reference (Quast et al., 2013; Yilmaz et al., 2014). Sequences tagged as potential chimeras by both *de novo* and reference-based analyses were discarded. Retained sequences were then clustered into operational taxonomic units (OTUs) at a similarity threshold of 95%. Clusters containing only one sequence across all samples (i.e., global singletons) represent likely sequencing artifacts (Tedersoo et al., 2010) and were removed to reduce OTU inflation. The most abundant sequence from each remaining cluster was selected as a representative sequence for that OTU.

2.4. Taxonomic assignment

Taxonomic assignment of the representative sequences was carried out using two methods: (1) BLAST + MEGAN v. 5.8.4 (MEtaGenome ANalyzer, Center for Bioinformatics, Tübingen, Germany) (Huson et al., 2011); and (2) the Ribosomal Database Project's (RDP) naïve Bayesian classifier (NBC) (Wang et al., 2007). In the first method, which is similarity-based, representative sequences were queried against a local installation of the GenBank nonredundant database using BLAST 2.2.30 + and the blastn algorithm (Altschul et al., 1997) with an e-value threshold of 10^{-10}. BLAST results were imported into MEGAN with the following parameters: minimum support = 1, minimum score = 100, top percent = 1.0, and winscore = 0.0. Using a lowest common ancestor (LCA) algorithm (Huson et al., 2007, 2011) and the established NCBI

taxonomy, MEGAN parses BLAST hits for a query and assigns the queried sequence to the lowest taxonomic rank supported. Though this method has been shown to be accurate in placing short fungal LSU reads even at lower taxonomic levels (Porter and Golding, 2012), novel sequences are often placed only to high-level classifications (e.g., 'Fungi') or not classified at all (Kunin et al., 2008).

The second taxonomic assignment method used, the RDP Classifier, compares 8 bp fragments of the queried sequence against reference sequences in a curated training set and calculates a score at genus level. Statistical support for the placement of a query sequence in a given genus is then estimated from 100 bootstrap replicates. Representative sequences from this study were classified using the RDP classifier v2.10 trained with LSU fungal training set 11 both with and without a bootstrap threshold of 50% (referred to as '50% cutoff' and 'best-match' analyses, respectively). For partial short reads $\leq$250 bp, a threshold of 50% bootstrap support has been shown to be accurate at placing fungal LSU sequences to genus level (Liu et al., 2012; Porras-Alfaro et al., 2014), but the 'best-match' analysis allows for provisional identification for groups that are poorly represented in databases, such as aquatic and early-diverging fungi. Taxonomic assignments made by the RDP Classifier were manually edited to reflect current accepted taxonomies [e.g., *Rozella* assigned to Cryptomycota instead of Chytridiomycota (Jones et al., 2011b); recently described phyla and sub-phyla within the former 'Zygomycota' (Hibbett et al., 2007)]. Results from the 'best-match' RDP classification were compared to those from the BLAST + MEGAN analysis and examined for concordance. When taxonomic assignments between the two methods differed, the RDP assignment was chosen.

2.5. Phylogenetic placement of most abundant OTUs

Sequences from the 50 most abundant fungal OTUs across all sites were aligned to the kingdom-wide nucLSU dataset from James et al. (2006a) using the '–add fragments' function in MAFFT v.7 (Katoh and Standley, 2013). Alignments were then refined by eye and ambiguously aligned regions were excluded. Maximum likelihood (ML) trees were inferred using RAxML v.8.0.0 (Stamatakis, 2014) under the GTRCAT model of nucleotide substitution with 1000 rapid bootstrapping replicates.

2.6. Diversity analyses

Because per-sample read totals varied significantly after the removal of non-fungal taxa, diversity metrics were assessed using the full eukaryotic dataset subsampled to 10,781 sequence reads, the lowest number of reads across samples. Alpha-diversity measures (corrected Chao index, and Shannon and Simpson biodiversity indices) for each sample were calculated in QIIME. To assess sampling completeness, rarefaction curves were generated for each sample using the complete eukaryotic dataset, also in QIIME. The distribution of OTUs across both habitats and seasons was visualized through Edwards' Venn diagrams generated using jvenn (Bardou et al., 2014).

3. Results

3.1. Sequence filtering and OTU clustering

Of the 654,728 raw input sequences, 355,102 (54.2%) were retained for OTU clustering and downstream analysis with most discarded sequences failing to meet the 200–400 bp length requirements. Previous microbial diversity surveys using the Ion Torrent sequencing platform have reported similarly high rates of low-quality sequences (Brown et al., 2013; Kemler et al., 2013). During OTU clustering, an additional 3701 chimeric or singleton sequences were identified and removed, resulting in a filtered dataset of 351,366 sequences. Per-sample read counts ranged from a minimum of 10,781 to a maximum of 36,653, with a mean of 21,960. Clustering of filtered sequences at 95% similarity generated 4379 non-singleton eukaryotic OTUs, 770 of which (17.6%) were assigned to 'Fungi' by both taxonomic assignment methods (BLAST + MEGAN and RDP without a bootstrap cutoff, or 'best match'). These 770 OTUs encompassed 56,005 reads (15.9% of total filtered reads), which were unequally distributed among samples (Table 1), with the highest read values generated from plankton samples taken at Piver's Island and intertidal sand samples collected from Bird Shoal. For the complete eukaryotic dataset, the number of OTUs was weakly positively correlated with sample read count (Table 2; Pearson's $r = 0.35$). For all but one sample, corrected Chao1 OTU estimates were higher than observed OTU counts, but per-sample OTU estimates recapitulated observed richness (e.g., the highest Chao1 estimates corresponded to the samples with the highest observed OTU richness). Rarefaction curves for each sample failed to reach plateaus, suggesting that the communities from each site were incompletely sampled (Fig. S1).

