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Beyond dikarya: 28S metabarcoding uncovers cryptic fungal lineages across a tidal estuary

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Abstract

Fungi are key drivers of biogeochemical processes, yet marine fungi remain understudied and under-characterized due to primer biases and database gaps. In this study, we conducted a metabarcoding survey targeting the small and large subunit rRNA genes and the internal transcribed spacer region of fungi (18S, 28S, and ITS2) in the sediment and surface water of salt and brackish marshes in the North Inlet—Winyah Bay estuarine system (Georgetown, South Carolina, USA). The universal 18S/16S primer set (515F-Y and 926R) identified few fungal taxa. The ITS2 primer set (ITS3mix and ITS4) revealed high diversity among Dikarya but failed to capture the full extent of early diverging fungi (EDF). In contrast, the 28S primer set (LR0R and LF402) excelled at identifying EDF lineages, including Chytridiomycota, Mucoromycota, Zoopagomycota, and Blastocladiomycota, many of which dominated the brackish marsh sampling site but were less prevalent in the salt marsh sampling sites. Over half of the fungal OTUs identified by the 28S primer set were from EDF lineages. Copy-normalized 28S qPCR showed that EDF were more abundant in brackish sediments than in the salt marsh. Several putative denitrifying fungi, primarily species from *Trichoderma* and *Purpureocillium*, were also detected, suggesting overlooked functional guilds that may contribute to estuarine nitrogen cycling. A FUNGuild analysis found that most lineages were saprotrophic. Overall, our findings show that EDF are key contributors to community differences across salinity gradients and may play more important functional roles in coastal biogeochemistry than is currently understood. The 28S primer set is ideal for marine fungal metabarcoding because it provides comprehensive taxonomic coverage and enables phylogenetic analysis.

Keywords Marine fungi, Metabarcoding, Salt marsh, Brackish marsh, Early diverging fungi, Dikarya

Introduction

Fungi play crucial roles in biogeochemical processes in marshes, contributing to carbon remineralization [155, 156] and phosphorus and nitrogen cycling [7, 107, 112]. In aquatic ecosystems, most fungi function as decomposers, breaking down complex organic materials such as macrophyte litter, dead algae, and other organic debris [12]. Like terrestrial fungi, many marine fungi produce extracellular enzymes that degrade complex organic matter such as lignin and cellulose while also assimilating nitrogen in forms such as ammonium or nitrate, which are common in plants, phytoplankton,

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and microbes [31, 112]. Additionally, some fungi play a key role in denitrification, removing nitrate from the environment and producing nitrous oxide, a potent greenhouse gas [74, 83].

Despite the growing interest in marine fungi, our understanding of their diversity in marine environments remains limited. One reason for this is the infrequent use of shotgun metagenomics, as fungal reads often represent a low percentage of community DNA [113]. To address this gap, amplicon-based metabarcoding surveys continue to constitute a key method for expanding our knowledge of marine fungal diversity. Most studies conducted in marine and coastal ecosystems have focused on sequencing the internal transcribed spacer (ITS) region—specifically, ITS1, ITS2, or the entire ITS region (ITS1, 5.8S, and ITS2)—which has been recognized as the recommended DNA barcode marker for fungi by the Consortium for the Barcode of Life since 2011 [137]. The ITS region generally provides high taxonomic resolution and has the highest probability of correct identification for broadly sampled fungi [137]. Although the ITS region provides high taxonomic resolution, challenges such as high variability, primer biases that exclude some groups, and the lack of early diverging fungal (EDF) sequences in ITS databases can complicate alignment and classification [62, 124, 127]. In comparison, the large subunit (LSU) ribosomal RNA (rRNA) (28S) region has received increasing interest because it offers greater taxonomic resolution than the 18S small subunit (SSU) rRNA region does [174]. The 28S region also enables phylogenetic reconstruction, which could improve the classification of novel fungal lineages. Projects such as the Fungal 28S Ribosomal RNA (LSU) RefSeq Targeted Loci Project have facilitated the use of 28S for taxonomic classification [129]. Additionally, testing universal primer sets, such as the one developed by [108, 109], offers the potential to quantify the relative abundance of both prokaryotes and eukaryotes, although this approach has rarely been applied to fungi and prokaryotes simultaneously [93].

Coastal salt marshes, which are highly productive habitats, provide essential ecosystem services, acting as nurseries for fisheries, protecting shorelines from erosion, and reducing nutrient loading in coastal waters [140]. As of 2017, 29% (94.7 million) of the U.S. population lived along the coastline, with 13.6% residing on the Atlantic coast [157]. Salt marshes, which serve as sinks for anthropogenic contamination, are significantly influenced by nutrient inputs [40, 89]. These ecosystems store an estimated 100–260 g of carbon per m^2 per year through the burial of organic carbon by plants and phytoplankton [86, 97]. Microbes, including fungi, utilize buried carbon for aerobic and anaerobic metabolism, which releases sequestered carbon [86], making salt marshes critical sites for carbon sequestration [36, 86] and biogeochemical cycling [155].

While most marine fungi identified belong to Ascomycota and Basidiomycota [7], recent environmental sequencing data have revealed EDF lineages, such as Blastocladiomycota, Zoopagomycota, and Chytridiomycota, in coastal environments. However, despite these findings, the functions and contributions of these early lineages to nutrient cycling in estuarine ecosystems remain poorly understood [30, 37, 69, 115, 116]. Furthermore, there is a need to validate the presence of these groups using complementary quantitative tools such as qPCR.

This study aimed to (1) determine which rRNA region is most suitable for studying marine fungal diversity, and (2) address the critical knowledge gap of the functions and contributions of fungi to nutrient cycling in estuarine ecosystems by focusing on the diversity and ecological roles of rarely described fungi in marsh ecosystems. We conducted a metabarcoding survey targeting the 18S SSU, 28S LSU, and ITS2 rRNA regions to analyze fungal community composition in the North Inlet–Winyah Bay (NI-WB) estuarine system in South Carolina, USA, during the summer and winter of 2020 and 2021. We combined this with copy-normalized 28S qPCR to confirm the abundance of EDF across sites and sample types and used ITS2-based detection to reveal the presence of putative denitrifying fungi. Our results revealed significant variation in fungal identification and diversity between primer sets in both salt and brackish marsh surface water and sediment samples. We also identified key parameters influencing community composition. We hypothesized that fungal diversity would vary significantly between primers and that salinity and seasonality would have substantial effects on community composition and diversity. By investigating taxa that are often underrepresented in traditional surveys, we enhance our understanding of their biodiversity and functions within these habitats. Our findings suggest that EDF may play significant roles in processes such as organic matter degradation and nutrient turnover, ultimately contributing to biogeochemical cycling in marsh environments.

Materials and methods

Study area

The North Inlet-Winyah Bay (NI-WB) estuarine system is located in Georgetown, SC, and hosts a National Oceanic and Atmospheric Administration (NOAA) National Estuarine Research Reserve (NERR) with four long-term monitoring sites, part of the NERR System-Wide Monitoring Program: Oyster Landing (OL), Clambank (CB), Debidue Creek (DB), and Thousand Acre (TA) (Fig. 1; [5]). The NI-WB NERR consists of approximately 19,000

Fig. 1 Locations of the sampling sites within North Inlet–Winyah Bay National Estuarine Research Reserve, South Carolina, USA. Image from Google Maps

acres of tidal marshes of varying salinity. Winyah Bay (TA sampling site) is a brackish, river-dominated estuary controlled by the Waccamaw, Sampit, Black, and Pee Dee Rivers (7.5 ± 10.4 psu) and receives approximately 557 m^3/s freshwater input annually [110]. At TA, which is located alongside Winyah Bay, the vegetation in the low marsh is dominated by *Spartina alterniflora*, whereas the high marsh is home to *Spartina cynosuroides*, *Juncus roemerianus*, *Typha angustifolia*, *Scirpus americanus*, and *Eleocharis engelmannii* [8, 76, 77, 145, 146]. The other three sampling sites—CB, OL, and DB—are located in different reaches of the North Inlet estuarine system, a relatively pristine, high-salinity, ocean-dominated salt marsh (29.6 ± 5.9 psu) also dominated by *Spartina alterniflora* [76, 77].

Sample collection

Seawater and sediment samples (48 of each) were collected from the NI-WB in June 2020, August 2020, February 2021, and November 2021 to study seasonal fungal diversity across the selected sites (Fig. 1). June and August represent summer months, and February and November represent winter months. Samples were taken near high tide at every sampling date. Water physicochemical parameters (temperature, salinity, pH, and chlorophyll *a* concentration) at each site were monitored by the NI-WB NERR using YSI EXO2 sondes (SKU: 599,502–00) [35] and obtained from the NERR System Centralized Data Management Office [102]. At each sampling location, surface sediment samples were taken in 1.5 mL tubes, and approximately 50 mL of surface water was filtered through a 0.22 µm Sterivex filter (EMD Millipore #SVGP01050, Burlington, MA). Three biological replicates were collected at each site during each sampling campaign. Samples were stored on ice in a cooler for approximately two hours while being transported to the laboratory and stored in a -80 °C freezer until DNA extraction.

DNA extraction

In the laboratory, DNA was extracted from the sediment via the DNeasy PowerSoil Pro Kit, following the manufacturer's protocol except for the first step (cell lysis). The sediment (0.50 ± 0.01 g) was weighed into MN bead type A tubes (Macherey–Nagel #740,786.50), which were bead-beaten in a Biospec Mini-BeadBeater-16 for 60 seconds. The remaining extraction steps followed the DNeasy PowerSoil Pro kit protocol without further modifications.

Water sample DNA was extracted from the Sterivex filters using the DNeasy PowerWater extraction kit, following the kit's protocol except for the first step (cell lysis).

Using sterilized scissors, half of each Sterivex filter paper was cut into 2 mm squares. The filters were placed into MN bead tubes type A (Macherey–Nagel #740,786.50) and beaten in a Biospec Mini-BeadBeater-16 for 60 s. The remaining extraction steps followed the DNeasy PowerWater kit protocol without further modifications. The concentration of the isolated DNA was determined via a Qubit fluorometer, and the DNA samples were stored at − 20 °C until amplicon sequencing.

Amplicon library construction and sequencing

Three PCR primers were used to target different fungal regions within the samples. Two sediment negative controls and two surface water negative controls for each primer set were processed through DNA extraction and amplicon library construction. The ZymoBIOMICS Microbial Community Standard and Standard II (log distribution) (ZymoBIOMICS #D6300) were processed through DNA extraction and amplicon library construction, and the ZymoBIOMICS Microbial Community DNA Standard and Standard II (log distribution) (ZymoBIOMICS #D6311) were included in the amplicon library (Supplementary Figure S1). We followed Illumina's 16S library protocol (Illumina #15,044,223 Rev.B; [113]), which is a two-step process: the first step involves amplicon PCR, and the second step involves index PCR.

To sequence the V4-V5 regions of the 16S and 18S rRNA genes, the universal primer set, which should amplify both the 16S rRNA and 18S rRNA gene sequences, 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') were used [108, 109]. The primers ITS3tagmix (Supplementary Table S1) [151] and ITS4tag001 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGAGTGCGTTCCTSCGCTTATTGATATGC-3') [152] were used to sequence the ITS2 region. The LR0R (5'-ACSCGCTGAACTTAAGC-3') and LF402 (5'-TTCCCTTTYARCAATTTCAC-3') primers were used to sequence the 28S LSU region of fungi [151].

A 10 µL PCR was performed for each sample in triplicate, with the following mixture: 0.1 µL of forward primer (10 µM), 0.1 µL of reverse primer (10 µM), 2 µL of 5 × GC Buffer (Thermo Scientific #F530S, Vilnius, LT), 0.2 µL of 10 mM dNTPs (Thermo Scientific #R0181, Vilnius, LT), and 0.1 µL of Phusion High-Fidelity DNA Polymerase (Thermo Scientific #F530S, Vilnius, LT). The thermal cycling settings for the universal primer set were as follows: initial denaturation at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 45 s, with a final elongation at 72 °C for 10 min. For the 28S primer, the settings were as follows: initial denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 53 °C for 30 s, and 72 °C for 45 s, with a final elongation at 72 °C for 10 min. For the ITS2 primers, the settings were as follows: initial denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 45 s, with a final elongation at 72 °C for 10 min. All PCR products were verified using gel electrophoresis.

