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A High-Resolution Time Series Reveals Distinct Seasonal Patterns of Planktonic Fungi at a Temperate Coastal Ocean Site (Beaufort, North Carolina, USA)

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ABSTRACT There is a growing awareness of the ecological and biogeochemical importance of fungi in coastal marine systems. While highly diverse fungi have been discovered in these marine systems, still, little is known about their seasonality and associated drivers in coastal waters. Here, we examined fungal communities over 3 years of weekly sampling at a dynamic, temperate coastal site (Pivers Island Coastal Observatory [PICO], Beaufort, NC, USA). Fungal 18S rRNA gene abundance, operational taxonomic unit (OTU) richness, and Shannon's diversity index values exhibited prominent seasonality. Fungal 18S rRNA gene copies peaked in abundance during the summer and fall, with positive correlations with chlorophyll *a*, SiO_4, and oxygen saturation. Diversity (measured using internal transcribed spacer [ITS] libraries) was highest during winter and lowest during summer; it was linked to temperature, pH, chlorophyll *a*, insolation, salinity, and dissolved inorganic carbon (DIC). Fungal communities derived from ITS libraries were dominated throughout the year by *Ascomycota*, with contributions from *Basidiomycota*, *Chytridiomycota*, and *Mucoromycotina*, and their seasonal patterns linked to water temperature, light, and the carbonate system. Network analysis revealed that while cooccurrence and exclusion existed within fungus networks, exclusion dominated the fungus-and-phytoplankton network, in contrast with reported pathogenic and nutritional interactions between marine phytoplankton and fungi. Compared with the seasonality of bacterial communities in the same samples, the timing, extent, and associated environmental variables for fungi community are unique. These results highlight the fungal seasonal dynamics in coastal water and improve our understanding of the ecology of planktonic fungi.

IMPORTANCE Coastal fungal dynamics were long assumed to be due to terrestrial inputs; here, a high-resolution time series reveals strong, repeating annual patterns linked to *in situ* environmental conditions, arguing for a resident coastal fungal community shaped by environmental factors. These seasonal patterns do, however, differ from those observed in the bacterioplankton at the same site; e.g., fungal diversity peaks in winter, whereas bacterial diversity maxima occur in the spring and fall. While the dynamics of these communities are linked to water temperature and insolation, fungi are also influenced by the carbonate system (pH and DIC). As both fungi and heterotrophic bacteria are thought to be key organic-material metabolizers, differences in their environmental drivers may offer clues as to which group dominates secondary production at this dynamic site. Overall, this study suggests the unique ecological roles of mycoplankton and their potentially broad niche complementarities to other microbial groups in the coastal ocean.

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As a key component of the biosphere, fungi fulfill a wide range of important biogeochemical and ecological functions in terrestrial and aquatic ecosystems (1–3). While fungal diversity and abundance in soils, plant-associated habitats, and freshwater environments have been well-documented (4–12), their ecological role and environmental drivers remain poorly characterized in marine ecosystems.

Planktonic marine fungi have long been known to exist as unicellular forms (e.g., marine yeasts), parasites of other organisms, and saprophytes attached to particulate organic matter (13–16). However, their metabolically active living forms, i.e., mycelia, have only recently been reported in the ocean water column (15–17). Currently, the predicted biogeochemical roles of marine fungi, including decomposition of detrital organic matter, are largely extrapolated from those of their terrestrial and freshwater counterparts (17, 18), with some direct evidence for extracellular enzymatic activity and the capacity to hydrolyze organic polymers (19).

In spite of a need for more clarity about the biogeochemical roles of marine fungi, their biomass has been reported to be comparable to that of bacterioplankton, suggesting they are understudied relative to their potential biogeochemical importance in coastal ecosystems (14). Since fungi were first cultured from the oceans several decades ago, they have been found throughout the oceans, from the deep sea to pelagic waters off the Northwest Antarctic Peninsula (20–25). And yet, due to the limitations of cultivation methods, only a few hundred fungal species have been isolated from the ocean water column, primarily members of the phyla *Ascomycota*, *Basidiomycota*, and *Zygomycota* (16). Previous, culture-independent molecular analyses of marine fungi have revealed both spatial diversity and novel, uncultured phylotypes of planktonic fungi in the ocean waters. Molecular diversity estimates also suggested that marine fungal diversity was low compared to that in other ecosystems, but this lower diversity could also be due to undersampling or other methodological biases, including primer choice and DNA extraction method (17, 26, 27). Nevertheless, in general, surface coastal waters appear to contain greater diversity than open-ocean samples (13, 14, 26). Based on these studies, fungal diversity appears to be linked to phytoplankton (e.g., primary productivity and specific phytoplankton taxa), nutrients (nitrate, nitrite, orthophosphate, and silicic acid), and temperature (16, 18). Thus, the limited available evidence suggests that marine planktonic fungi can be an important, metabolically active component of water column microbial communities (13, 14, 16, 18, 26). However, identifying the linkages between planktonic fungal abundance and diversity and environmental variables remains an important challenge to clarify the ecology of these organisms in coastal marine ecosystems.