3.2. Taxonomic assignment

'Best-match' analysis placed reads to all eight phyla (Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Cryptomycota, Entomophthoromycota, Glomeromycota, Neocallimastigomycota) and four 'zygomycete' sub-phyla (Kickxellomycotina, Mortierellomycotina, Mucoromycotina and Zoopagomycotina) (Fig. 2A). Reads were binned to 33 classes, 89 orders, 174 families, and 318 genera. The dominant groups observed were the Ascomycota (66.8% fungal reads), Chytridiomycota (19.4%), and Basidiomycota (7.0%).

When employing a 50% bootstrap confidence level, the proportions of unclassified sequences across all sites ranged from 10.9% (phylum) to 31.5% (genus). Sequences were binned to only 173 genera from 108 families, 65 orders, 24 classes, and 5 phyla (in addition to two sub-phyla from the 'Zygomycota'). Across all samples, the dominant fungal phyla observed were Ascomycota (66.6% fungal reads), Chytridiomycota (15.4%), and Basidiomycota (6.7%). OTUs allied to the 'Zygomycota' (12, 0.33%), Blastocladiomycota (1, 0.02%), and Neocallimastigomycota (1, 0.01%) were minimally abundant. The remaining 224 OTUs (representing 10.9% of fungal reads) could not be identified beyond 'Fungi'.

Notably, lineages that are better represented in the RDP's LSU fungal training set 11—typically the Ascomycota and Basidiomycota—were more likely to be identified to both higher and lower taxonomic levels (Fig. 3). For example, 99.7% of all 'best-match' Ascomycota sequences and 96.9% of all 'best-match' Basidiomycota sequences could be assigned to their respective phyla under the 50% confidence threshold. At the genus level, 62.4% of ascomycete reads and 76.9% of basidiomycete reads could be binned. By comparison, only 79.5% of 'best-match' Chytridiomycota sequences could be binned at the phylum level, and just 5.7% could be binned to genus. Among the other zoosporic lineages, <2% of Blastocladiomycota sequences and <0.5% of Neocallimastigomycota sequences could be binned to genus. None of the sequences binned to the Cryptomycota, Entomophthoromycota, and Glomeromycota in the 'best-match' analysis could be placed to any taxonomic level with 50% bootstrap support.

3.3. Per-site diversity

OTU counts differed across the four sites (Table 1; Table S3), ranging from a high of 440 fungal OTUs at the intertidal sand flats of Bird Shoal, to a low of 376 OTUs in the marine sediments from Cape

Table 1
Total number of filtered sequence reads, eukaryotic OTUs, fungal sequence reads, and fungal OTUs from samples collected seasonally from four coastal North Carolina sites. Values in parentheses indicate percentage of total reads/OTUs observed.

Site	Season	Filtered reads	Total OTUs	Fungal Reads		Fungal OTUs	
Piver's Island	Winter	32,715	707	14,473	(44.2%)	246	(34.8%)
plankton (PT)	Spring	23,032	443	465	(2.0%)	63	(14.2%)
	Summer	29,742	1155	4159	(14.0%)	240	(20.8%)
	Fall	21,139	523	2854	(13.5%)	151	(28.9%)
Town Marsh	Winter	36,653	1248	6300	(17.2%)	318	(25.5%)
wetland sediments (WS)	Spring	28,098	635	261	(0.9%)	72	(11.3%)
	Summer	22,034	258	351	(1.6%)	58	(22.5%)
	Fall	12,891	391	6796	(52.7%)	142	(36.3%)
Bird Shoal	Winter	10,781	371	4319	(40.1%)	141	(38.0%)
intertidal sand (IS)	Spring	22,306	864	1011	(4.5%)	85	(9.8%)
	Summer	15,958	792	437	(2.7%)	62	(7.8%)
	Fall	31,188	1016	10,550	(33.8%)	318	(31.3%)
Cape Lookout Bight	Winter	14,747	931	1010	(6.8%)	176	(18.9%)
sediment core (SC)	Spring	13,684	978	775	(5.7%)	162	(16.6%)
	Summer	20,522	1143	944	(4.6%)	175	(15.3%)
	Fall	15,876	1097	1300	(8.2%)	209	(19.1%)

Table 2
Diversity metrics for coastal marine samples.

Site	Season	Total OTUs	Chao1	Shannon	Simpson
Piver's Island	Winter	707	782	4.988	0.904
plankton (PT)	Spring	443	497	3.804	0.811
	Summer	1155	1119	6.470	0.962
	Fall	523	538	4.821	0.917
Town Marsh	Winter	1248	1267	7.016	0.964
wetland sediments (WS)	Spring	635	700	4.926	0.848
	Summer	258	359	0.793	0.138
	Fall	391	572	4.438	0.903
Bird Shoal	Winter	371	450	4.782	0.900
intertidal sand (IS)	Spring	864	941	7.326	0.985
	Summer	792	1001	6.724	0.957
	Fall	1016	1054	7.536	0.981
Cape Lookout Bight	Winter	931	1168	6.405	0.947
sediment core (SC)	Spring	978	1236	6.813	0.964
	Summer	1143	1241	6.907	0.972
	Fall	1097	1279	6.520	0.912

Lookout Bight. Estuarine sediments on Town Marsh harbored 400 OTUs, and 399 OTUs were observed from the microplankton sampled at Piver's Island. Seven of the eight phyla and all four 'zygomycete' sub-phyla reported were found in all four sampling sites (Fig. 4; Table S3); members of the Cryptomycota were observed only in samples from Bird Shoal and Cape Lookout Bight (Fig. 2B). For all sites, the Ascomycota was the most speciose phylum reported; however, most taxa that were recovered were found at relatively low abundances. For all sites except Piver's Island, fungi were more diverse and constituted a larger fraction of the total eukaryotic community observed in the cooler months (Table 1).