The triplicates were pooled and cleaned using sparQ PureMag beads at a 1:1 ratio of beads to PCR products following the sparQ PureMag bead clean-up protocol (Quantabio #95,196/IFU-124.1 REV 03). The cleaned PCR products were quantified via the high-sensitivity 1 × DNA Qubit assay. Different indices were used to identify each sample after sequencing with the Nextera XT DNA Library Preparation Kit (Illumina #FC-131–1096). The indexed amplicon libraries were cleaned again via the same bead clean-up protocol described above. A total of 293 amplicon libraries were pooled to reach a concentration of 2 nM and sent to the Duke Genome Center for sequencing in one lane of an S-Prime flow cell (250 bp PE) on the Illumina NovaSeq 6000 platform, which generated a total of 468,658,500 raw reads.

Sequence analysis

A modified protocol from Fuhrman et al. [51] was used to analyze the 18S rRNA sequencing results [99, 108, 109]. The raw reads were quality-filtered via bbduk.sh (ktrim = r ordered minlen = 51 minlenfraction = 0.33 mink = 11 tbo tpe rcomp = f k = 23 hdist = 1 hdist2 = 1 ftm = 5 pigz = t unpigz = t zl = 4 ow = true) and Trimmomatic (ILLUMINACLIP:$adapters:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:200). Using USEARCH v11.0.667, the filtered forward and reverse reads were merged with the "-fastq_mergepairs" function. The unmerged reads represented the 18S reads, and the merged reads represented the 16S reads. The 18S forward and reverse reads do not overlap using this primer set. All nonmerged reads were trimmed to 190 bp via Trimmomatic (CROP:190 MINLEN:190). The nonmerged reverse reads were converted to their reverse complements with the "seqtk seq -r" function in seqtk. An "N" was added to the end of the forward nonmerged sequences as a placeholder for the missing base pairs between the nonmerged forward and reverse complement reads, and an "F" was added to the end of the nonmerged forward quality scores. Forward and reverse complement reads were merged via the "-fastq_mergepairs" function and filtered with maxEE set to 1 via the "-fastq_filter" function in USEARCH v11.0.667. The reads were dereplicated via the "-fastx_unique" function and clustered into 3,762 97% operational taxonomic units (OTUs, "-cluster_otus" function) via USEARCH v11.0.667 (Supplementary Table S2). This function also removes chimeric sequences. The nucleotide sequences of the 18S primer set were classified —using a naïve

Bayesian classifier [123] implemented in QIIME 2 against the SILVA SSU 138–99 [25].

For the ITS2 sequencing results, the raw reads were merged and trimmed to the ITS2 region via ITSxpress. With USEARCH v11.0.667, the merged reads were filtered with maxEE set to 1 via the "-fastq_filter" function. The reads were dereplicated via the "-fastx_unique" function and clustered into 18,389 97% operational taxonomic units (OTUs, "-cluster_otus" function) via USEARCH v11.0.667 (Supplementary Table S2). The ITS2 OTUs were classified using a combination of a naïve Bayesian classifier trained on the UNITE 9.0 database for eukaryotes [101] using QIIME2 v2022-11 [25] and a homology-based classification on the UNITE Advanced Analyses on the PlutoF platform [1].

For 28S sequencing reads, raw reads were quality-filtered via bbduk.sh (ktrim = r ordered minlen = 51 minlenfraction = 0.33 mink = 11 tbo tpe rcomp = f k = 23 hdist = 1 hdist2 = 1 ftm = 5 pigz = t unpigz = t zl = 4 ow = true) and Trimmomatic (ILLUMINACLIP:$adapters:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:200). With USEARCH v11.0.667, the filtered forward and reverse reads were merged with the "-fastq_mergepairs" function and filtered with maxEE set to 1 via the "-fastq_filter" function. The reads were dereplicated via the "-fastx_unique" function and clustered into 8,461 97% operational taxonomic units (OTUs, "-cluster_otus" function) via USEARCH v11.0.667 (Supplementary Table S2). The nucleotide sequences of the 28S primer set OTUs were classified via the RDP classifier 2.14 [163, 164].

Quantitative PCR on the 28S region

Fungal 28S primers, LR0Rngs (5'-ACSCGCTGAACT TAAGC-3') and LF402 (5'-TTCCCTTTYARCAATTTC AC-3') from Integrated DNA Technologies (Coralville, Iowa, USA), were utilized [151]. The 20-µL quantitative PCR (qPCR) reactions included 5 µL 2×SYBR-Green Master Mix (Applied Biosystems, Waltham, Massachusetts, USA), 5 µL ultrapure water, and 0.6 µL of each primer. The remaining reaction volume included the appropriate amount of DNA template and ultrapure water to ensure the final concentration of template DNA was 0.75 ng μL^{-1}. For samples whose template concentration was too low to ensure this, the maximum volume of template (8.8 µL) was used and corrected during data processing. Touchdown qPCR, a method that can improve the detection of low-abundance targets in environmental samples [175, 176], was performed on the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). Cycling settings were as follows: 50 °C for 2 min, per Applied Biosystems SYBR-Green protocol to prevent any carry-over contamination; an initial denaturation at 95 °C for 3 min; followed by the "touchdown" cycles of denaturation at 95 °C for 20 s and annealing at 66 °C for 10 s, decreasing the annealing temperature by 3 °C for four cycles; this was succeeded by the amplification stage that included denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 60 s, for 60 cycles; with a final melt curve analysis that occurred at 95 °C for 15 s, 60 °C for 60 s, then increasing to 95 °C at 0.15 °C s^{-1}, measuring every 0.15 °C.

QPCR was conducted with six technical replicates per sediment sample, distributed across three independent qPCR runs. Occasionally, individual technical replicates displayed anomalously high or low quantification cycle (Cq) values relative to other replicates. In addition to the threshold-based criteria described for standards, outlier replicates were identified using Grubb's test, a statistical method widely applied in qPCR workflows, implemented with default parameters in the R Statistical Software "outliers" package [20, 55, 73]. Due to limitations in template DNA availability, a reduced number of technical replicates (≤ 3) were performed for water samples. Ten surface water samples, primarily from TA (n = 8), yielded insufficient Cq data or melt curve quality and were therefore excluded from downstream quantification of 28S rRNA gene copies. For more detail on the qPCR methods see the Supplementary Information (Supplementary Figures S2, S3).

Copy-normalized fungal abundance calculations

Copy-normalized fungal abundances were quantified by first normalizing raw 28S rRNA gene copy numbers from qPCR to sample mass or volume (g or mL) and scaling them by the proportion of fungal reads recovered with the 28S primer set. This value was then corrected for the fraction of the amount of DNA template used in qPCR reactions, yielding an estimate of fungal 28S copies per mass or volume. To convert gene copies to cell numbers, one representative genome per fungal species was downloaded from NCBI, 28S rRNA genes were extracted with Barrnap [136], and copy numbers were recorded; the full taxonomic ranks of each species were retrieved via the NCBI Entrez *efetch* API, and mean 28S copy numbers were calculated for each taxonomic rank. Sequenced 28S OTUs were matched to these reference genomes at the lowest possible taxonomic level. Then using the relative abundance of the corresponding OTU, the samplelevel 28S copy estimates were converted into copy-normalized fungal genome equivalent counts (GE). We used GE as a proxy for fungal abundance because this metric accounts for variability in rRNA gene copy number among taxa, providing a closer approximation of the actual number of fungal cells and facilitating meaningful comparisons across diverse lineages. Finally, differences in fungal

abundance between brackish and salt marsh sediments were assessed with a Kruskal–Wallis test.

Identification of putative fungal denitrifiers

Fungal ITS2 sequences from Maeda et al. [83] and Lazo-Murphy et al. [74] were used to determine putative denitrifying fungi from the ITS2 primer set. ITSx was used to trim the reference sequences to the ITS2 region (–preserve T –save_regions ITS2). Then, the "cd-hit-est" function in CD-HIT [50] was used to match ITS2 OTUs from this study to these reference sequences at 100% (-c 1.0 -n 10). The relative abundance of these OTUs were calculated via the phyloseq package in R [87].

Phylogenetic analysis

All fungal sequences from the 28S region and two outgroup sequences from the SAR supergroup (*Frustulia* sp. and *Amphora commutata*; sequences from NCBI) were aligned (using MUSCLE [39]) and manually trimmed (using Jalview). Maximum-likelihood trees were inferred with IQ-TREE 2 v2.2.6 using the option -m MFP + MERGE, which first runs ModelFinder Plus to search across the full set of time-reversible substitution models and select the best-fit model for each partition under the Bayesian Information Criterion (BIC) [70, 91]. The tree was visualized via the interactive tree of life [75], with the respective phylum of each OTU shown in various colors via a color strip dataset.

Statistical analyses

The relative abundance of each OTU was calculated via the phyloseq package in R [87]. The full OTU abundance table was rarefied to a depth of 21,362 reads, chosen as the second lowest sequencing depth across all samples to maximize comparability while minimizing sample exclusion. [23]. These rarefied OTUs were used to calculate Shannon diversity (using the diversity function in the vegan package in R), Simpson diversity (using the diversity function in the vegan package in R; [105]), richness (using the specnumber function in the vegan package in R), and evenness (Shannon diversity divided by the log of richness in R) for each sample. Hutcheson t tests (using the Hutcheson_t_test function in the ecolTest package in R) were performed to determine the significance of the differences in Shannon diversity, Simpson diversity, and evenness among the different primers. *P*-values were adjusted for multiple comparisons using Benjamini-Hochberg (p.adjust function in R stats package method = "BH"). The environmental parameters of the surface water, including salinity, temperature, pH, and chlorophyll *a* content at each station at the day of sampling, were investigated via weighted unifrac principal coordinates analysis (PCoA) to determine their roles in shaping the microbial community composition in the surface water via the ape package in R. A PERMANOVA (vegan package in R) for PCoA was conducted to determine the significance of the environmental variables.

The raw fungal OTU counts from the 28S region were normalized via the transfer matrix method (EdgeR package in R; [130]). Differential abundance analysis was performed on these normalized OTU counts based on three salinity ranges (low: < 10 ppt, moderate: 10–25 ppt, and high: > 25 ppt). The normalized counts per million of the top 32 (based on relative abundance) differentially regulated OTUs were visualized and grouped by sample type (sediment or surface water) and sampling location using the ggplot2 package in R [170]. FUNGuild [100, 118] was used to hypothesize the function of the fungi that were differentially abundant in the salt and brackish marshes identified by the 28S primer set.

Results and discussion

Environmental context

The February and November samples were collected at nearly the lowest temperatures, whereas the June and August samples were collected near the highest temperatures of the entire sampling period (Fig. 2). At CB (North Inlet), the water temperature ranged from 6.8 °C to 34.2 °C, with averages of 21.2 °C ± 6.3 °C in 2020 and 20.7 °C ± 6.9 °C in 2021 (Fig. 2A). Salinity ranged from 6.5 ppt to 34.5 ppt in 2020 and 9.2 ppt to 41.6 ppt in 2021 (Fig. 2E). The average pH was 7.7 ± 0.2 in 2020 and 7.6 ± 0.2 in 2021 (Fig. 2I). Chlorophyll *a* peaked in May 2020 (77.1 µg/L) and July 2021 (22.2 µg/L) (Fig. 2M). Similar trends were observed at DB and OL (both are situated in the North Inlet; Fig. 2B, C, F, G, J, K), with chlorophyll *a* maxima occurring in August 2020 and 2021 (Fig. 2N, O).

The brackish marsh, TA (Winyah Bay), exhibited seasonal variation, with average temperatures of 20.3 °C ± 6.6 °C in 2020 and 20.7 °C ± 6.9 °C in 2021 (Fig. 2D). Salinity ranged from 0.1 to 26.7 ppt (Fig. 2H), whereas pH values averaged 6.7 ± 0.3 in 2020 and 7.1 ± 0.4 in 2021 (Fig. 2L). The chlorophyll *a* maxima were observed in August 2020 (172.1 µg/L) and July 2021 (96.9 µg/L) (Fig. 2P). The August time point was taken shortly after the August 2020 phytoplankton bloom (Fig. 2).