High-resolution time series can assess microbial responses to environmental changes, as they both span the range of seasonal environmental conditions and allow measurement of time-lagged biological responses, thus potentially providing clues about physical, chemical, or biological forcing on microbial community structure (28, 29). In this study, we present data for fungal diversity and dynamics from 3 years of weekly samples from a coastal, mesotrophic ocean site (the Pivers Island Coastal Observatory [PICO]), located at the Beaufort Inlet, Beaufort, NC, USA, where water from the Newport River Estuary mixes with waters from the coastal Atlantic Ocean (30). Thus, this site integrates both marine and terrestrial influences on mycoplankton. This location is adjacent to the second largest estuarine system on the U.S. East Coast (Neuse-Pamlico) and situated in a temperate environment (30). This site was chosen due to the availability of a long-term, weekly monitoring station (2011 to present) with comprehensive metadata. Inferences can be drawn regarding how mycoplankton assemblages may potentially interact with the environment, as well as with other groups of organisms, using the corresponding time series data for bacterioplankton and phytoplankton at PICO through an association network approach. The goals of the current study were to understand the seasonal variability of mycoplankton, to identify

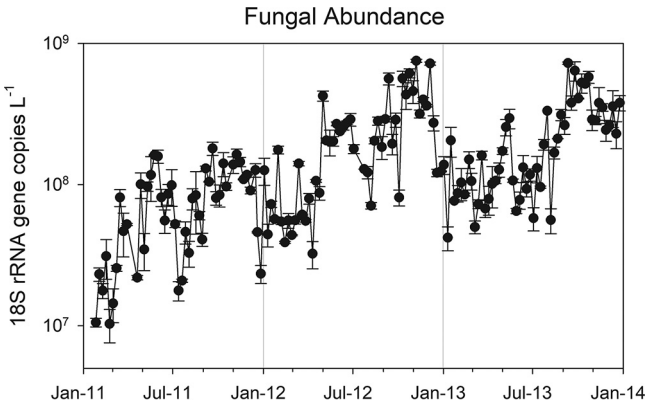

FIG 1 Seasonal changes in fungal abundance estimated by using fungus-specific 18S rRNA gene Q-PCR analysis of weekly environmental DNA samples at Pivers Island Coastal Observatory (PICO) from 3 years of observations (2011 to 2013).

environmental factors affecting mycoplankton communities, and to compare fungal diversity and dynamics with those of other microbial communities at a coastal ocean time series (PICO).

RESULTS

Fungal abundance and diversity. Fungal abundance was estimated by using fungal 18S rRNA gene quantitative PCR (Q-PCR) analysis of DNA samples, and fungal 18S rRNA gene sequence abundances at PICO generally ranged from 0.10×10^8 to 7.54×10^8 copies liter^{-1} and exhibited an annual pattern (Fig. 1). Two peaks in abundance were observed yearly during the summer and fall, but the timing varied from year to year, with the highest fungal 18S rRNA gene sequence abundances observed in 2012 and 2013. The dynamic patterns of fungal 18S rRNA gene sequence abundances were significantly correlated with chlorophyll *a* ($r = 0.393$), SiO_4 ($r = 0.318$), and oxygen saturation ($r = 0.215$) ($n = 148$, $P < 0.01$) (see Table S1 in the supplemental material).

To investigate the diversity of these fungi, the internal transcribed spacer (ITS) region was amplified (31, 32), sequenced, and analyzed. Of the 14,535 operational taxonomic units (OTUs), 2,796 (19%) could be linked to known cultured and uncultured fungi, representing 27 classes and 84 orders from 5 phyla (*Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Glomeromycota*, and *Neocallimastigomycota*) and 4 subphyla (*Mucoromycotina*, *Kickxellomycotina*, *Entomophthoromycotina*, and *Mortierellomycotina*) (Fig. S1). Using the entire data set of known and potential fungi, the OTU richness and Shannon's diversity were highest during winter and lowest during summer (Fig. 2), and both were significantly correlated with a range of environmental parameters ($n = 144$, $P < 0.01$), including temperature ($r = 0.731$ and $r = 0.630$, respectively), pH ($r = 0.598$ and $r = 0.533$), salinity ($r = 0.347$ and $r = 0.390$), insolation ($r = 0.416$ and $r = 0.418$), chlorophyll *a* ($r = 0.454$ and $r = 0.324$), and dissolved inorganic carbon (DIC) ($r = 0.298$ and $r = 0.316$), suggesting the importance of environmental factors in shaping fungal community structure at PICO (Table S1).