Although the microplankton samples collected at Piver's Island comprise the greatest number of fungal reads of all sites considered, very few taxa were major contributors to the overall fungal community. Ascomycota comprised 93.7% of all sequencing reads (Fig. 2B), with the Dothideomycetes, Lecanoromycetes, and Eurotiomycetes alone contributing 86.3% of total reads (Table S1). The primary Dothideomycete representatives were in the Capnodiales (*Mycosphaerella*) and Pleosporales (*Phaeosphaeria*, *Preussia*) (Table S2). The Lecanoromycetes was the single most abundant class in the microplankton tows, driven largely by the presence of the lichen genus *Buellia,* which was the dominant taxon recovered from the Piver's Island samples. Nearly all of the sequences belonging to Eurotiomycete-aligned OTUs were binned to a single genus, *Exophiala*, in the Chaetothyriales. Other groups contributing to the fungal community were the Malasseziales (*Malassezia*) and the Spizellomycetales (*Spizellomyces*). Piver's Island samples contained the fewest reads that could not be identified to phylum or beyond using a 50% confidence threshold (1.5%) (Fig. 5).

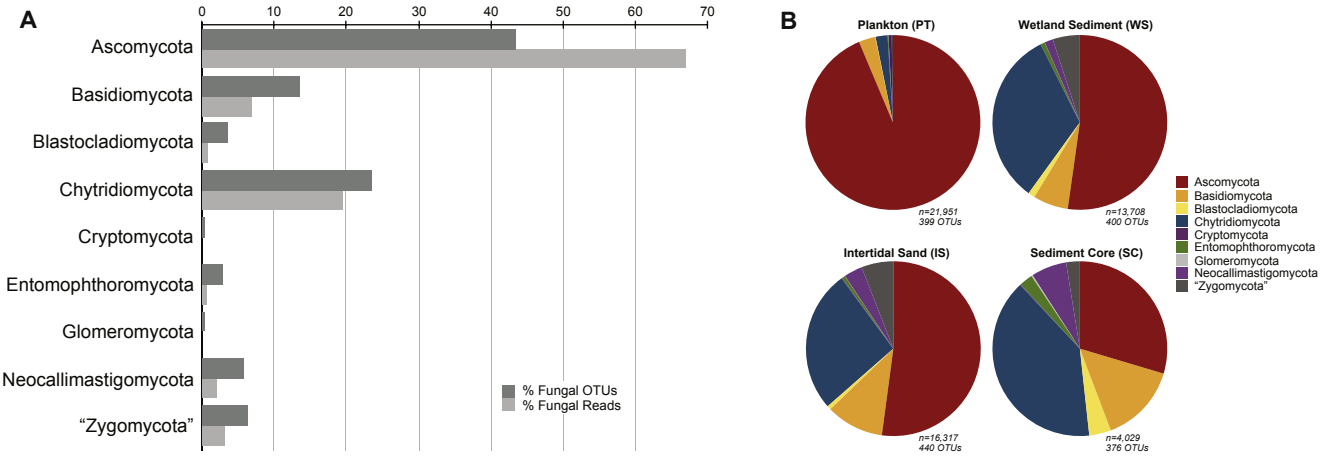

Fig. 2. (A) Contribution of each fungal phylum to total observed diversity, as both proportion of fungal OTUs and proportion of fungal sequences. (B) 'Best-match' taxonomic composition of fungal sequences observed at four sites in coastal North Carolina.

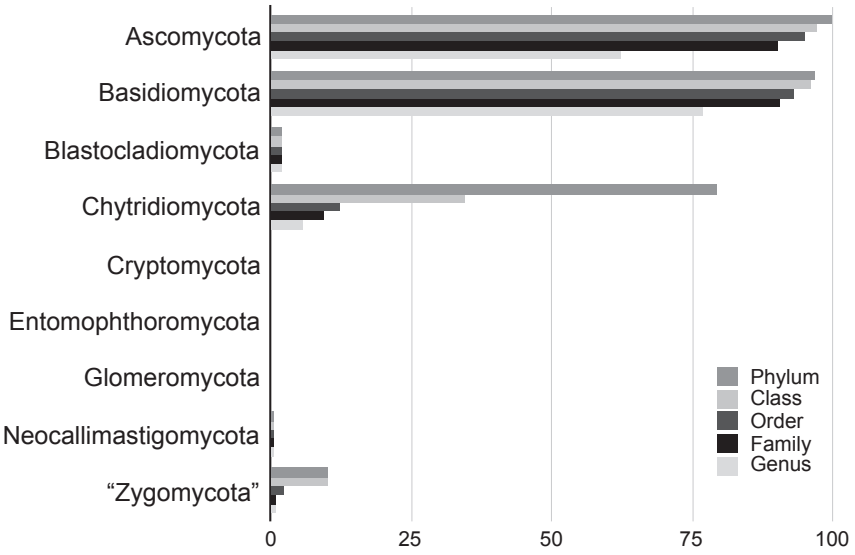

Fig. 3. Proportions of reads from each fungal phylum assigned to each taxonomic level with bootstrap support ≥50% using the RDP Classifier. Phylum names are based on ‘best-match’ assignments.