Fungal community composition identified by the universal SSU primer set (18S and 16S rRNA)

In this study, 82.1% of the reads generated by the universal primer set (9,767,053 in total) were aligned with 16S OTUs. The remaining reads (1,746,250) mapped to 18S OTUs, of which only three (one *Pleosporaceae*, one *Saccharomycetaceae*, and one *Malasseziaceae*) were

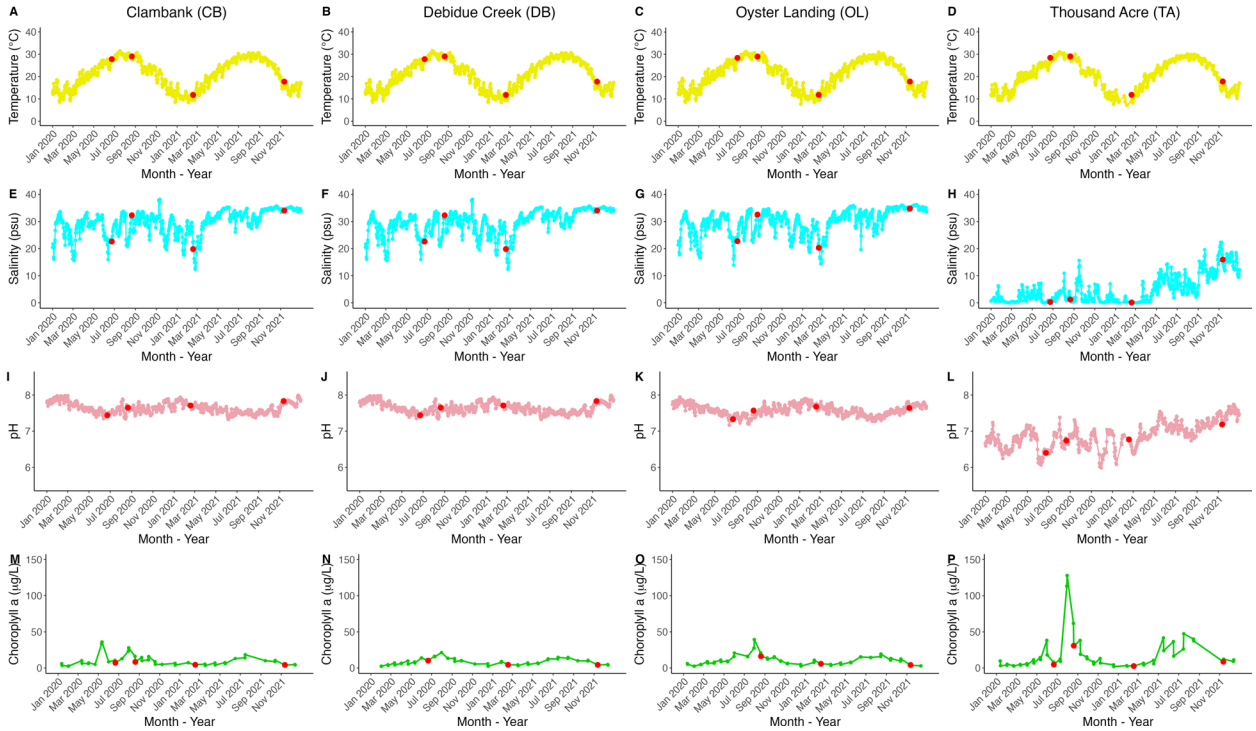

Fig. 2 Surface water temperature (yellow; **A**-**D**), salinity (blue; **E**–**H**), pH (pink; **I**-**L**), and chlorophyll *a* (green; **M**-**P**) measurements from CB (**A**, **E**, **I**, **M**), DB (**B**, **F**, **J**, **N**), OL (**C**, **G**, **K**, **O**), and TA (**D**, **H**, **L**, **P**) from January 2020 to January 2022. The red dots indicate the sampling dates. Data were collected via the National Estuarine Research Reserve's (NERR) System-Wide Monioring Program and obtained from the NERR's Centralized Data Management Office

identified as fungi (Supplementary Table S3), none of which belongs to EDF. The percentage of these fungal OTUs in the 18S universal primer set library ranged from 0.003 to 0.03%. These findings demonstrate that this universal primer set is not suitable for assessing fungal diversity, even though it has the potential to assess both prokaryotic and eukaryotic microbial communities simultaneously [88]. This is because the amount of fungal DNA is much lower than that of bacterial DNA, so the vast majority of the sequencing reads are bacterial. Additionally, primer bias may have resulted in lower detection of fungal sequences. Universal primers are often optimized to preferentially bind to conserved regions in bacterial or archaeal 16S rRNA genes, which may result in inefficient amplification of eukaryotic 18S sequences, particularly fungal taxa [166]. Several studies have shown that even minor mismatches at primer binding sites can lead to large discrepancies in amplification efficiency across taxa [88, 144]. In marine environments, where fungi often represent a minor fraction of the total microbial biomass, such biases may be amplified, leading to underrepresentation in sequencing results. Fungi-specific 18S primers are likely better suited for studying marine fungal diversity by targeting the small subunit (SSU) rRNA [11].

Comparison of the ITS2 and 28S primer sets using ZymoBIOMICS standards

When the ZymoBIOMICS DNA standard community is evaluated via the ITS2 and 28S primer sets, the expected relative abundance ratio of *Saccharomyces cerevisiae* to *Cryptococcus neoformans* should be 1:1. This ratio was closely reflected in the community composition obtained via the 28S primer set (Supplementary Figure S1A). However, when the community was analyzed with the ITS2 primer set, the relative abundance of *C. neoformans* was 64.4% (Supplementary Figure S1B). Although the analysis was based on a single replicate, this discrepancy suggests that the ITS2 primer may not perform as effectively as the 28S primer in accurately representing the relative abundances of fungal taxa.

For the ZymoBIOMICS Community (cells) Standard, where the same expected 1:1 ratio of *S. cerevisiae* to *C. neoformans* should apply, neither the 28S nor ITS2 primer sets resulted in *C. neoformans* reaching 50% relative abundance (Supplementary Figure S1). This deviation may be caused by a lower rRNA copy number in *C. neoformans* than in *S. cerevisiae* within the cell mixture provided by ZymoBIOMICS. Alternatively, the cell wall of *C. neoformans* may present greater resistance to lysis due to the presence of a large polysaccharide capsule,

which has been reported for this species [17]. In addition to mechanical lysis, lysis can be supplemented with enzymatic digestion of the fungal cell wall [72, 172]. These findings suggest that our study, along with other metabarcoding surveys of environmental samples, could exhibit a community compositional bias against microorganisms recalcitrant to cell lysis [42, 49].

Fungal community composition identified by the ITS2 region primer set

A total of 8236 fungal OTUs (clustered at 97% similarity level) were detected in at least one sample from our metabarcoding survey targeting the ITS2 region and used for subsequent analyses and visualizations (Figs. 3, 4, 7). Ascomycota and Basidiomycota dominated all the samples and constituted, on average, 85.8% and 4.8% of the

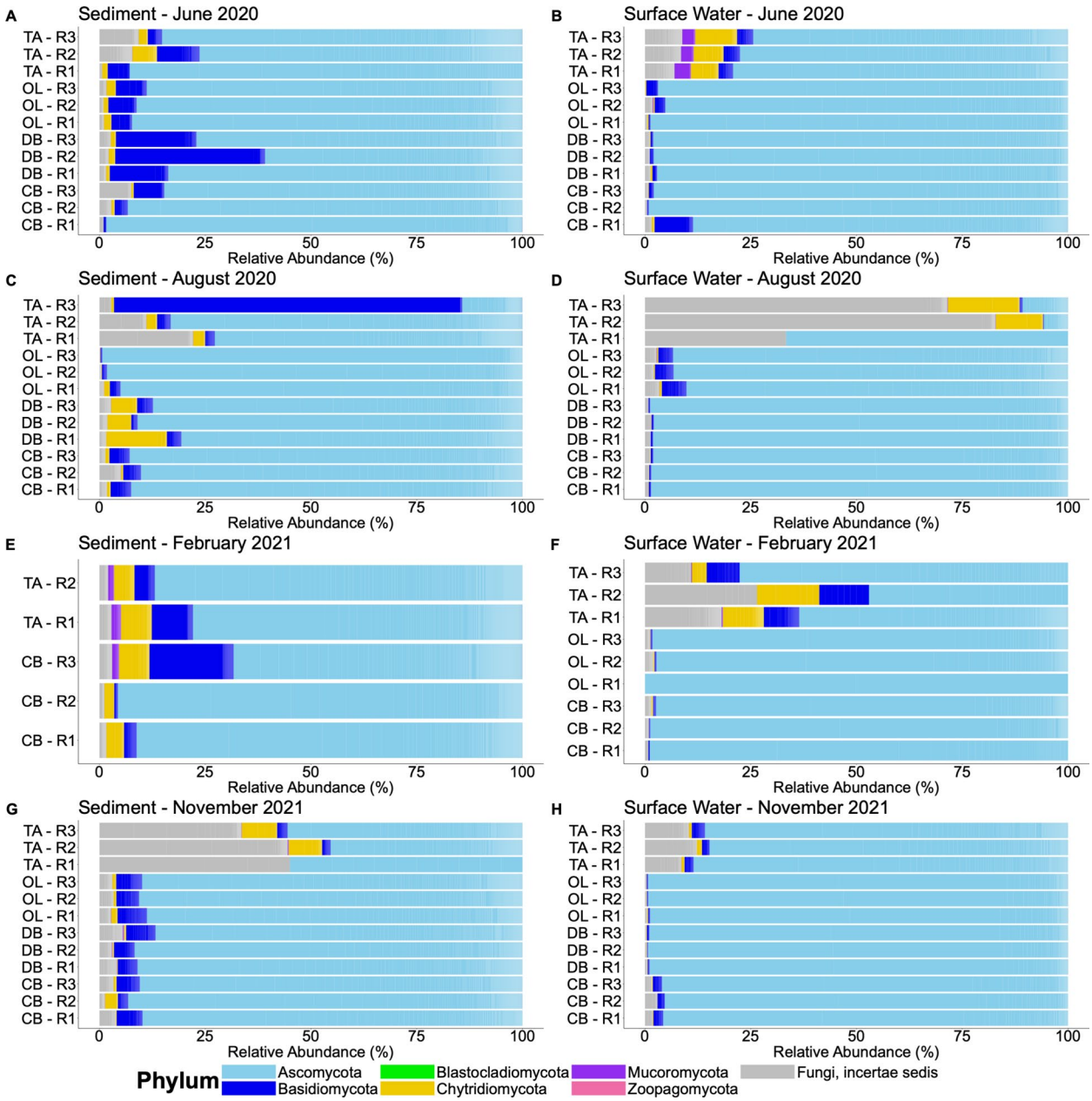

Fig. 3 Relative abundance of the 8236 fungal ITS2 OTUs across all stations: **A** sediment in June 2020, **B** surface water in June 2020, **C** sediment in August 2020, **D** surface water in August 2020, **E** sediment in February 2021, **F** surface water in February 2021, **G** sediment in November 2021, and **H** surface water in November 2021