Fungal community seasonality and environmental drivers. The fungal communities at PICO were dominated throughout the year by *Ascomycota*, with contributions from *Basidiomycota*, *Chytridiomycota*, and *Mucoromycotina*, and their relative abundances exhibited seasonal patterns throughout the time series (Fig. 3). *Basidiomycota* was absent at some time points and displayed peaks associated with declines in the relative abundance of *Ascomycota*, while *Chytridiomycota* and *Mucoromycotina* mostly occurred in winter (Fig. 3). Consistent with trends in community diversity (Fig. 2), winter communities at PICO were composed of the most diverse composition of fungal phyla/subphyla, while the summer communities were dominated by the phylum *Ascomycota* (Fig. 3). At higher taxonomic resolution, within the *Ascomycota*, *Pleosporales*, *Capnodiales*, and *Hypocreales* were the most ubiquitous orders (Fig. 4), with

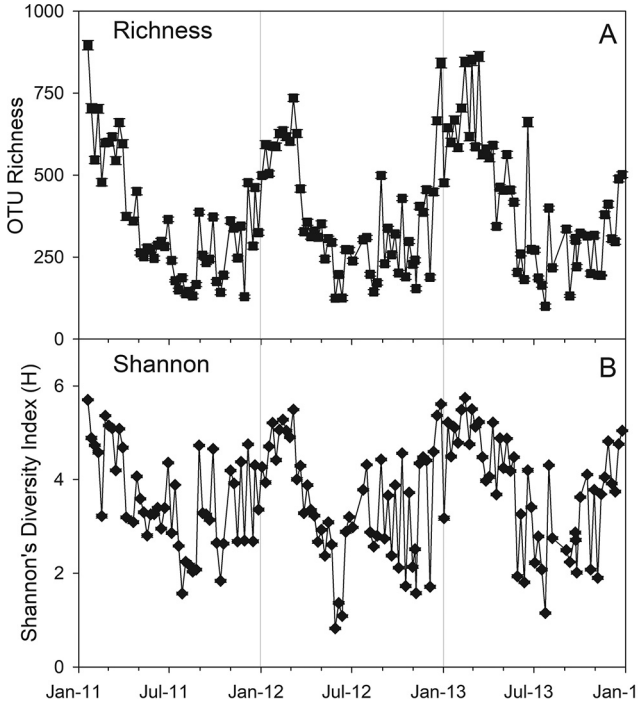

FIG 2 Seasonal changes in fungal internal transcribed spacer (ITS) OTUs with 96% identity from 3 years of weekly environmental sampling at the Pivers Island Coastal Observatory.

striking contributions from *Hypocreales* during the periods of May 11 to June 8, June 29 to July 6, and July 20 in 2011. Finally, in the phylum *Chytridiomycota*, *Rhizophydiales* was the most abundant order and was highest in relative abundance during the winter (Fig. 4).

In order to uncover the key factors driving the dynamics of fungi in the coastal ocean, changes in relative abundance at the phylum/subphylum level in relation to

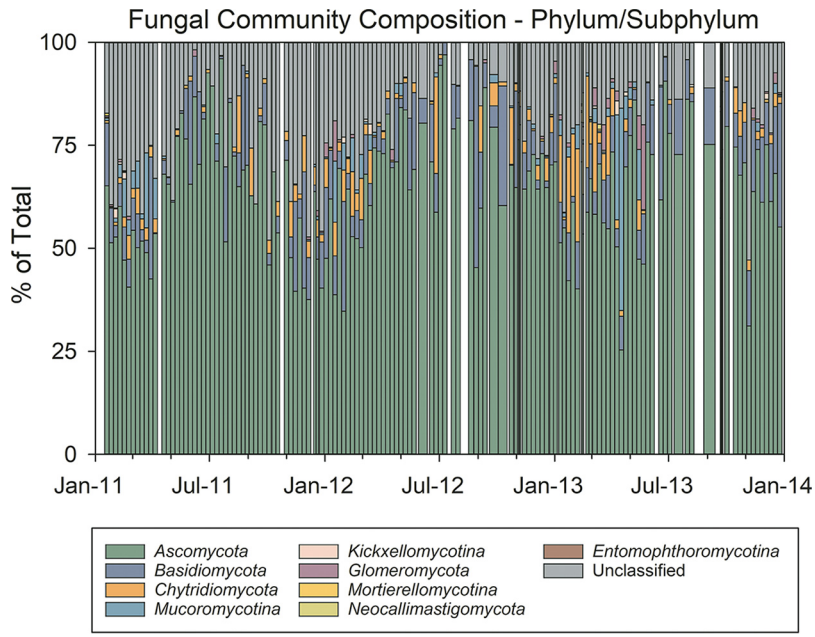

FIG 3 Seasonal changes in OTUs identified as fungi classified at phylum/subphylum level over 3 years of weekly environmental sampling at the Pivers Island Coastal Observatory (PICO). The data shown are rarified to 500 sequences per library at each time point.