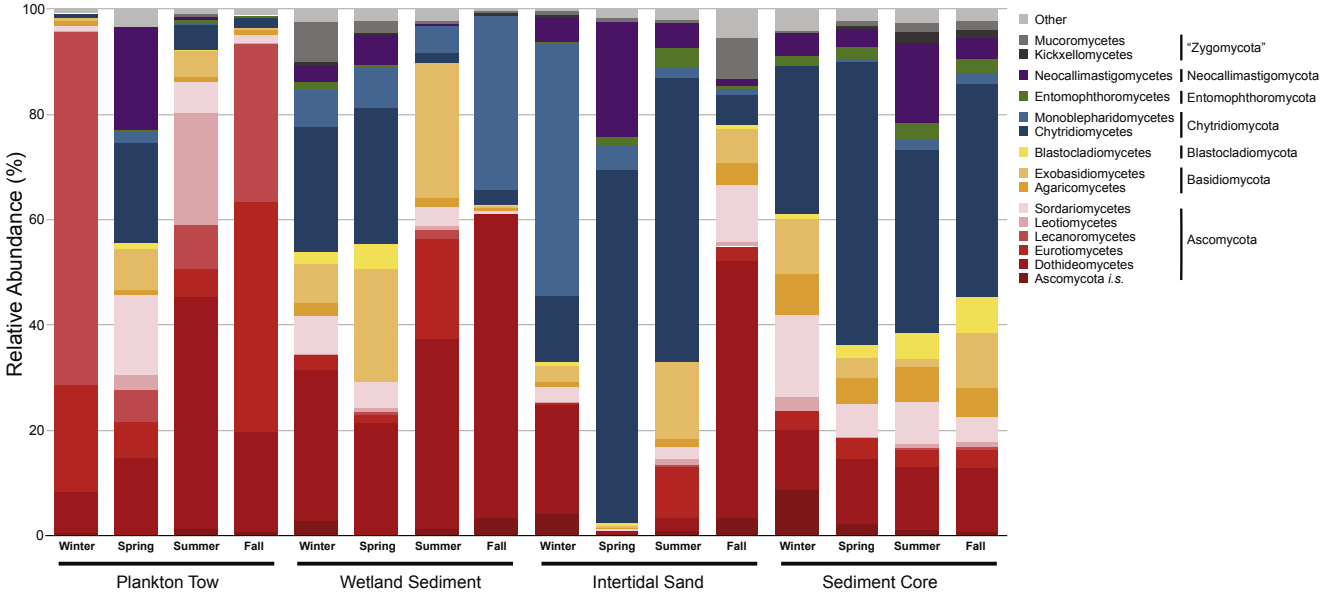

Fig. 4. Relative abundances of fungal sequences from seasonal sampling of four coastal marine sites. Assignments to fungal classes are based on ‘best match’ taxonomic designations made using the RDP fungal database for classification. Note: these data are not normalized due to the wide variation of fungal sequences across sites and seasons. See Table 2 for per-sample read counts.

In the wetland sediments of Town Marsh, the most abundant fungal classes were the Dothideomycetes (Ascomycota), Exobasidiomycetes (Basidiomycota), Chytridiomycetes (Chytridiomycota), and Monoblepharidomycetes (Chytridiomycota) (Fig. 4; Table S1). Collectively, these groups comprise 80.6% of all sequence reads for Town Marsh samples. The fungal community in these sediments was dominated by the Pleosporales (*Phaeosphaeria, Phaeodothis*), Malasseziales (*Malassezia*), Capnodiales (*Mycosphaerella*), Chytridiales (*Entophlyctis, Karlingiomyces*), Spizellomycetales (*Spizellomyces*), and Monoblepharidales (*Oedogoniomyces*) (Table S2). The samples collected from Town Marsh also contained considerable novel diversity, with 110 OTUs (27.5% of OTUs observed in site; 12.2% of total site reads) unidentifiable to phylum or beyond when implementing a 50% bootstrap constraint (Fig. 5).

The dominant classes in the intertidal sand flats on Bird Shoal mirrored those in the microplankton tows from Piver's Island and the sediments from Town Marsh (Fig. 4; Table S1). The Dothideomycetes, Chytridiomycetes, Monoblepharidomycetes, and Exobasidiomycetes, with the addition of the Neocallimastigomycetes, comprised 68.9% of the total sequencing reads from the annual sampling at the site. As on Town Marsh, the Pleosporales (*Phaeosphaeria, Phaeodothis*), Malasseziales (*Malassezia*), and the zoosporic orders Monoblepharidales (*Oedogoniomyces*) and Spizellomycetales (*Spizellomyces*) were among the dominant groups. Under a 50% confidence threshold, 16.3% of total site reads, most allied to the Chytridiomycota and Neocallimastigomycota in ‘best-match’ analyses, could not be identified beyond ‘Fungi’ (Fig. 5).

Finally, the fewest OTUs were reported from the oxygen-

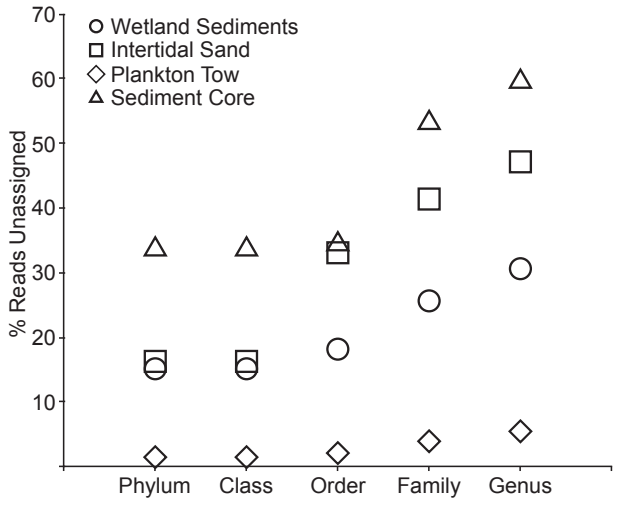

Fig. 5. Percentage of unassigned fungal sequences for each taxonomic rank when implementing a 50% bootstrap cutoff using the RDP fungal database for taxonomic classification.