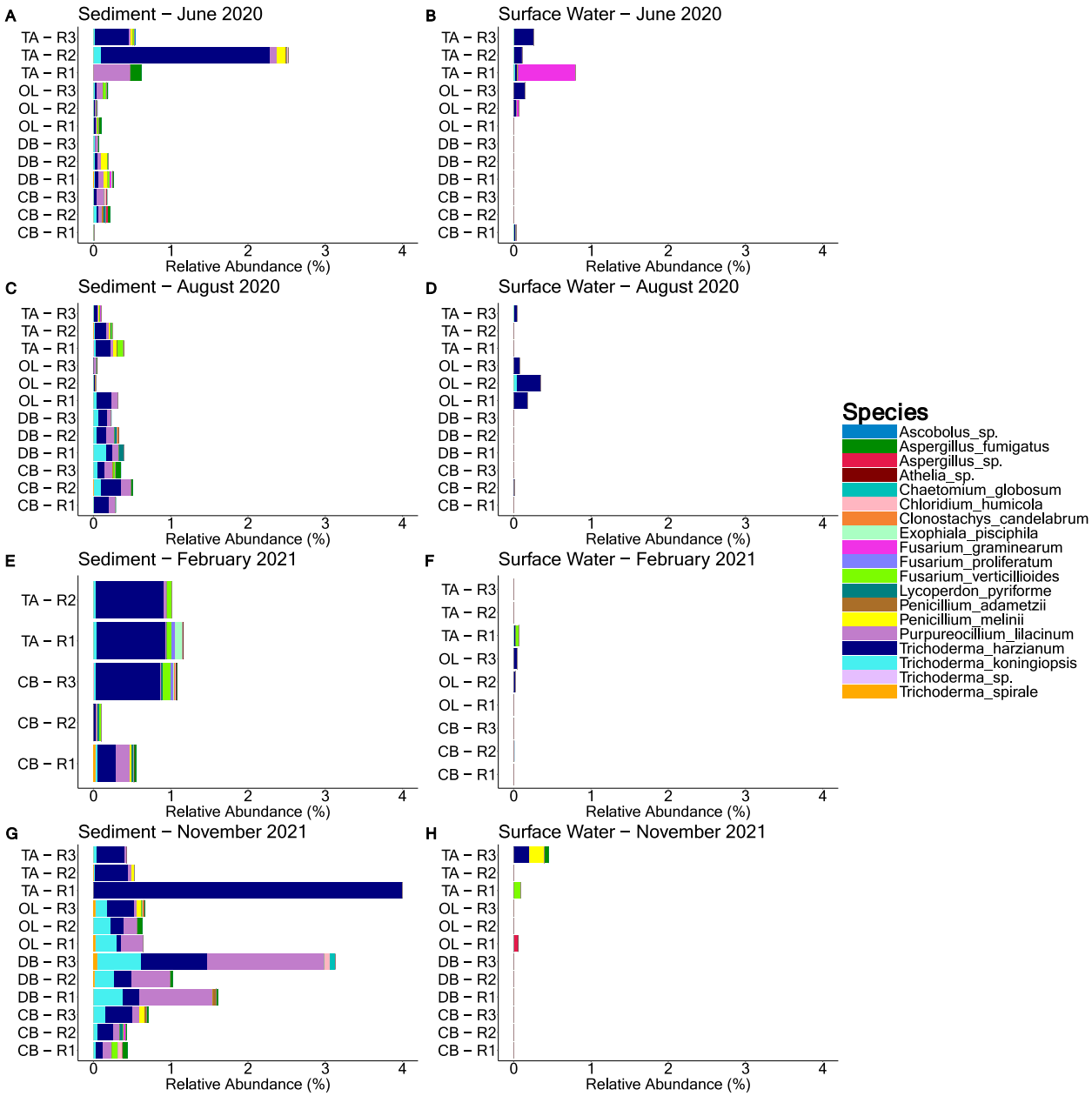

Fig. 4 Relative abundance of putative denitrifying fungal ITS2 OTUs across all stations: **A** sediment in June 2020, **B** surface water in June 2020, **C** sediment in August 2020, **D** surface water in August 2020, **E** sediment in February 2021, **F** surface water in February 2021, **G** sediment in November 2021, and **H** surface water in November 2021. The putative denitrifying fungi were identified from Maeda et al. [83] and Lazo-Murphy et al. [74]

fungal community, respectively. Unclassified fungi (only classified at the kingdom level as fungi) made up an average of 6.8% (ranging between 0.0 and 82.8%) of all fungal reads. The composition and diversity of the fungal community assessed by ITS2 sequencing significantly varied between sample types (sediment and surface water; PERMANOVA $p = 0.001$), marsh type (brackish and salt marsh; Supplementary Table S4), and sampling dates (except Nov. 2021 vs. June 2020; Supplementary Table S5) (Fig. 3). Ascomycota dominated most samples from both the sediment and surface water, whereas Basidiomycota dominated in only one TA sediment sample in August 2021 (Fig. 3). The relative abundance of EDF was low (< 2.4% on average). Chytridiomycota were present in all the samples, with an average relative abundance of 2.5% in the sediment and 1.9% in the surface water (Fig. 3).

Other EDF phyla (Blastocladiomycota, Mucoromycota, and Zoopagomycota) were detected, but all were present at extremely low levels (< 0.2% relative abundance on average).

Most marine studies using primers targeting the ITS2 region (Table 1) consistently detected Ascomycota, Basidiomycota, Chytridiomycota, and a notable proportion of unclassified fungi, each representing over 1% relative abundance [32, 38, 71, 80, 113, 115, 126, 162, 175, 176]. These taxa dominate marine fungal communities, reflecting the high prevalence of Dikarya (Ascomycota and Basidiomycota) across a range of ecosystems, whereas Chytridiomycota typically represents a smaller but consistent component. The substantial proportion of unclassified fungi underscores the complexity and novelty within marine fungal communities, revealing limitations in current databases to fully resolve this diversity [7]. Studies targeting the ITS1 region (Table 1) have identified more Zoopagomycota than those using the ITS2 primer set [10, 28, 78, 79, 149], suggesting that the choice of the ITS primer set can significantly influence the detection of certain fungal groups. However, similar to the ITS2 primer set, the majority of the fungal community identified by the ITS1 primers included Dikarya, Chytridiomycota, and unclassified fungi (Table 1) [10, 27, 28, 78, 79, 106, 120, 148, 149, 158, 165, 167]. This highlights the dominance of these groups across different ITS markers, although subtle differences in community composition may emerge depending on the primer region targeted.

We identified putative denitrifying fungi by matching our ITS2 OTU sequences (at 100% identity level) to published fungal taxa known to denitrify [74, 83]. Sediment samples contained a higher relative abundance of these putative denitrifying fungi than the surface water, consistent with the expectation that denitrification is more active in the low-oxygen, organic-rich conditions typically found in benthic environments [85, 90, 134]. Members of *Trichoderma* dominated the putative denitrifying fungi (Fig. 4). *Trichoderma harzianum* was dominant in the brackish marsh samples, reaching up to 4% of the relative fungal community, in June 2020, February 2021, and November 2021 (Fig. 4). This seasonal recurrence suggests a potentially stable role for *T. harzianum* in removal of nitrate within brackish marsh sediment. Its high abundance, combined with its known capacity to reduce nitrate under anaerobic conditions [83], points to a potential ecological role in marsh sediment nitrogen cycling. The salt marsh samples were more variable and dominated by *T. harzianum*, *Trichoderma koningiopsis*, and *Purpureocillium lilacinum*, especially during August 2020 and November 2021 (Fig. 4). These species have also been associated with nitrate reduction and may be adapted to higher salinity environments compared to their brackish counterparts [74].

While denitrification is usually attributed to prokaryotes, many studies point to a poorly understood role for fungi in marine systems denitrification [74, 83, 138, 147]. However, fungi lack the nitrous oxide reductase gene (nosZ), so they are unable to complete the final step of denitrification (reducing N_2O to N_2 gas) [4]. As a result, fungi likely contribute disproportionately to N_2O emissions, a potent greenhouse gas [4, 33, 138]. This has important implications for wetland and estuarine nitrogen cycling, where fungal denitrification may act as a source of N_2O. The detection of putative fungal denitrifiers, especially in marsh sediments, suggests that fungi may contribute to nitrogen loss and gaseous N outputs in ways previously underestimated. However, further work, including cultivation, transcriptomic, metagenomic, or stable isotope labeling approaches, is needed to confirm their activity and quantify their contribution to N_2O fluxes under in situ conditions. These preliminary observations underscore the importance of considering fungi in broader assessments of estuarine nitrogen cycling and greenhouse gas production.

Fungal community composition identified by the 28S LSU region primer set

A total of 3624 fungal OTUs (clustered at 97% similarity level) were detected in at least one sample from our metabarcoding survey targeting the D1 region of the large subunit rRNA and were used for downstream analyses (Figs. 5, 6, 7). To investigate the phylogenetic diversity of fungi in the surface water and sediments of North Inlet–Winyah Bay, a maximum-likelihood tree was constructed using the 3624 fungal OTUs identified by the 28S primer set, along with two outgroup sequences from the SAR supergroup (*Frustulia sp*. and *Amphora commutata*; Fig. 5). Over half (51.0%) of the fungal OTUs were classified as EDF. Among these EDF lineages, 22.7% were identified as Chytridiomycota, 15% as Zoopagomycota, 6.9% as Blastocladiomycota, 3.7% as Neocallimastigomycota, and 2.7% as Mucoromycota (Fig. 5). The remaining 48.8% of the OTUs were classified as Dikarya fungi, with Ascomycota accounting for 37.5% and Basidiomycota accounting for 11.3% (Fig. 5). Most Ascomycota OTUs were from the classes Dothideomycetes (13.7%) and Sordariomycetes (11.7%). Overall, there was strong agreement between taxonomic classifications and phylogenetic placements. However, phylogenetic analysis was used to curate and refine the classifier-based results. The seven OTUs that could not be assigned to any fungal phylum by the RDP classifier clustered clearly with Ascomycota in the phylogenetic tree (Fig. 5) and were accordingly reclassified as Ascomycota based on their

Table 1 The presence of marine fungal phyla in studies from coastal and open oceans (water and sediment) using different primer sets

Study	Primer	Dikarya	Blastocladio-mycota	Chytridio-mycota	Crypto-mycota	Mucoro-mycota	Olpidio-mycota	Neocallimastigo-mycota	Zoopago-mycota	Unclassified Fungi
This Study	28S	***	**	***		**			***	
	ITS2	***		**		*			*	***
	18S	***								
[2]	28S	***		**		***		***	***	
[116]	28S	***	**	***		**		**	***	
[47]	ITS2	***		***	*		*			***
	28S	***	*	**	*	**			*	**
[10]	ITS1	***		**					*	**
[27]	ITS1	***		*						
[28]	ITS1	***								***
[79]	ITS1	***		*		*			**	***
[78]	ITS1	***		**				*	***	***
[106]	ITS1	***								
[120]	ITS1	***								***
[148]	ITS1	***		*			*			***
[149]	ITS1	***		*					**	***
[158]	ITS1	***		*		*				***
[165]	ITS1	***		**	*	*				***
[167]	ITS1	***						*		***
[32]	ITS2	***								***
[71]	ITS2	***		**						**
[80]	ITS2	***		*		*				***
[113]	ITS2	***		**						***
[117]	ITS2	***		*						***
[126]	ITS2	***		**	*	*			*	***
[162]	ITS2	***		***						***
[175, 176]	ITS	***		*					*	***
[37]	ITS1 & ITS2	***		***		*				
[38]	ITS1 & ITS2	***								***
[138]	18S	***		*	**					*
	ITS1	***		*	*					***
	ITS2	***		*		**				*
[11]	18S	***		***	**	*			**	*
[59]	18S	***		***						**
[60]	18S	**		**						***
[122]	18S	***		**	***					**

Table 1 (continued)

Study	Primer	Dikarya	Blastocladio-mycota	Chytridio-mycota	Crypto-mycota	Mucoro-mycota	Olpidio-mycota	Neocallimastigo-mycota	Zoopago-mycota	Unclassified Fungi
[131]	18S	***	*	**	**	*			*	

Three asterisks indicate that the relative abundance of the respective fungal phylum is greater than 10% on average across all samples

Two asterisks indicate that the relative abundance of the respective fungal phylum is less than 10% and greater than 1% on average across all samples

One asterisk indicates that the relative abundance of the respective fungal phylum is less than 1% and greater than 0% on average across all samples

The [116] study relative abundance was based on the analyses we performed (Supplementary Figure S4)