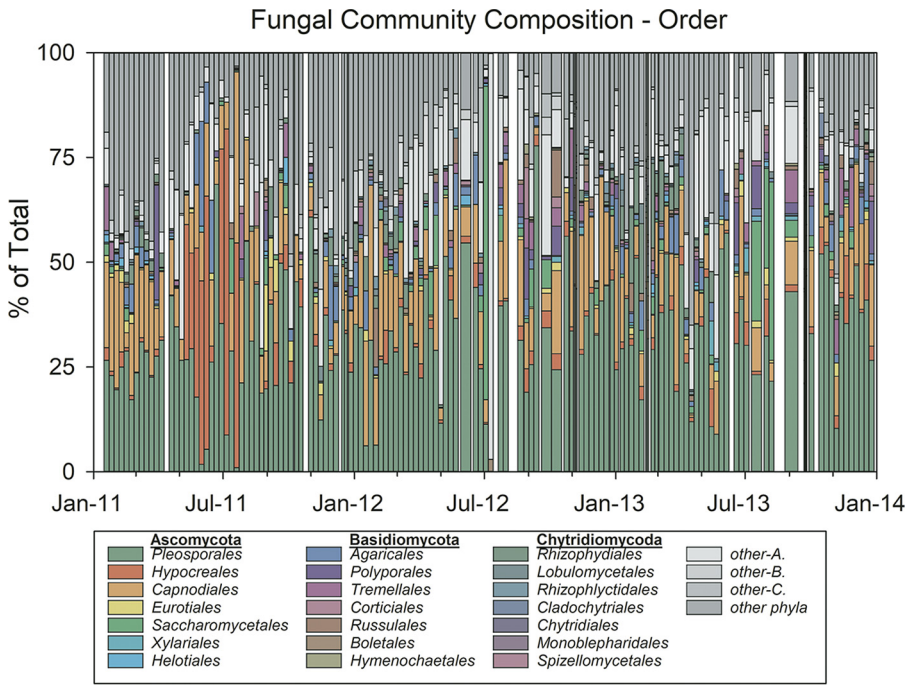

FIG 4 Seasonal changes in OTUs identified as fungi classified at the order level over 3 years of weekly sampling at the Pivers Island Coastal Observatory (PICO). The data shown are rarified to 500 sequences per library at each time point.

environmental variables were investigated. Interestingly, phyla/subphyla appeared to respond to distinct environmental variables. For example, the relative abundance of *Ascomycota* was significantly correlated with salinity ($r = 0.294$), temperature ($r = 0.522$), insolation ($r = 0.469$), chlorophyll *a* ($r = 0.263$), and pH ($r = 0.275$) ($n = 134$, $P < 0.01$) (Table S2.1), while the abundance of *Chytridiomycota* was significantly related to salinity ($r = 0.288$), temperature ($r = 0.338$), and insolation ($r = 0.265$) ($n = 134$, $P < 0.01$) (Table S2.1). The abundances of *Kickxellomycotina* ($n = 134$, $P < 0.01$) and *Mucoromycotina* ($n = 134$, $P < 0.01$) were significantly correlated with temperature ($r = 0.230$ and $r = 0.181$) and pH ($r = 0.211$ and $r = 0.255$), respectively (Table S2.2). Other groups, such as *Basidiomycota*, *Glomeromycota*, and *Entomophthoromycotina*, were not significantly correlated with any of the environmental variables measured at PICO (Tables S2.1 and S2.2).

Although the fungal community generally followed a repeating annual pattern, the winter-summer community transition is shown on the first redundancy analysis (RDA) axis, while the late-winter–early-spring and late-fall–early-winter communities are separated along the second RDA axis (Fig. 5). To identify the environmental factors driving transitions between these communities, we compared changes in community composition to a constrained set of environmental variables. Although the first two axes only explained 25% of the community variation, surface water temperature, insolation, pH, and dissolved inorganic carbon were significantly associated with the changes of fungal communities ($P < 0.01$) (Fig. 5). In comparison, the bacterial community composition in these same samples was significantly associated with temperature and light (isolation), as for the fungi, but also salinity (32).

Cooccurrence network of fungi and environmental variables. In order to explore potential drivers of the observed diversity and dynamics, a cooccurrence network was constructed for the fungal community and environmental variables. After removing OTUs observed in less than one-third of the samples, 134 OTUs, representing 44.76% of the total sequences, were included in this analysis. The resulting network was composed of 71 nodes, including 68 OTUs (11 culturable fungi, 25 uncultured fungi, and 32 potential fungi) and 3 environmental parameters (Fig. 6). The network reveals potential

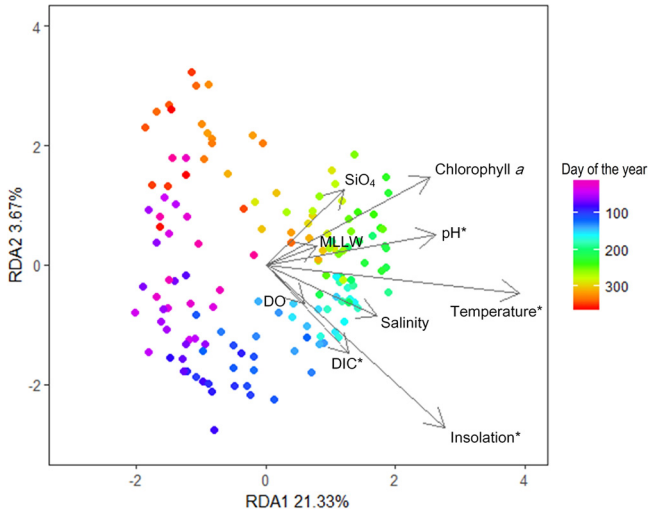

FIG 5 Redundancy analysis (RDA) biplot linking fungal community composition to environmental variables for each sample at PICO over 3 years of weekly sampling (2011 to 2013). Each circle represents the fungal community composition at a specific time point. Environmental variables marked with asterisks are statistically significant ($P < 0.01$), as assessed by the marginal effects of terms. DO, dissolved oxygen (oxygen saturation); MLLW, mean lower low water (tidal height); DIC, dissolved inorganic carbon.