deficient marine sediments at Cape Lookout Bight (Table S3), which also contained the fewest fungal reads (7.2% of all fungal reads) (Table 1). As seen in the other sampling sites, the Dothideomycetes, Sordariomycetes, and Exobasidiomycetes were among the more abundant classes recovered (Table S1). However, unlike most sites located near the Beaufort Inlet, the overall dominant classes in the Cape Lookout sediments belonged to zoosporic lineages: the 110 OTUs binned to the Chytridiomycetes, Monoblepharidomycetes, and Neocallimastigomycetes (34.8% of total OTUs from the sediment core samples) encompass 50.3% of the total reads for this site (Fig. 4). At finer taxonomic scales, the dominant taxa in these sediments included the Chytridiales (*Entophlyctis, Mesochytrium*), Spizellomycetales (*Spizellomyces*), Neocallimastigales (*Cyllamyces*), Malasseziales (*Malassezia*), Blastocladiales (*Catenomyces*), and Entomophthorales (*Basidiobolus*) (Table S2). Although the largest fraction of OTUs were placed taxonomically to the Ascomycota (146 OTUs, 38.8% total site diversity), only ten genera—*Aspergillus, Chaetomidium, Immersiella, Mycosphaerella, Phaeodothis, Phaeosphaeria, Preussia, Saccharata, Cladosporium,* and *Trichothecium*—contributed $\geq$ 1.0% each to total read abundance. No individual ascomycete genus comprised more than 2.6% of total read abundance. Finally, when employing a 50% bootstrap cutoff for taxonomic assignment, sediments from Cape Lookout harbored the highest percentage of putatively novel OTUs (Fig. 5), which comprised 33.6% of total read abundance and are heavily weighted toward the early-diverging lineages.

Although many of the dominant taxa were shared across habitats, each site harbored unique diversity (Fig. S2a). Over 40% of the fungal OTUs observed (317) were observed at a single site. The intertidal sand flats of Bird Shoal had the highest number of unique OTUs (103, comprising 6.5% of site reads), while the sediments collected from the persistent wetlands on Town Marsh contained the fewest (67, 1.9% of site reads). Unlike the Beaufort Inlet sites where unique OTUs represented only a small fraction of the communities at each site, the 73 OTUs unique to the sediments at Cape Lookout Bight comprised 12.1% of all site reads. Despite the proximity between Town Marsh, Bird Shoal, and Piver's Island, only 49 OTUs were shared among all three sites. OTUs unique to their respective sites were typically rare or nominally abundant. By contrast, the 50 most abundant OTUs across all habitats, comprising 79.8% of total fungal reads, were widespread across sampling locations, with 38 being found in all four locales. Only 2 of the 50 most abundant OTUs were localized to a single site. Fungi in these coastal sites may also exhibit seasonality: fungal communities were more diverse and more abundant in cooler months (Fig. S2b), although the principal taxa at each site were more likely to be present year-round. Across all fungal OTUs, 37.1% (286 OTUs) were observed during a single season, with the winter and fall having the highest number of unique OTUs (105 and 107, respectively). By contrast, only 121 OTUs (15.7%) were observed throughout the year (Fig. S2b). The numbers of ascomycete and basidiomycete OTUs fluctuated widely across seasons (Table S4), while the species richness of early-diverging groups was less variable.

Notably, the 50 most abundant OTUs were not exclusively from the Ascomycota or Basidiomycota, but rather originated from groups across the fungal tree (Fig. 6). All major phyla were represented, although Ascomycota and Chytridiomycota were the most diverse with 28 and 10 representatives, respectively.

4. Discussion

Culture-dependent and molecular studies of fungal diversity and ecology have documented the critical roles fungi play as primary decomposers, parasites, and symbionts in terrestrial environments. By contrast, the diversity and functional roles of fungi in aquatic environments, and especially marine habitats, are poorly understood (Wurzbacher et al., 2010). The application of next-generation sequencing technologies to microbial surveys of under-sampled aquatic habitats has revealed considerable novel diversity across the fungal kingdom, including the poorly characterized early-diverging lineages (Grossart et al., 2016). In the case of coastal marine habitats, classical culturing studies suggest that marine fungi are relatively rare, localized to the coasts, and primarily allied to the Dikarya (Hyde et al., 1998). In the present study, amplicon libraries derived from sediment and water samples collected seasonally from four coastal marine habitats in North Carolina revealed that marine fungal communities are considerably more diverse than culture-dependent studies have found, with sites harboring OTUs from all major phyla and sub-phyla. Despite high species richness, only a few taxa were consistently abundant across all sites and/or seasons. Particularly noteworthy was the finding that the zoosporic fungi are among the more abundant and species-rich taxa represented, which is contrary to historical surveys indicating that marine fungi are dominated by the Ascomycota and Basidiomycota. A significant fraction of total fungal reads from surveyed sites represented novel lineages that are only distantly related to sequences in curated reference databases. Furthermore, the vast majority of these novel phylotypes were most closely allied to taxa in the zoosporic fungi and the lineages that formerly comprised the 'Zygomycota'.

4.1. Plankton sampling (Piver's island)

Plankton samples collectively contributed the greatest number of fungal sequences of any habitat sampled; however, those sequences were distributed across only a small fraction of site OTUs. The single most abundant OTU from this site – and across all sites surveyed – was allied to the crustose microlichen genus *Buellia,* whose species are largely terricolous, and can be found growing in coastal areas. Other prominent Ascomycota included parasites of *Spartina* spp. (*Phaeosphaeria*, *Mycosphaerella*) common to the eastern U.S. coast (Buchan et al., 2002; Lyons et al., 2010), plant pathogens (*Sclerotinia*, *Saccharata*), and animal parasites (*Exophiala*). Among the ten most abundant OTUs at this site, only one basidiomycete (*Malassezia*) and one chytridiomycete (a putative member of the order Spizellomycetales) were represented.