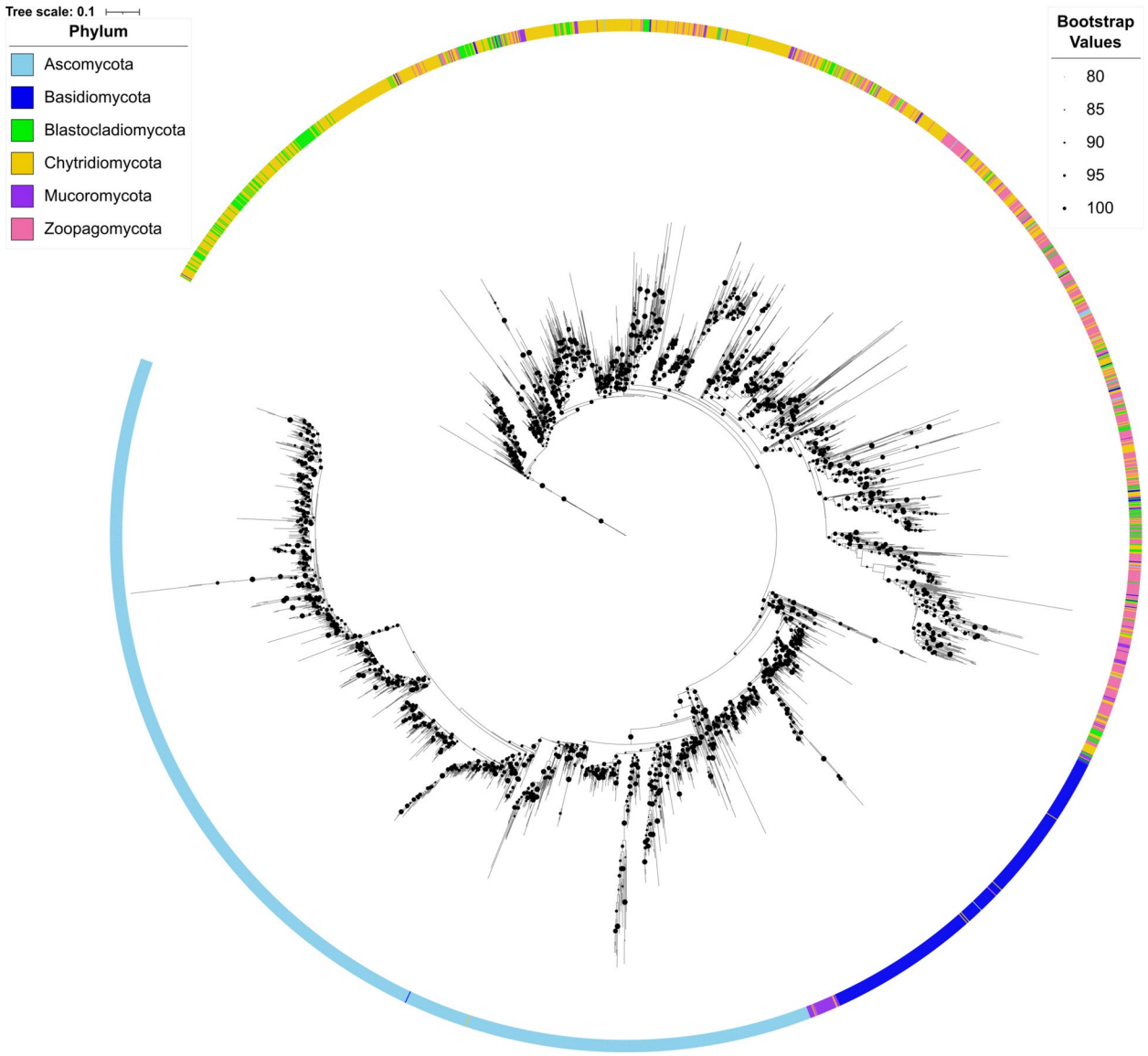

Fig. 5 A maximum likelihood phylogenetic tree reconstructed with the 3624 28S rRNA OTUs. Sequences from the SAR supergroup (*Frustulia sp.* and *Amphora commutata*) were included as an outgroup. The color indicates the phylum of each OTU classified by the RDP classifier [163]. Branches supported by bootstrap values greater than 80 are marked with a filled circle

placement. These revised classifications were also supported by NCBI BLASTn results. All results presented below reflect this curated classification framework.

The maximum-likelihood phylogenetic tree supported the monophyly of Ascomycota and Basidiomycota, confirming their shared descent from a common ancestor. These results align with those of previous studies [64, 66, 143], which revealed that Ascomycota and Basidiomycota form a clade of Dikarya fungi, diverging from other EDF lineages [64]. In line with earlier findings [66, 103, 119, 168], over half of the sequences identified in this study were from EDF, many of which are polyphyletic (Blastocladiomycota, Chytridiomycota, Neocallimastigomycota, and. Zoopagomycota). Previous work has shown that EDF in marine environments can represent between 1.7 and 72% of fungal reads, primarily from Chytridiomycota or Mucoromycota [28, 116, 128].

Across all seasons (Supplementary Table S6) and sample types (water and sediment; PERMANOVA $p = 0.001$), the 28S rRNA gene revealed significant differences in fungal community composition between salt and brackish marshes (Supplementary Table S7). In the salt marsh sediment samples, Ascomycota was consistently predominant and consisted of, on average, 80.0% of the salt

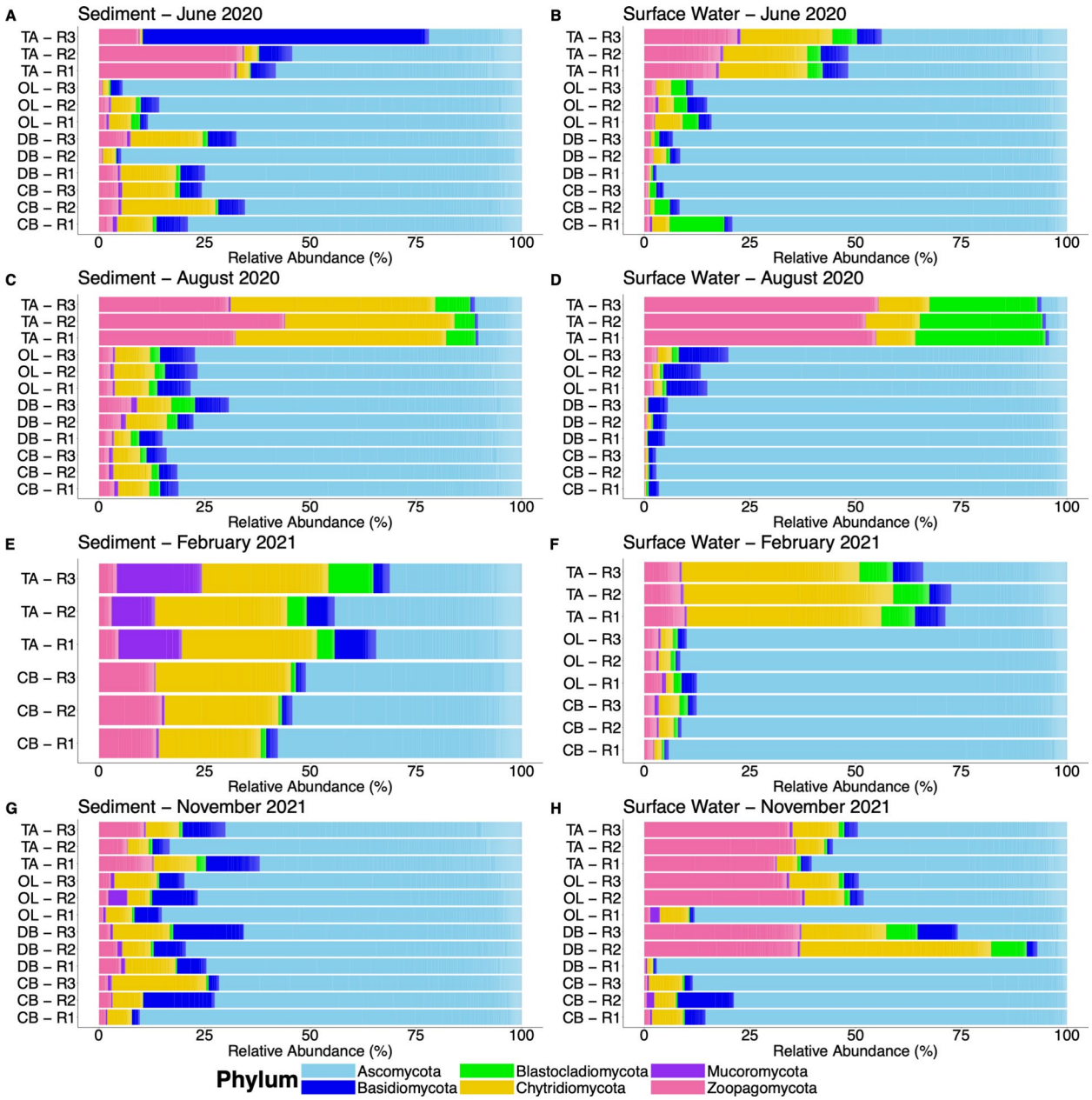

Fig. 6 Relative abundance of the 3624 fungal 28S OTUs across all stations: **A** sediment in June 2020, **B** surface water in June 2020, **C** sediment in August 2020, **D** surface water in August 2020, **E** sediment in February 2021, **F** surface water in February 2021, **G** sediment in November 2021, and **H** surface water in November 2021

marsh fungal community (Fig. 6). Various Sordariomycetes dominated the sediment samples (Supplementary Figures S5 & S6), whereas Capnodiales and Pleosporales (two Dothideomycetes) dominated the surface water samples (Supplementary Figures S5 & S7). In the brackish marsh, while Ascomycota was still the dominant phylum (37.9% on average), Zoopagomycota and Chytridiomycota accounted for 23.8% and 21.5% of the fungal community, respectively (Fig. 6). Zoopagomycota (mostly Entomophthorales, see Supplementary Figure S8) were more prevalent in the sediment and surface water in the summer of 2020 than in the winter of 2021, and their relative abundance reached 52.3% in August 2020 in Thousand Acre surface water. Chytridiomycota were present in most samples, with an average relative abundance of 14.1% in the sediment and 9.4% in the

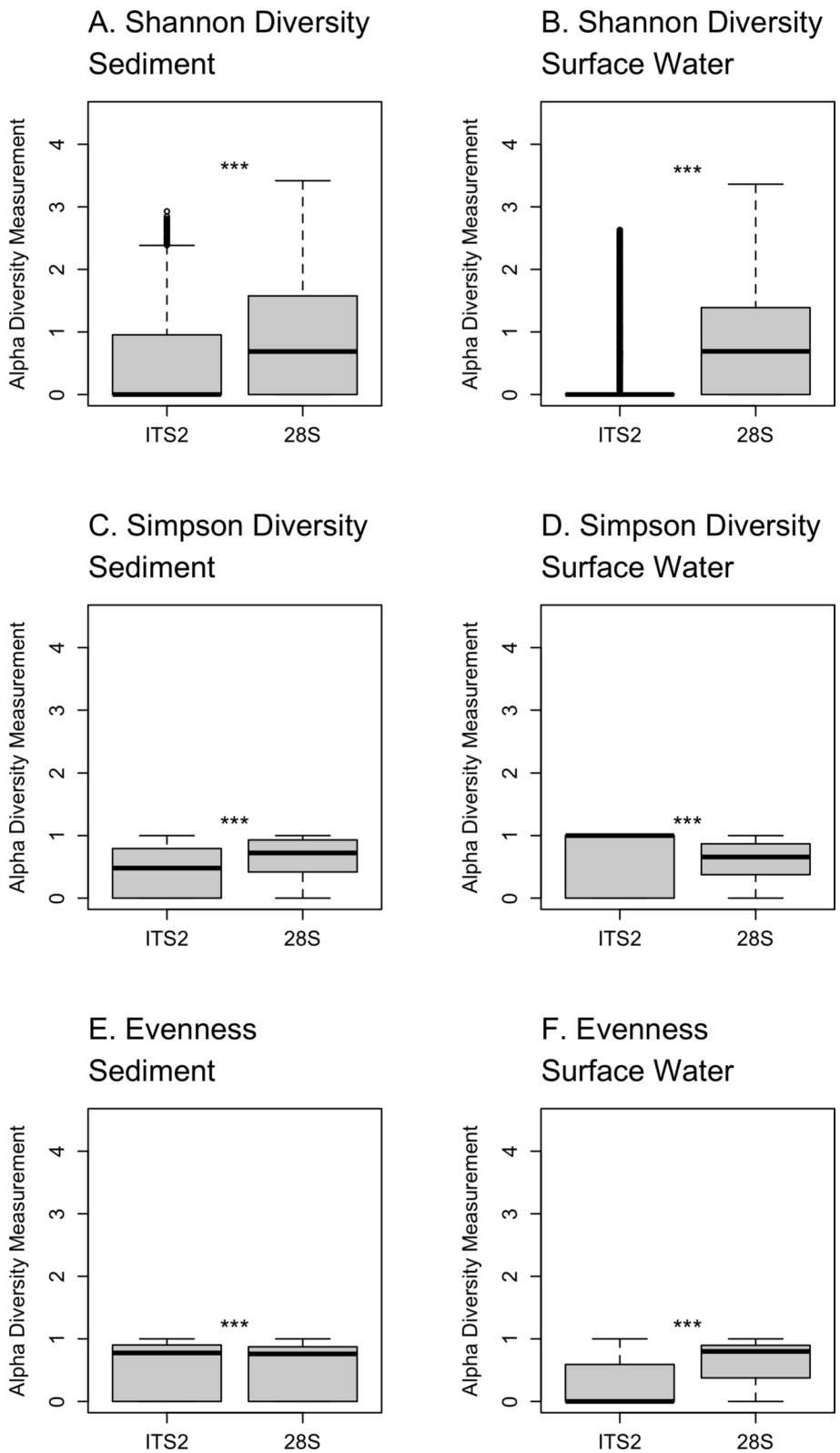

Fig. 7 Calculated Shannon diversity (**A** and **B**), Simpson diversity (**C** and **D**), and evenness (**E** & **F**) indices of fungal OTUs from sediment and surface water based on ITS2 and 28S rRNA sequencing. Three stars (***) indicate *p*-values < 0.001 (from the Benjamini–Hochberg adjusted Hutcheson t tests *p*-value)

surface water (Fig. 6). Blastocladiomycota (mostly *Catenariaceae*, see Supplementary Figure S9) was predominant in the brackish marsh water column, with a relative abundance of 10.4%, and was present in most samples, with an average relative abundance of 3.1%. Mucoromycota were detected, but were present at low levels (1.1% relative abundance on average).