associations of individual OTUs with environmental factors. For example, OTU-21 (uncultured fungus) was closely related to insolation and OTU-23 (potential fungus) with chlorophyll *a*; overall, 15 OTUs were negatively correlated with temperature, again highlighting the importance of temperature in determining fungal community structure at PICO. Furthermore, the network revealed two main clusters (Fig. 6). The first cluster contained 29 nodes, which included 20 OTUs of potential fungi, 3 OTUs of uncultured fungi, and 6 OTUs of cultured fungi (*Ascomycota*). Although in this cluster, most associations were cooccurrences (Fig. 6, green lines), some potential competition/mutual exclusion relationships (Fig. 6, red lines) were observed. The second cluster consisted of 39 nodes, which included 12 OTUs of potential fungi, 22 OTUs of uncultured fungi, and 5 OTUs of cultured fungi (four *Ascomycota* and one *Chytridiomycota*). Within this cluster, competition/mutual exclusion relationships dominated (Fig. 6, red lines) between OTU-112 (uncultured *Agaricomycetes* in the *Basidiomycota*) and 34 other OTUs, suggesting that OTU-112 could play a keystone role in the fungal community at PICO, with the most edges in the network (33, 34). In contrast, other OTUs (e.g., OTU-14 [potential fungus], OTU-43 [*Dothideomycetes* in the *Ascomycota*], and OTU-89 [uncultured *Chytridiomycetes*]) had only one neighbor, and thus, they were not predicted to interact with other fungal OTUs to shape the fungal community.

Cooccurrence network of fungi and phytoplankton. As fungi have been previously linked to phytoplankton, a cooccurrence network was constructed for both of these taxa to reveal potential relationships. After removing OTUs observed in less than one-third of the total samples, 134 fungal OTUs (representing 44.76% of the total sequences) and 335 phytoplankton OTUs (representing 91.29% of the total sequences) were included in this analysis. The resulting network was composed of 223 nodes, including 57 OTUs of fungi (45 cultured/uncultured and 12 potential fungi) and 166 OTUs of phytoplankton (Fig. 7). Phytoplankton nodes (OTUs) included 109 *Stramenopiles*, 23 *Synechococcales*, 13 *Cryptophyta*, 8 *Haptophyceae*, 6 unassigned chloroplasts, 5 *Chlorophyta*, 1 member of *Chroococcales*, and 1 unassigned cyanobacterium. The majority (92 of 146 edges) of associations between fungi were competition/mutual exclusion (Fig. 7, red lines), while cooccurrences (Fig. 7, green lines) were dominant within the phytoplankton (403 of 678 edges). Although most edges between fungi and phytoplankton were competition/mutual exclusion (Fig. 7, red lines) (498 edges), cooccurrence (Fig. 7, green lines) (12 edges) did occur between fungi (5 OTUs of