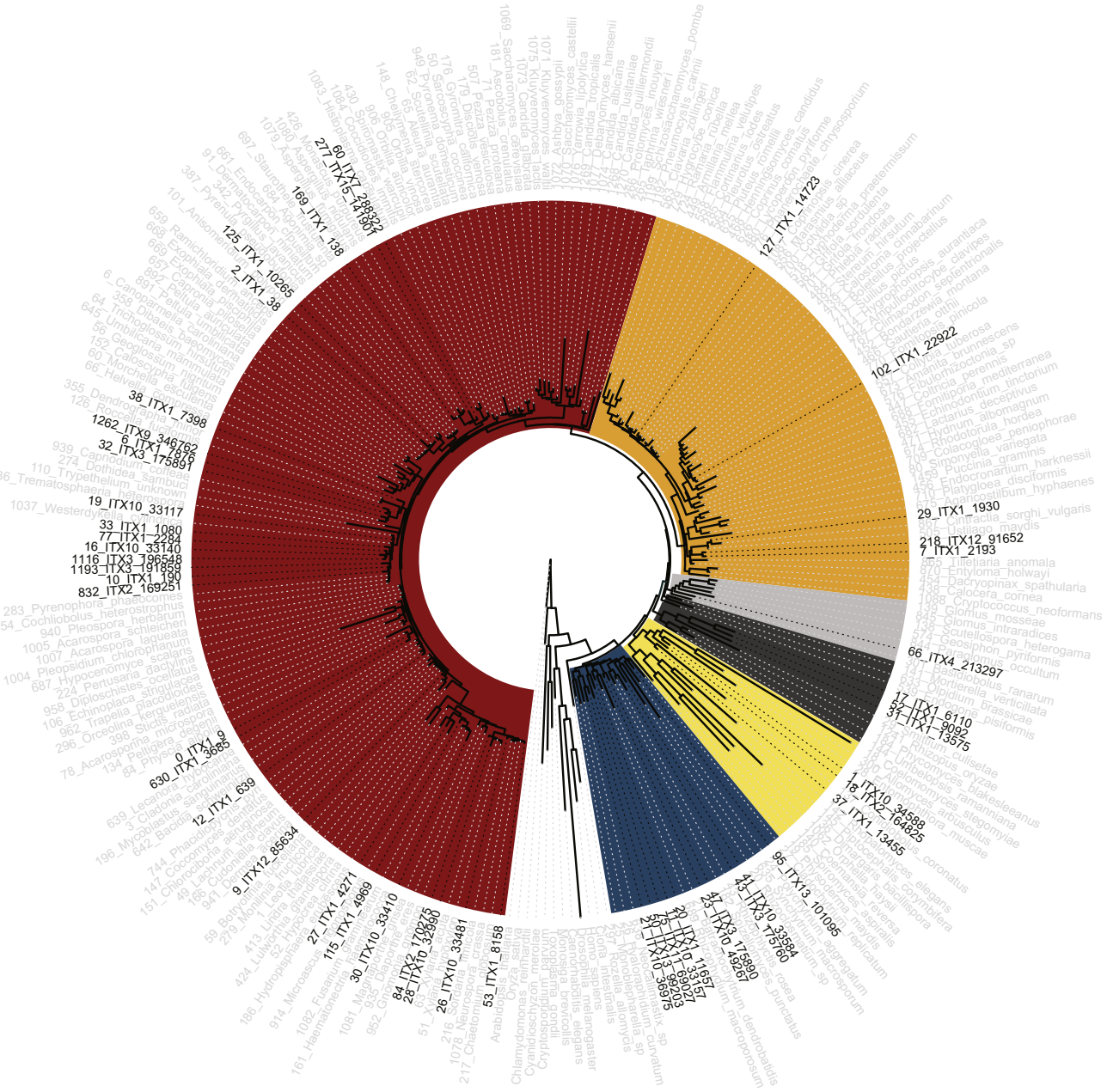

Fig. 6. Maximum likelihood tree of 50 most abundant OTUs. Gray and black fonts denote reference and amplicon sequences, respectively. Colors indicate major fungal phyla, clockwise from left: Ascomycota (red); Basidiomycota (orange); Glomeromycota (light gray); 'Zygomycota 1' (dark gray); Blastocladiomycota + 'Zygomycota 2' (yellow); Chytridiomycota + Neocallimastigomycota (blue).

Malassezia, which is primarily known as a human pathogen but has also been shown to be widely distributed across marine habitats (Amend, 2014), was also recovered at all sites in the present study. The relatively low abundance and diversity of the zoosporic fungi in plankton samples was particularly surprising. In freshwater habitats, chytrids play dual roles as saprobes/parasites of phytoplankton (Kagami et al., 2007; Rasconi et al., 2012) and nutrient-rich food sources for zooplankton, forming an oft-ignored component of the microbial food web called the mycoloop (Kagami et al., 2014). Although considerably less is known about the mycoloop in marine environments, recent high-throughput sequencing studies of pelagic fungal communities in Arctic (Comeau et al., 2016; Hassett and Gradinger, 2016) and temperate (Richards et al., 2015; Comeau et al., 2016; Jeffries et al., 2016) waters have shown a predominance of novel, chytrid-like phylotypes. Moreover, parasitization of marine phytoplankton by chytrid fungi has been observed directly (Hassett et al., 2016; Hassett and Gradinger, 2016; Scholz et al., 2016), suggesting that these fungi play a similarly critical role in nutrient-cycling in the marine realm (Jephcott et al., 2016). Several factors likely contributed to the poor sampling of zoosporic fungi from Piver's Island, with the principal one being the mesh size of the plankton net used. While zoosporic fungi are common parasites of larger desmids and diatoms in freshwater environments (Kagami et al., 2014), the mesh size used in the present study (80 μm) was too large to capture many smaller marine algal hosts (Hassett and Gradinger, 2016) and free-swimming zoospores. Deploying a plankton net with a finer mesh or direct filtration of unfractionated seawater may be a better strategy for sampling planktonic

zoosporic fungi more thoroughly. In addition to mesh size, the relatively small amount of seawater sampled in the present study and the collection site's proximity to shore likely account for the over-representation of largely terrestrial lichen taxa.