A very small number of studies investigating marine fungal communities have utilized the 28S primer set, highlighting a gap in the literature [2, 47, 116] (Table 1). A previous survey of fungal diversity targeting 28S rRNA in coastal sediments approximately three hundred kilometers away in North Carolina revealed similar fungal community compositions [116]. Given the similar physical environments between the sampling sites in our study and those in Picard [116], the similar results in terms of fungal diversity suggest that surveys targeting 28S rRNA are likely reproducible. By applying our 28S analysis pipeline to a previous survey of fungal diversity in coastal sediments from North Carolina [116] via the updated RDP 2.14 classifier [163], we successfully classified all fungal OTUs at the phylum level (Supplementary Figure S4). Among the studies that have employed this marker, taxa from a range of fungal phyla, including Chytridiomycota, Mucoromycota, Zoopagomycota, and Dikarya, were consistently identified, indicating that the 28S region can capture broad phylogenetic diversity. Interestingly, Blastocladiomycota, a lesser-known group of EDF, was detected in two [47, 116] results from our pipeline) of the three studies focused on marine environments. These findings suggest that the 28S primer set has the potential to detect not only common marine fungal taxa but also more rare and ecologically significant groups. Additionally, these studies reported that fewer than 10% of fungal reads were unclassified [2, 47, 116], which contrasts with the often higher proportion of unclassified reads observed with other ribosomal markers in marine fungal surveys. This relatively low percentage of unclassified reads highlights the robustness of the 28S primer set in accurately assigning taxonomic identities, further supporting its utility in marine fungal research.

Variation in fungal diversity across primer sets

Diversity was significantly higher with the 28S (LSU) primers across all conditions—depth, site, and season ($p < 0.01$; Fig. 7, Supplementary Table S8). In sediment, mean Shannon diversity was 0.9 ± 0.9 for the 28S primers compared to 0.6 ± 0.7 with ITS2. In surface water, the 28S primers yielded a mean Shannon diversity of 0.8 ± 0.8, while ITS2 yielded 0.2 ± 0.5. Simpson diversity followed similar patterns (Fig. 7C, D), and evenness was also generally higher with the 28S primers, particularly in surface water (Fig. 7E, F). The ITS2 primer set identified more unique taxa at the class, order, family, and genus levels (Supplementary Table S9), whereas the 28S primer set yielded higher overall Shannon and Simpson diversity. This contrast is likely due to the conserved nature of the 28S region, which limits taxonomic resolution but captures a more even community structure [14, 81, 151]. In comparison, the ITS2 data, while richer in taxa, showed lower evenness due to the dominance of a few highly abundant taxa.

The ITS2 primer set detected low relative abundances for Blastocladiomycota (0.0002%), Mucoromycota (0.2%), and Zoopagomycota (0.0008%), which were present in the brackish marsh at average abundances of 7.1%, 2.2%, and 23.8%, respectively, as revealed by the 28S primer set (Supplementary Table S10). The ITS2 set identified a greater proportion of Ascomycota in both the brackish (65.6%) and salt marshes (93.6%) than did the 28S primer set (37.6% in the brackish and 79.9% in the salt marshes).The ITS2 primer set identified significantly more Ascomycota in the salt marsh than the 28S primer set (Welch's t-test, $t = -3.152$, $df = 121.28$, p-value $= 0.002$). Compared with the ITS2 primer set, the 28S primer set consistently detected a significantly greater number of EDF in both the salt (Welch's t-test, $t = 24.2$, $df = 62.3$, p-value $< 2.2 \times 10^{-16}$) and brackish marsh (Welch's t-test, $t = 16.9$, $df = 23.2$, p-value $= 1.6 \times 10^{-14}$) and no unclassified fungi (Fig. 6, Supplementary Table S10). Although EDF often constitute less than half of the total fungal reads in marine environments, their ecological importance is substantial, contributing to nutrient cycling, decomposition, and symbiotic interactions with marine organisms such as algae and invertebrates [116]. The underestimation of these lineages in previous studies is frequently attributed to the limitations of culture-based methods and biases in molecular techniques, particularly when targeting ribosomal RNA regions that do not fully capture the diversity of EDF [116]. While the ITS2 primer set identified 8,236 fungal OTUs—more than twice as many as did the 28S primer set—it predominantly detected Ascomycota and Basidiomycota, with an average of 6.8% unclassified fungi of all fungal reads and 22.5% of ITS2 OTUs unclassified above the phylum level (Supplementary Table S10). Previous studies utilizing long 18S-ITS-28S sequences suggest that many undescribed taxa belong to EDF lineages [150, 153]. Additionally, marine fungal studies employing ITS primer sets have reported that 20–80% of fungal OTUs are unclassified at the phylum level [32, 79, 80, 126]. These discrepancies may stem from limitations in ITS-region databases [124], as ITS primer sets were developed primarily from terrestrial sequences, resulting in a bias toward terrestrial Dikarya [7].

The 28S primer set provides several advantages, including broader taxonomic coverage, by capturing EDF lineages such as Zoopagomycota and Blastocladiomycota, which are often underestimated by the ITS2 primer set (Table 1; Supplementary Table S10). This broader phylogenetic scope is critical for capturing the full extent of fungal diversity in marine ecosystems, where EDF lineages play essential ecological roles. Additionally, studies utilizing the 28S primer set report a lower proportion of unclassified reads—less than 10%—than the higher percentages observed with ITS primer sets, likely due to biases in databases that are more focused on terrestrial fungi [2, 47, 116]. These findings indicate that the 28S region is more effective at detecting fungal taxa across various habitats and environmental conditions, including salinity gradients and seasonal shifts.

The fractions of fungal reads generated by the ITS2 and 28S primer sets were comparable (nearly half, Supplementary Figure S11), as were the proportions of the total number of OTUs (Supplementary Table S2). Notably, despite the 28S primer set being designed for fungal specificity [151], approximately half of the reads were nonfungal. This observation underscores a critical consideration for experimental design when targeting fungal communities. Specifically, experimental designs including sequencing depth per sample should consider allocating resources to generate twice the number of reads necessary for fungal-specific analysis to adequately account for nontarget amplification.

Copy-normalized fungal abundance using 28S qPCR

The majority of studies aiming to quantify fungal abundance from environmental samples have relied on 18S and ITS primers [141]. This has largely been due to historical precedence as the 18S region is highly conversed across dominate terrestrial lineages (Ascomycota and Basidiomycota), and as ITS has offered a greater degree of species level variability within these lineages [141]. Because the 28S region provides a good balance between sequence conservation and variation and it enables phylogenetic analysis, covered fungal diversity well (Fig. 6), and the variation in the number of 28S rRNA genes is smaller than that of ITS2 [137] we decided to use the 28S primers for qPCR. Beyond this choice of marker, our study addresses an important but often overlooked issue: most previous studies quantifying fungi via qPCR assume that all amplicons are fungal, even though unspecific amplification can occur with virtually all commonly used primers [151]. To overcome this, we used the same 28S primer set for both qPCR and metabarcoding, allowing us to accurately determine the proportion of qPCR products that are truly fungal. This combined approach provides a more reliable estimate of fungal abundance and can serve as a framework for future qPCR-based quantification of fungal communities in environmental samples.

Fungal abundances were significantly higher in brackish marsh sediments than in salt marsh sediments (Kruskal–Wallis test, $\chi^2 = 23.3$, df = 1, $p < 0.001$; Fig. 8). For example, the brackish site (TA) reached mean abundances up to $5.9 \times 10^5 \pm 1.6 \times 10^5$ (mean ± standard error) genome equivalent (GE)/g in June 2020 and $2.9 \times 10^6 \pm 5.5 \times 10^5$ GE/g in February 2021, compared to salt marsh sites such as CB, which averaged $6.5 \times 10^4 \pm 2.2 \times 10^4$ GE/g in June 2020 and $4.3 \times 10^5 \pm 1.1 \times 10^5$ GE/g in February 2021 (Fig. 8, Supplementary Table S12). In contrast to sediments, surface water samples had much lower fungal abundances. There was a significant difference between surface water samples from the salt marsh sites and the brackish marsh site (Kruskal–Wallis test, $\chi^2 = 6.6$, df = 1, $p = 0.01$; Fig. 8). The salt marsh DB (DB R3) site had up to $1.3 \times 10^5 \pm 1.3 \times 10^4$ GE/mL in August 2020, while CB ranged from 3.9×10^4 GE/mL in August 2020 to ~ 111 GE/mL in June 2020. The brackish marsh (TA) mean abundances ranged between 87 ± 9 GE/mL in replicate 3 (R3) in February 2021 to 8.9×10^3 GE/mL in replicate 2 (R2) February 2021, but averaged 4.4×10^3 across all brackish marsh surface water samples (Supplementary Table S12).

In the water column, bacteria abundances ranged from 10^6 to 10^8 cells per mL [3, 13, 169], approximately three to five orders of magnitude higher than the estimated fungal abundance observed in the surface water at our NI-WB sites, underscoring the dominance of bacterial taxa in planktonic microbial communities within coastal marsh systems. The large variation in cell counts between bacteria and fungi likely reflects fundamental differences in growth rates, dispersal strategies, and nutrient utilization between fungi and bacteria, with fungi typically associated with particulate or host-derived substrates [7, 52, 106, 112]. Bacterial abundance in marsh sediments reported previously ranges between 1×10^8 and 4×10^8 GE/g [67, 111, 132], approximately three orders of magnitude greater than our estimated fungal abundance in the sediment marsh samples. The closer ratio of fungal to bacterial abundance in sediments may reflect the ecological niches fungi occupy in benthic environments, such as the decomposition of complex organic matter (e.g., plant detritus and lignocellulose) [7, 112] and interactions with plant roots, particularly in Spartina-dominated marshes [19, 22]. The reduced disparity suggests that fungi are not merely minor constituents of the sediment microbial community, but may contribute meaningfully to nutrient cycling and organic matter transformation. Thus, while bacteria numerically dominate both compartments, the higher proportional abundance of fungi in sediments compared to surface water implies a potentially greater

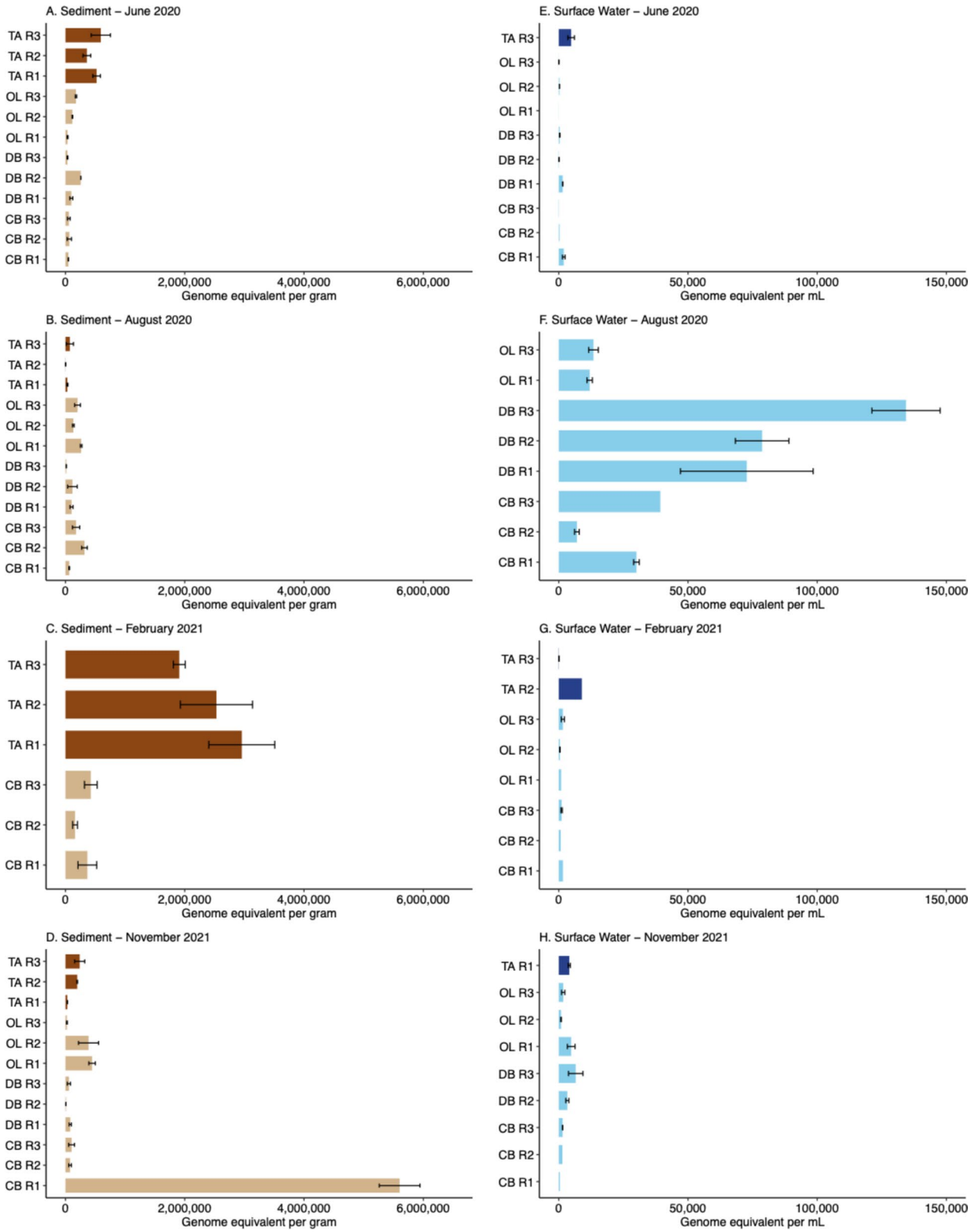

Fig. 8 Copy-normalized fungal abundances (genome equivalent per g or mL) estimated using 28S qPCR for **A** sediment and **B** surface water samples across salt marsh sites – Clambank (CB), Debidue Creek (DB), and Oyster Landing (OL)—and a brackish marsh site – Thousand Acre (TA)—from North Inlet – Winyah Bay. Brackish marsh (TA) samples are shaded. The error bars represent the standard error between technical replicates. In samples where only one technical replicate was performed no error bars were included

ecological role for fungal taxa in benthic marsh processes compared to their role in the overlying water column.