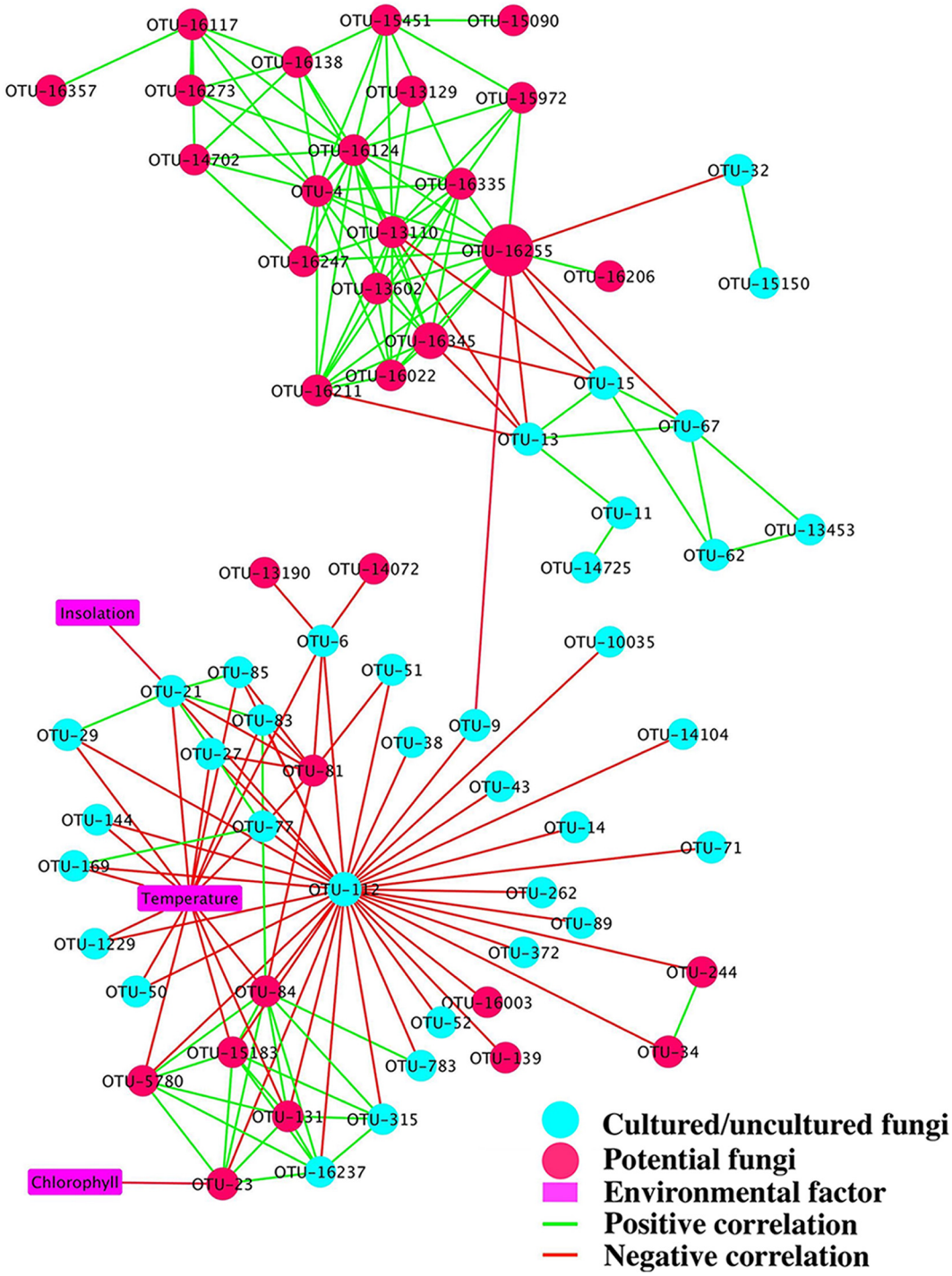

FIG 6 Network of identified and predicted new fungal OTUs and environmental variables at PICO. The OTUs chosen in this analysis were observed in more than one-third of the total sampling time points, allowing us to examine core interactions in the coastal ocean.

uncultured fungi and 5 OTUs of potential fungi) and phytoplankton (7 OTUs of *Synechococcales* and 5 OTUs of *Stramenopiles*). Within the competition/mutual exclusion relationships, phytoplankton OTU-95 (*Stramenopiles*) (30 edges) had the most connections with fungi and fungal OTU-6 (uncultured *Chytridiomycota*) (37 edges) the most links to phytoplankton. Furthermore, OTU-112 (uncultured *Agaricomycetes* in *Basidiomycota*) (33 edges) and OTU-84 (potential fungus) (30 edges) had more exclu-