4.2. Persistent wetland sediments (Town Marsh)

In coastal wetlands along the Atlantic coast of North America, the smooth cordgrass *S. alterniflora* is the dominant vegetation and thus an abundant food source for symbiotic and saprophytic microbes (Peterson and Howarth, 1987), both above-ground on senescent plant tissue and in marsh sediments. Predictably, many of the fungi recovered from the persistent intertidal wetlands between Town Marsh and Bird Shoal islands are plant-associated. Pathogens of *S. alterniflora* and cellulose decomposing hyphomycetes were the principal ascomycetes recovered, and increased proportions of the Mucoromycotina relative to other sites were attributable chiefly to the endomycorrhizal symbiont *Endogone.* In Beaufort Inlet, salt marsh productivity is also fueled by benthic microalgae such as cyanobacteria and, to a lesser degree, diatoms (Currin et al., 1995), perhaps explaining the predominance of algae-associated genera (e.g., *Entophlyctis, Mesochytrium, Olpidium*) among the zoosporic fungi. Of particular note with regard to the zoosporic fungi is the relative abundance of OTUs binned to the Monoblepharidomycetes (=monoblephs), a small class within the Chytridiomycota containing only six genera (James et al., 2006b) and 20–25 species. Monoblephs, the second most abundant class observed in wetland sediments (Fig. 4), have been isolated solely from freshwater habitats where they degrade plant material including twigs, leaves, and fruits (Sparrow, 1933). The profusion of cellulosic material in salt marsh sediments coupled with findings that early-diverging fungi (and specifically the Monoblepharidomycetes) have long had the capacity to decompose tissues from green plants (Chang et al., 2015), suggest that the monoblephs may constitute a previously unknown, but critical microbial component governing nutrient transfer in salt marshes.

4.3. Intertidal sand (Bird Shoal)

Arenicolous fungi, which are generally defined as fungi that live on or among sand grains, play similar roles to soil fungi in decomposing organic material (Kohlmeyer and Kohlmeyer, 1979). Relatively few arenicolous fungi have been described, nearly all of which belong to the Ascomycota and Basidiomycota, and are functionally characterized by their preferred substrata (e.g., driftwood, macroalgae, cellulose detritus, feathers). Intertidal sand samples collected from Bird Shoal harbored the most fungal species of all sites (Fig. S2a), though many taxa were shared with the wetland sediments from Town Marsh. The Ascomycota was both the most abundant and speciose phylum recovered, but the Chytridiomycota, which was the second most diverse group, had a higher per-OTU abundance. Just as observed in the wetland sediments, over half of all sequences binned to the Chytridiomycota belonged to the Monoblepharidomycetes.

The abundance of zoosporic fungi within these intertidal sand samples may at first seem surprising, but considering historical methods of surveying arenicolous fungi, it is clear that sampling strategies would have largely missed interstitial chytrid fungi. Studies assessing both diversity and abundance of sand fungi have mostly relied on microscopic examination of fruit bodies on incubated detritus or ungerminated spores collected from sea foam. These sampling methods preferentially select for taxa that (1) have specialized nutritional requirements, such as those that can degrade lignin and cellulose, and (2) have spores that are resistant to drying and/or adapted to passive dispersal onto suitable substrates, thus precluding the description of many early-diverging fungi (Kohlmeyer, 1966), particularly the chytrids whose zoospores lack chitinous cell walls. In addition to zoosporic fungi, higher proportions of other early-diverging groups, such as the Entomophthoromycota and Mucoromycotina were also observed (Fig. 4), suggesting that these microfungi target invertebrate hosts and particulate refractory materials embedded in sand.

4.4. Benthic marine sediments (Cape Lookout Bight)

Sediments collected from Cape Lookout produced the lowest number of total reads, the least fungal reads, and smallest number of fungal OTUs across all sites. The overall abundance and taxonomic composition observed in these marine sediments, however, were less variable throughout the year than at other sites, suggesting that the fungal communities are relatively stable across seasons. As seen at other sites, taxon diversity was high, but most taxa were rare, with over 80% of all OTUs from Cape Lookout being represented by ten or fewer sequences (data not shown). Because sediments act as a reservoir for spores, it is difficult to determine whether these OTUs are active rare taxa or simply those whose propagules were carried downstream in detritus. Proportions of the various 'Zygomycota' lineages were elevated in marine sediment samples, but did not comprise a substantial fraction of the community (Fig. 4). Zoosporic fungi were particularly abundant in these sediments (Fig. 4), though few could be placed taxonomically beyond phylum or class with $\geq$50% bootstrap support in RDU Classifier analyses. Putative representatives (or possible near-relatives) of the enigmatic zoosporic fungal genus *Olpidium,* whose phylogenetic placement is outside the Chytridiomycota but remains unresolved (James et al., 2006a; Sekimoto et al., 2011), were among the most abundant OTUs. The presence, and often predominance, of early-diverging 'near-chytrid' phylotypes in the Cape Lookout sediments reflects similar findings from other culture-independent surveys of marine sediments (Le Calvez et al., 2009; Nagano et al., 2010; Nagahama et al., 2011; Richards et al., 2012, 2015; Jeffries et al., 2016).