The observed differences in fungal community size and composition between brackish and salt marsh sediments highlight the influence of salinity and organic matter availability in coastal estuaries. Brackish marsh, characterized by lower salinity and greater freshwater and terrestrial inputs (resulting in higher nutrient concentrations; Fig. 2), appear to support larger fungal populations and higher relative abundances of EDF, such as Chytridiomycota and Zoopagomycota (Figs. 6, 8). Such groups may play key roles in decomposing complex organic matter and linking aquatic and terrestrial nutrient cycles in these transitional habitats [9, 54, 100, 118, 154]. In salt marsh sediments, the lower overall fungal abundances and dominance of Ascomycota (Fig. 6, 8) likely reflect stronger salinity constraints on fungal growth and survival. Many fungal lineages, particularly terrestrial-derived taxa, exhibit reduced growth rates, spore germination, or enzymatic activity at seawater salinity levels, with only a subset of halotolerant or halophilic fungi thriving under such conditions [56, 68, 135]. This interpretation differs from the patterns highlighted by the PCoA analysis (Fig. 9), which mainly emphasize shifts in community composition along salinity gradients. The qPCR data provide a complementary perspective, indicating that salinity not only shapes fungal community structure but also reduces absolute fungal abundance, a measure of their physiological well-being and biomass potential in high-salinity environments. Differences between sediments and surface waters further underscore the importance of benthic habitats as hotspots of fungal biomass and potential activity in coastal estuaries. While surface waters contained far fewer fungal cells, sediments may serve as reservoirs of active decomposers and propagules for dispersal [7]. Together, the findings suggest that shifts in salinity regimes—driven by sea-level rise or freshwater diversion [41, 63, 173]—could substantially alter the structure and function of coastal fungal communities, with important implications for carbon cycling and ecosystem resilience.

Fungal lineages drive community composition differences across salinity gradients

There were significant differences in fungal community composition between salinity ranges in both sediment and surface water (Fig. 9). These differences were observed in the sediment between high (>25 ppt) and low (<10 ppt) salinities, as well as between high and middle (10–25 ppt) salinities. In surface water, differences were found between middle and low salinities and between high and moderate salinities (Supplementary Table S13, S14, Supplementary Figure S11). Salinity significantly influenced fungal community structure, explaining 2.1% of the total variation (PERMANOVA, $R^2 = 2.1\%$, $p = 0.001$). Other significant factors included temperature ($R^2 = 5.4\%$), pH ($R^2 = 5.5\%$), chlorophyll *a* ($R^2 = 1.7\%$), sampling date ($R^2 = 3.3\%$), and marsh type (salt vs. brackish marsh, $R^2 = 10.9\%$) (all $p = 0.001$). Sampling station and sample type (sediment vs. surface water) also accounted for significant variation in community composition ($R^2 = 8.3\%$), particularly for the 28S primer set (Fig. 9).

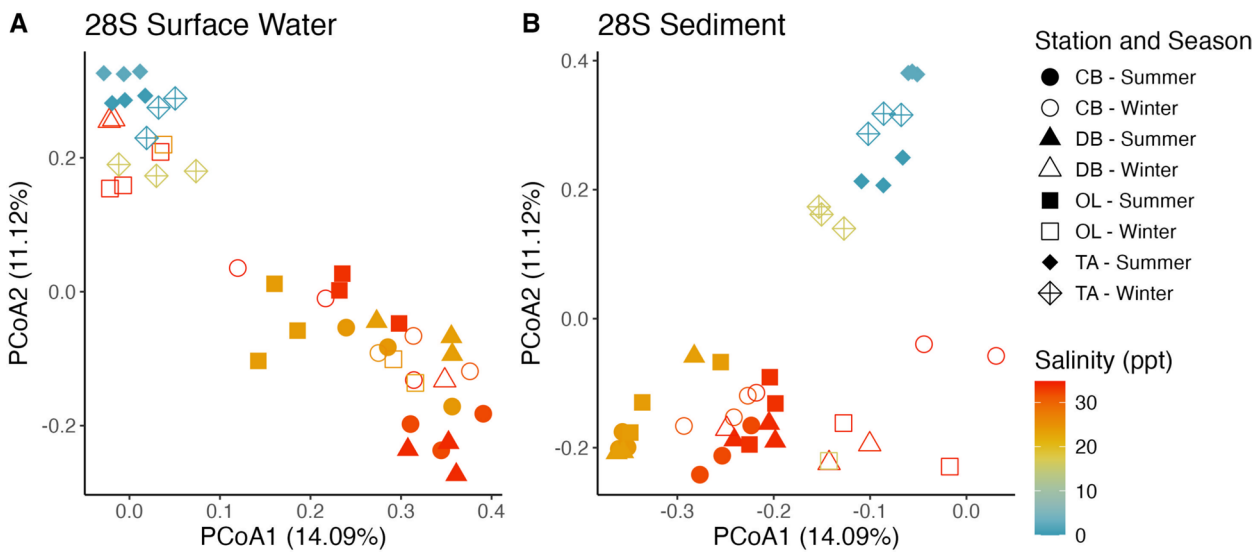

Fig. 9 Weighted unifrac principal coordinate analysis ordinations based on fungal 28S 97% OTUs (**A** & **B**) from all four study sites, comparing summer and winter in the surface water (**A**) and sediment (**B**)

The 28S primer set revealed that Ascomycota was the dominant phylum in most of the salt marsh samples, whereas EDF were more abundant in the brackish marsh samples. Salinity was clearly a key factor driving variations in fungal community composition (Fig. 9, PERMANOVA, $R^2 = 2.1\%$, $p = 0.001$), which is consistent with previous studies showing the impact of salinity on fungal diversity and community structure in marine and estuarine environments [94, 131, 160]. Salinity acts as a selective environmental filter, favoring fungi with specific physiological adaptations to osmotic stress, ionic imbalance, and desiccation [94, 114], thereby reducing overall fungal diversity while promoting the dominance of salt-tolerant species. This results in a fungal community that is often less diverse but highly specialized, reflecting the challenging conditions of high-salinity environments. Elevated salinity environments exert osmotic stress that can lead to cellular dehydration, membrane destabilization, and protein denaturation [114]. Fungi that persist under such conditions possess osmotolerance mechanisms, such as the accumulation of compatible solutes (e.g., glycerol, mannitol, trehalose), the expression of high-affinity ion transporters, and structural adaptations to membranes and cell walls that mitigate ionic toxicity and maintain cellular turgor [29, 44, 114]. Significant differences in diversity were identified between high salinity levels (> 25 ppt) and low salinity levels (< 10 ppt) in sediment, as well as between mid-range salinity levels (10–25 ppt) and low salinity levels in surface water (Supplementary Figure S11, Supplementary Table S14).

EDF play crucial roles in driving differences across salinity levels (Fig. 10). OTUs from EDF, such as Zoopagomycota (*Basidiobolus*), Chytridiomycota (*Spizellomyces, Entophlyctis, Olpidium,* and *Oedogoniomyces*), Mucoromycota (*Diversispora*), and Blastocladiomycota (*Catenomyces*), along with Basidiomycota, were more differentially abundant in the brackish marsh site than were Dikarya fungi (Supplementary Table S13; Fig. 10). Moreover, Sordariomycetes and Dothideomycetes (Ascomycota) were more differentially abundant in the salt marsh sites (Fig. 10; Supplementary Figure S5). OTUs, such as *Basidiobolus, Oedogoniomyces, Cryptococcus,* and *Magnaporthe*, which are abundant in both environments, were differentially abundant in both surface water and sediment. These findings suggest that salinity is a critical factor influencing the ecological dynamics of fungal communities. Below, we discuss the ecological implications of this salinity divide for fungal communities using FUNGuild to determine the putative roles of these fungi in the marsh.

Dikarya

Ascomycota is the largest fungal phylum, with over 64,000 known species [171]. It dominates most marsh ecosystems, contributing up to 95% of the fungal community composition [21, 76, 77, 94, 161], and is a predictor of carbon, nitrogen, and phosphorus cycling processes in salt marshes [76, 77]. Most Ascomycota fungi are metabolically versatile saprotrophs that secrete extracellular enzymes to break down complex organic matter [159]. *Aniptodera sp., Nectria sp., Kirschsteiniothelia sp., Pyrenochaeta sp.,* and *Mycosphaerella sp.* showed higher in both relative and absolute abundances in brackish than salt marsh sediments (Fig. 10, Supplementary Figure S12). These genera have been linked to the early stages of *Spartina* leaf decay [6, 18, 82]. For example, *Aniptodera sp.* averaged ~ 113 GE/g in brackish marsh sediment compared to 0 GE/g or mL in salt marsh sediment (Wilcoxon rank-sum test, $W = 35$, $p = 0.004$), while *Pyrenochaeta sp.* and *Kirschsteiniothelia sp.* were ~ 23,131 and ~ 11,463 GE/g, respectively, in brackish sediments but dropped to ~ 338 and ~ 33 GE/g in salt marsh sediments (Wilcoxon rank-sum test, $W = 32$, $p = 0.1$ (*Pyrenochaeta sp.*; $W = 34$, $p = 0.02$ (*Kirschsteiniothelia sp.*); Supplementary Figure S12). Conversely, *Lulworthia sp.*, *Ophiosphaerella sp.,* and *Ramulispora sp.* were differentially more abundant in salt marsh sediments (Fig. 10), suggesting these saprotrophic fungi may be better adapted to saline conditions. For instance, *Ramulispora sp.* had a mean abundance of ~ 19,399 GE/g in salt marsh sediments but only ~ 588 GE/g in brackish sediment, indicating some genera may thrive under a range of salinity conditions (Wilcoxon rank-sum test, $W = 17$, $p = 0.7$, Supplementary Figure S12).

Plant diversity plays a key role in shaping the abundance and richness of pathogenic fungi. In our study, we detected a greater abundance of pathogenic fungi, such as *Pithomyces* sp. and *Magnaporthe* sp., in brackish marsh sediment than in salt marshes (Fig. 10, Supplementary Figure S12). *Pithomyces* sp. had ~ 10,702 GE/g in the brackish marsh sediments and ~ 332 GE/g in the salt marsh sediments (Wilcoxon rank-sum test, $W = 28$, $p = 0.3$). *Magnaporthe* sp. was differentially abundant in the brackish marsh surface water (Fig. 10) and did not have sufficient qPCR results. This difference is likely due to the greater plant diversity in the brackish marsh, which offers a wider range of potential hosts for these fungi. In contrast, the monoculture of *Spartina alterniflora* in salt marshes limits fungal host availability, resulting in lower pathogenic fungal abundance [16, 26, 92, 177]. Previous studies have consistently shown that diverse plant communities create more opportunities for pathogen–host interactions, increasing the diversity and richness of pathogens, including fungi, across ecosystems [16, 92].