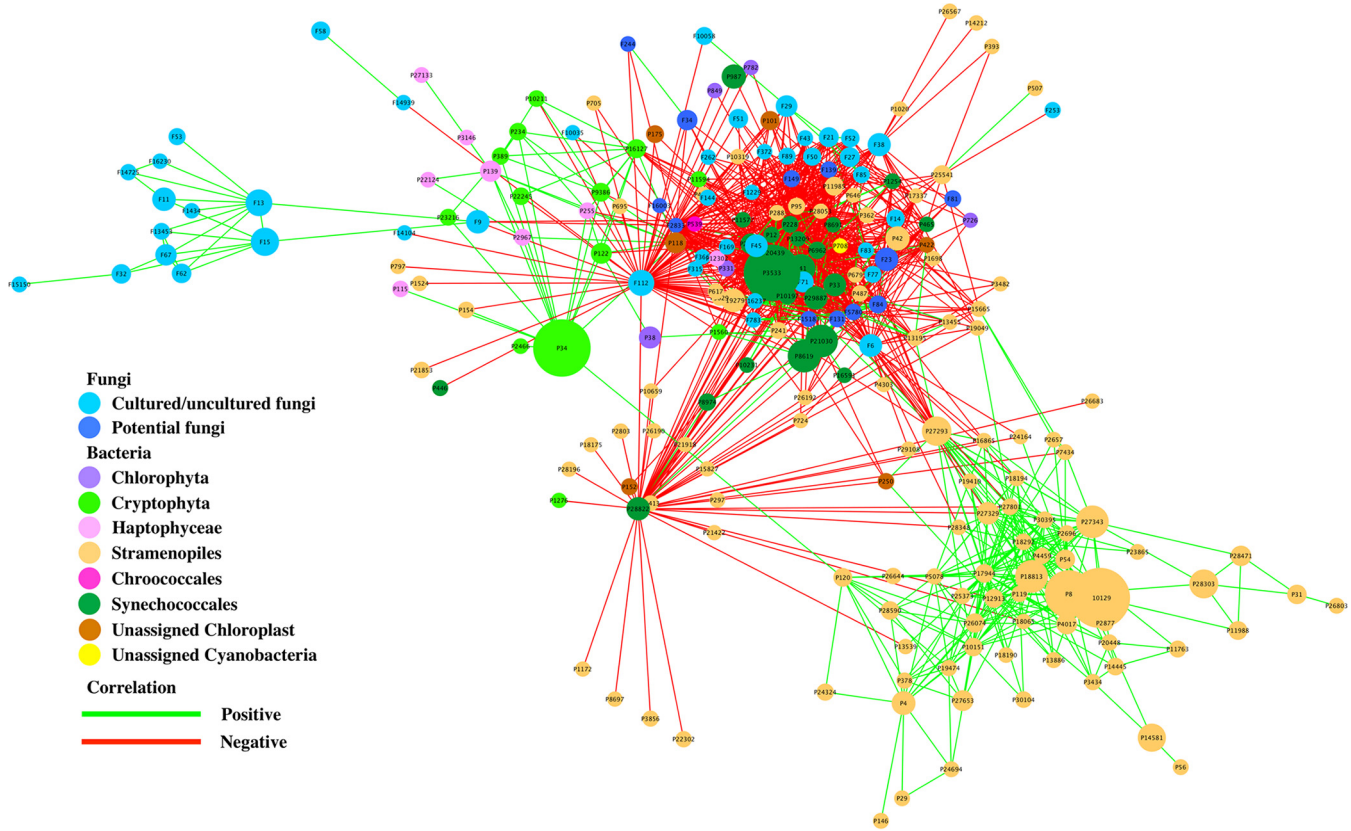

FIG 7 Network of fungal OTUs (identified by "F" and the OTU number) and phytoplankton OTUs (identified by "P" and the OTU number) at PICO. The OTUs chosen in this analysis were observed in more than one-third of the total sampling time points, allowing us to examine core interactions in the coastal ocean.

sion relationships with phytoplankton than other fungal OTUs did. The network centrality of phytoplankton OTU-95 (*Stramenopiles*) (30 edges), fungal OTU-6 (uncultured *Chytridiomycota*) (37 edges), OTU-112 (uncultured *Agaricomycetes* in *Basidiomycota*) (33 edges), and OTU-84 (potential fungus) (30 edges) could indicate the importance of interactions between fungi and phytoplankton in this system (33, 34).

DISCUSSION

Fungi found in the coastal ocean have often been assumed to be derived from terrestrial sources, but more recent culture-based and culture-independent studies have revealed the presence and potential biogeochemical importance of putative endemic coastal and open-ocean mycoplankton (27, 35). However, aside from a few notable exceptions, there is still a limited understanding of the abundance and diversity of marine fungi, their ecological niches, and their functioning in the ocean's carbon and nutrient cycles. In this time series study spanning 3 years, we have shown that the mycoplankton community displays consistent seasonal patterns, with specific fungal groups and taxa having distinct environmental correlates and associations with other marine community members, suggesting that these taxa are largely residents of the coastal ocean rather than transient terrestrial inputs.

Fungal abundance and diversity at PICO. The abundance of fungal 18S rRNA gene copies at PICO (0.10×10^8 to 7.54×10^8 18S rRNA gene copies liter^{-1}) (Fig. 1) is substantially higher than that measured in the Western English Channel (5.1×10^5 to 9.9×10^7 copies liter^{-1}) (18). Although operon number is poorly constrained in marine fungi, this metric does provide insight into fungal dynamics. PICO fungal 18S rRNA gene abundance was significantly correlated with chlorophyll *a*, SiO_4, and oxygen saturation (Table S1 in the supplemental material), whereas mycoplankton abundance in the Western English Channel was negatively correlated with salinity and positively corre-

lated with the concentrations of particulate organic carbon, ammonia, total particulate nitrogen, and particulate organic nitrogen (18). In contrast with the English Channel site, the PICO site is located at the mouth of an estuary, and thus, a larger terrestrial influence is likely; however, salinity at this site is generally high, ~33 practical salinity units (PSU), potentially explaining the lack of relationship between fungal 18S rRNA gene abundance and salinity (28, 30).