Another especially interesting result is the year-round presence of OTUs allied to the Neocallimastigomycota (i.e., the rumen fungi), which are currently understood to be obligate endosymbionts inhabiting the guts of primarily ruminant hosts (Gruninger et al., 2014). Neocallimastigomycota were also reported from plankton, estuarine sediment, and intertidal sand samples in low numbers, however, their occurrence can be attributed to the presence of feral horses in the RCERR. While the Neocallimastigomycota-like OTUs observed in the Cape Lookout sediments might also have originated from vertebrate hosts upstream, there is some evidence for symbiotic marine members of this group. Anaerobic fungal thalli and flagellated zoospores were observed in the gut and coelomic fluid of the coastal sediment-dwelling sea urchin *Echinocardium cordatum* (Thorsen, 1999), and also found in the guts of the algae-grazing marine iguana *Amblyrhynchus cristatus* (Mackie et al., 2004). Thus, sequences assigned to this phylum may have originated from resting spores awaiting ingestion by a marine invertebrate host. Alternatively, anaerobic zoosporic fungi may also be free-living in anoxic sediments and soils; molecular signatures of Neocallimastigomycota have been detected in landfill soils (Lockhart et al., 2006) and lacustrine (Wurzbacher et al., 2016) and estuarine (Mohamed and Martiny, 2011) sediments. While the functional roles these fungi play remain unclear, it has been proposed that in addition to cellulose decomposition, anaerobic fungi, including potential free-living relatives of the rumen fungi, may form symbiotic relationships with chemoautotrophic prokaryotes in deep-sea sediments, generating bioavailable hydrogen (Ivarsson et al., 2016). None of the 25 putative neocallimastigomycotan OTUs in

Cape Lookout sediments could be assigned to the phylum with ≥50% bootstrap support, suggesting that free-living anaerobic zoosporic fungi may be only distantly related to symbiotic taxa, or represent a separate clade entirely.

4.5. Methodological considerations

One of the primary challenges inherent in molecular surveys of broad-scale eukaryotic diversity is the selection of an appropriate DNA marker and, consequently, a corresponding primer pair. In high-throughput surveys of fungal diversity, three ribosomal loci are utilized: small subunit (SSU/18S), the internal transcribed spacers (ITS1 and ITS2), and large subunit (LSU/28S). For this study, I chose to target the D1 region of the ribosomal large subunit, which is sufficiently conserved for kingdom-wide sequence alignment (unlike the hyper-variable ITS), but also variable enough for finer scale phylogenetic delimitations (Porter and Golding, 2012). Although ITS has been adopted as the universal barcode for fungi due to its high levels of interspecific variability (Schoch et al., 2012), LSU can be used to infer deep relationships among fungi (James et al., 2006a), is a commonly employed marker in phylogenetic studies in zoosporic lineages (James et al., 2006b; Letcher et al., 2006; Porter et al., 2011), and when used in environmental sequencing surveys recovers community patterns congruent to those found by ITS (Brown et al., 2014, Porras-Alfaro et al., 2014).

Neither of the primers used in this study are fungal-specific, although the reverse primer was designed to amplify taxa across the fungal tree, including the Cryptomycota. However, as the vast majority of fungal species remain unknown and primer pairs may only amplify a subset of a target community (Stoeck et al., 2006), it is likely that the diversity reported here is but a snapshot of the fungal diversity present in the coastal sites surveyed. Furthermore, extant reference databases are biased toward terrestrial taxa—and the Dikarya in particular—confounding efforts to identify novel aquatic fungi (Panzer et al., 2015). In the first round of taxonomic identification in this study, which queried amplicons against the GenBank database and assigned taxonomy using MEGAN's lowest common ancestor algorithm, 1273 OTUs were tentatively identified to 'Fungi' (data not shown), although only 770 of those were also placed to the fungal kingdom by the RDP analyses. Many of these additional 503 OTUs were assigned to early-diverging fungal lineages and may represent true fungal species, but in light of the current limitations of taxonomic databases, I adopted a conservative approach and excluded them from downstream analyses.

Due to additional methodological considerations such as sample size and study design (Lindahl et al., 2013), the results presented herein cannot draw firm conclusions about the structure of marine fungal communities through space and time, nor do they provide an exhaustive catalogue of marine fungi from these sites. Rather, these data offer preliminary insight into the breadth of fungal diversity present in these historically undersampled marine habitats, and demonstrate that our current understanding of marine fungal diversity and ecology is largely incomplete. I conclude that coastal marine fungi are considerably more diverse than previously thought, especially among the poorly understood early-diverging lineages. The findings from this study will contribute to a more complete understanding of marine and estuarine fungi and help inform future studies of their occurrence and the functional roles they play in their respective ecosystems.

Acknowledgments

This study was funded through a Graduate Research Fellowship awarded to KTP by the Mycological Society of America and Duke Biology Grant-in-Aid. KTP was supported by a National Science Foundation Graduate Research Fellowship, an NSF Doctoral Dissertation Improvement Grant (NSF-DDIG 1311540), a Ford Foundation Pre-Doctoral Fellowship, and a Duke University Dean's Graduate Fellowship. This article is part of a doctoral dissertation by KTP completed under the direction of Kathleen M. Pryer, who provided guidance and many helpful edits. KTP wishes to thank the crew of the R/V Susan Hudson at Duke Marine Lab and Megumi Shimizu for making the Cape Lookout Bight sampling possible.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.10.006.

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