Fig. 10 The normalized abundance (counts per million, calculated by EdgeR) of the 32 most abundant operational taxonomic units (rows) that were differentially abundant across low (< 10 ppt), middle (10–25 ppt), and high (> 25 ppt) salinity ranges. The data are from all samples (columns) collected from North Inlet–Winyah Bay salt and brackish marsh sediment and surface water via the 28S primer

This highlights the strong link between plant diversity and microbial community structure, particularly in estuarine environments.

Basidiomycota, the second-largest fungal phylum, includes approximately 40,000 species, including mushrooms, plant pathogens, symbiotic fungi, and saprotrophic fungi [61]. In our study, Basidiomycota were present in all the salt and brackish marsh samples, particularly in the sediments from June 2020 to November 2021. Among the Basidiomycota, Agaricomycetes was

the dominant class across both the sediment and surface water samples during most seasons (Supplementary Figure S13). Agaricomycetes are well known for their ability to degrade plant material, particularly lignin and cellulose, which are the toughest components of plant cell walls [45]. This class includes a wide range of saprotrophic fungi, such as mushrooms and polypores, that play crucial roles in nutrient cycling by breaking down organic matter [45]. In salt and brackish marshes, where plant detritus is abundant, Agaricomycetes likely contribute significantly to the decomposition of plant material, thus driving carbon cycling and organic matter turnover. All the differentially abundant genera within the Basidiomycota phylum were from the Agaricomycetes class (Fig. 10). However, in November 2021, Tremellomycetes became the dominant class in sediments at CB and OL, indicating possible seasonal shifts in fungal community composition (Supplementary Figure S13). Despite this, the consistent presence of Agaricomycetes highlights their essential ecological function as decomposers in these marsh ecosystems.

Cryptococcus sp. was more abundant in salt marsh sediments (~6720 GE/g on average in the salt marsh and ~1116 GE/g on average in the brackish marsh; Wilcoxon rank-sum test, $W = 14$, $p = 0.4$; Supplementary Figure S12), whereas *Datronia sp.* (~134 GE/g on average in the brackish marsh and ~63 GE/g on average in the salt marsh; Wilcoxon rank-sum test, $W = 27$, $p = 0.4$; Supplementary Figure S12) and *Rhizopogon sp.* (~3353 GE/g on average in the brackish marsh and ~717 GE/g on average in the salt marsh; Wilcoxon rank-sum test, $W = 28$, $p = 0.4$; Supplementary Figure S12) were more abundant in brackish marsh sediments (Fig. 10). *Cryptococcus sp.* and *Datronia sp.* are saprotrophic fungi that degrade leaf and woody material [100, 118]. *Rhizopogon sp.*, a symbiotrophic fungus associated with *Pinaceae* species, likely originates from nearby coastal pine forests (dominated by *Pinus taeda*) [5, 100, 118]. The presence of *Rhizopogon* and *Datronia* DNA in brackish marsh sediments may represent spores from nearby pine trees. Additionally, *Malasseziaceae*, commonly found in marine environments [7], was detected at low abundance across all primers used in this study (average relative abundance: 0.0004% from ITS, 0.32% from 28S, and 0.33% from 18S; Supplementary Figures S14 & S15).

Early diverging fungi

Zoopagomycota was the dominant EDF phylum in many brackish marsh samples, particularly in June and August 2020, in both sediment and surface water (Fig. 6). Entomophthorales was the dominant Zoopagomycota order in North Inlet–Winyah Bay (Supplementary Figures S8). Zoopagomycota are nonzoosporic fungi (i.e., they do not produce free-swimming spores) [98], and most are animal parasites, although some are saprotrophs [118]. We found five *Basidiobolus* OTUs that were significantly more abundant in brackish marsh sediment (~38,925 GE/g; Supplementary Figure S12) than in salt marsh sediment (~213 GE/g; Wilcoxon rank-sum test, $W = 39$, $p = 0.003$; Supplementary Figure S12) (Fig. 10). *Basidiobolus sp.* are often saprotrophic fungi known to degrade plant material [100, 118]. Tidal marshes are among the most productive ecosystems worldwide [104], often releasing large accumulations of dead organic material to the surrounding surface waters and sediment (e.g., wrack; [57]). In this system, *Basidiobolus* may contribute to the breakdown of fibrous plant tissues [65, 84], such as leaf litter and rhizomes. Their activity could be integral to early stages of detrital decay and carbon turnover in brackish marsh sediment, supporting microbial food webs and nutrient recycling. Previous studies have also revealed *Basidiobolus sp.* to be dominant in North Carolina coastal sediments, albeit in euhaline settings [116].

Chytridiomycota were present in all samples collected from NI-WB but were particularly prominent in the sediment of the brackish marsh in August 2020 and in the surface water of the brackish marsh in June 2020 and February 2021 (Fig. 6; Supplementary Figure S16). Some lineages, particularly those in Blastocladiomycota and Chytridiomycota, are known to cause infectious diseases in various hosts, including phytoplankton (particularly diatoms and Cyanobacteriota), amphibians, and salt-marsh plants [43, 48, 53, 125, 142]. Chytridiomycota are abundant in many coastal ecosystems [34]. The samples taken in June and August 2020, shortly after algal blooms (Fig. 2), likely provided a host for these Chytridiomycota. *Entophlyctis* sp. are known to parasitize algae [100, 118]. Two *Entophlyctis sp.* OTUs were significantly more abundant in the brackish marsh (~3583 GE/g or mL in the brackish marsh and ~4 GE/g or mL in the salt marsh; Wilcoxon rank-sum test, $W = 30$, $p = 0.02$; Supplementary Figure S12, Fig. 10). *Entophlyctis sp.* have been previously found to parasitize algae in various freshwater systems [133, 139]. *Entophlyctis sp.* OTUs may be parasitizing algae in NI-WB.

Spizellomyces sp. is commonly a saprotrophic fungus known for degrading pollen [100, 118], with approximately 29% of known Chytridiomycota being pollen saprotrophs [118]. One *Spizellomyces sp.* OTU was abundant in the brackish marsh sediment (~118,700 GE/g in the brackish marsh sediment and ~0 GE/g in the salt marsh sediment; Wilcoxon rank-sum test, $W = 25$, $p = 0.3$; Supplementary Figure S12). In this estuarine system, *Spizellomyces* may contribute to the seasonal turnover of pollen that accumulates in marsh sediment from inflowing rivers [15, 24, 96]. Its increased abundance in the brackish

site suggests that pollen inputs, potentially from a more diverse plant community, provide a specialized niche for this taxon. *Spizellomyces* has been previously found to degrade pollen in soils [46, 58] and found in marshland sediments [58]. *Oedogoniomyces sp.*, another likely saprotrophic fungus known for degrading plant and algal material [100, 118], was also significantly more abundant in the brackish marsh than in the salt marsh (~46,320 GE/g or mL in the brackish marsh and ~103 GE/g or mL in the salt marsh,Wilcoxon rank-sum test, $W=38$, $p=0.006$; Supplementary Figure S12) (Fig. 10). Brackish marshes support more diverse plant ecosystems than salt marshes do [5, 8, 76, 77, 145, 146], which results in a wider variety of organic matter inputs (e.g., decaying leaves, roots, pollen, and other plant debris). Saprotrophic fungi, such as *Spizellomyces* sp. and *Oedogoniomyces* sp., thrive on organic matter, including macrophyte and algal material. A more diverse plant community produces a richer and more varied substrate for these fungi to degrade, leading to their increased abundance in the brackish marsh.

Blastocladiomycota were present in most of the sediment and surface water samples from NI-WB, with a particularly high relative abundance in the brackish marsh surface water in August 2020 and February 2021 (Fig. 6). The family *Catenariaceae* was the dominant Blastocladiomycota group identified in NI-WB (Supplementary Figures S9). Blastocladiomycota are primarily found in soil and freshwater environments as saprotrophs and parasites of invertebrates, plants, algae, oomycetes, and other blastoclads [121]. Fewer than 200 species of Blastocladiomycota have been documented [95]. Three *Catenomyces sp.* OTUs were more abundant in the sediment and surface water of the brackish marsh than in those of the salt marsh (~25,222 GE/g or mL in the brackish marsh and ~27 GE/g or mL in the salt marsh; Wilcoxon rank-sum test, $W=33$, $p=0.06$; Supplementary Figure S12). *Catenomyces sp.* are often saprotrophs that degrade plant material in soil ecosystems [100, 118]. Since Blastocladiomycota are found primarily in freshwater systems, we hypothesize that they may outcompete Ascomycota for organic matter in low-salinity environments.

Mucoromycota were highly abundant in brackish marsh sediment in February 2021 (Fig. 6). *Diversisporaceae* was the dominant Mucoromycota family in the brackish sediment in February 2021 (Supplementary Figure S17), whereas *Mucoraceae* (Supplementary Figure S12) and *Endogonaceae* (Supplementary Figure S12) dominated the remaining samples (Supplementary Figure S18). One *Diversispora sp.* OTU was abundant in the brackish marsh sediment (~167,594 GE/g in the brackish marsh sediment and ~0 GE/g in the salt marsh sediment;Wilcoxon rank-sum test, $W=23.5$, $p=0.5$; Fig. 10; Supplementary Figure S12). *Diversispora sp.* are typically endosymbiotrophs that form symbiotic relationships with plant roots [100, 118]. Mucoromycota are primarily soil saprotrophs, comprising approximately 80% of the group [118], although 1.5% are known to be endophytes [118].

While EDFs have long been understudied in coastal marshes compared to Dikarya, our results suggest that EDFs, including Zoopagomycota, Chytridiomycota, Blastocladiomycota, and Mucoromycota, likely contribute critical but poorly characterized functions in brackish and salt marsh ecosystems. These phyla dominate the fungal community (Figs. 5, 6). Specifically, they may act as saprotrophs degrading complex plant and algal material (e.g., *Basidiobolus*, *Spizellomyces*, *Oedogoniomyces*, *Catenomyces*), parasites regulating algal populations (e.g., *Entophlyctis*), and symbiotrophs forming root associations (e.g., *Diversispora*) [100, 118]. Many of these EDFs remain uncultured and poorly described, but using FUNGuild [100, 118], we inferred their likely ecological roles and functional guilds based on taxonomic identity. These functional roles would directly influence carbon turnover, nutrient release, and food web dynamics, especially in the brackish marsh with higher plant diversity and more varied organic inputs. By explicitly linking EDF taxonomic diversity to these key ecosystem processes, our study helps fill an important functional knowledge gap and underscores the need for deeper experimental and cultivation-based work to validate the ecological roles of these mostly uncultured EDF lineages in estuarine ecosystems.

Conclusions

This study shows that the primer set targeting the D1 region of the 28S large subunit rRNA gene can identify a broader and richer marine fungal community, including many EDF, in salt and brackish marsh surface water and sediments than the commonly used ITS2 primer set. This difference likely stems from gaps in EDF representation in ITS2 databases [11] and the bias of the ITS2 primer set toward terrestrial Dikarya [7]. This study represents a significant advancement in our understanding of marine fungal diversity by demonstrating that the 28S large subunit rRNA primer set can reveal a richer community of fungi, particularly EDF lineages, in salt and brackish marshes. Phylogenetic analyses and copy-normalized qPCR confirmed that EDF are key contributors to the distinct fungal communities between brackish and salt marshes, with notably higher EDF cell abundances in brackish sediments. Meanwhile, the ITS2 region revealed the unexpected presence of several putative denitrifying fungi, including *Trichoderma* and *Purpureocillium* species, emphasizing its value for detecting functional guilds related to nitrogen cycling. Together, these

metabarcoding and qPCR results underscore the overlooked saprotrophic and functional roles of EDF in estuarine ecosystems and demonstrate the complementary strengths of each marker. Collectively, these results demonstrate that the 28S large subunit rRNA primer set is a more robust and comprehensive tool for characterizing the full extent of marine fungal diversity, particularly for uncovering EDF lineages that remain underrepresented by ITS2-based approaches.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00786-3.

Supplementary Material1.

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Madeleine A. Thompson: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; Birch Maxwell Lazo-Murphy: Methodology, Writing – original draft, Writing – review & editing; Bruce W. Pfirrmann: Methodology, Project administration, Resources, Writing – review & editing; William H.J. Strosnider: Methodology, Project administration, Resources, Writing – review & editing; James L. Pinckney: Methodology, Resources, Writing – review & editing; Xuefeng Peng: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Data availability
Raw reads generated in this study are available at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA1158776 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1158776).

Declarations

Competing interests
The authors declare no competing interests.

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