Our study shows that fungi are a highly diverse group of microbes in the coastal ocean, but comprehensive databases are still lacking for this group, precluding precise identification. As observed previously (27, 36), more than 80% of our sequences could not be attributed to any named fungi in the UNITE database using BLAST, suggesting poor representation of aquatic/marine fungi in public databases that are predominantly derived from soil environments (37). For those sequences that can be assigned to specific taxa, we observed that the dominant phyla were *Ascomycota*, *Basidiomycota*, and *Chytridiomycota*, as observed previously (18, 38–43), with *Mucoromycotina* also abundant. The dominance of *Dikarya* in marine habitats is consistent with previous studies (13, 17, 39, 42, 44, 45). Many marine organisms in this clade cause or are associated with disease, and thus, they are assumed to be parasitic agents in marine environments (17). Marine yeasts belong to both the *Ascomycota* and *Basidiomycota* (46); they are common in both coastal and pelagic waters and generally associated with nutrients (47), suggesting they are also saprotrophs in the marine environment (17). As in this study, other molecular surveys have revealed an unexpectedly large diversity of *Chytridiomycota* in a number of marine habitats (39, 41, 43, 46, 48, 49), and they are often assumed to be either facultative or obligate parasites of marine algae (46, 50). *Mucoromycotina* has also been reported to be a major component of fungal communities in oxygen-depleted environments (42, 51–53), suggesting that they may have physiological adaptations to low-oxygen environments and here might be sourced from benthic resuspension. In addition to the above-named taxa, we observed that *Glomeromycota* can be abundant during spring and late autumn/early winter at this temperate site. Some *Glomeromycota* live exclusively as obligate symbionts of photoautotrophs, including not only vascular plants and bryophytes but also cyanobacteria (46, 54). Considering the nutritional benefits of these fungal symbionts to their hosts, the *Glomeromycetes* observed here could engage in symbiotic relationships with cyanobacteria or algae (55). The higher fungal diversity in this coastal time series compared with those of pelagic Pacific waters (13) and Hawaiian coastal waters (26) suggests the importance of nutrients/productivity or potentially terrestrial/freshwater inputs in supporting planktonic fungal diversity (35).

Fungal and bacterial community comparison. Our findings reveal that the fungal 18S rRNA gene copy abundance and diversity metrics in this temperate coastal site exhibit strong seasonality (Fig. 1, 3, and 4), which is consistent with a previous coastal time series study in the upwelling system off Chile (14). However, at this same site, bacterial abundance peaked in the summer, while diversity was highest in the spring and late fall, whereas fungal diversity peaked in the winter, suggesting unique ecological roles and potentially broad niche complementarity between these two groups. Similarly, the composition of the fungal community at PICO displayed seasonal patterns (Fig. 3, 4, and 5) and links to environmental variables, as observed for the bacterioplankton previously (28). Both bacterial and fungal community compositions were linked to water temperature and insolation, but fungal communities were also likely driven by changes in the carbonate system (e.g., pH and DIC) (Fig. 5), while bacterial community composition was associated with changes in salinity. In some ways, this observation is surprising, as previous studies have linked fungi to terrestrial and riverine inputs (17, 18). However, the generally high salinity at PICO, ~33 psu, means that the site is likely representative of the coastal ocean, without a strong freshwater influence.

In fungal and bacterial communities, consistent changes in the relative abundances of some dominant taxa, such as *Ascomycota* (fungi) and SAR11 (bacteria), drive the seasonal patterns in diversity at higher taxonomic levels. However, when assessed at

the finer OTU level, bacterial communities exhibit switches between taxa with strong preferences for either summer or winter (28). These microdiversity-driven seasonal patterns were not apparent in the coastal fungal community, which could be due to several possible factors, as follows: (i) the fungal community is not structured through temperature-driven fine-scale diversification, (ii) the fungal ITS sequence does not cluster sequences at the same phylogenetic resolution as bacterial 16S rRNA gene sequences, or (iii) differences in how bacterial and fungal OTUs are operationally constrained make cross-comparisons difficult. However, due to the potential linkages between fungi and other taxa, specifically phytoplankton, we sought to investigate this further using network analysis.

Association networks between fungi and between fungi and phytoplankton at PICO. Beyond simple temporal progression, our findings highlight potential interactions between fungi and between fungi and phytoplankton (Fig. 6 and 7). While the fungus-only cooccurrence network highlights the role of temperature as a key environmental driver and of OTU-122 as a potential keystone organism, we turned to the phytoplankton-fungus network to identify potential pathogenic or host relationships in this data set. Phytoplankton are the dominant primary production in marine ecosystems, constituting the base of the aquatic food web (56). They are susceptible to a number of fungal parasites (48). Some taxa of the early-diverging fungal phylum *Chytridiomycota*, mainly the members of *Chytridiomycetes* (chytrids) (50), are virulent parasites (57–62) that can attenuate phytoplankton productivity. In addition to parasitism, some studies have shown that marine fungi metabolize phytoplankton-derived organic matter (13, 14, 63). The importance of interactions in driving eukaryotic diversity has been suggested for the global ocean (64), as well as for fungi. Phytoplankton play an important role in determining mycoplankton diversity in Hawaiian coastal waters (26), and high fungal biomass and phytoplankton biomass cooccurred in the upwelling system off Chile (14). At this temperate site, fungal OTU-6 (uncultured *Chytridiomycota*) was the most connected to phytoplankton (37 coexclusion edges), which is consistent with previous studies that have showed *Chytridiomycota* to be associated with phytoplankton blooms (18, 60, 65). This group includes saprotrophs and parasites (66, 67) and, thus, may be involved in bloom termination or phytoplankton cell death. Interestingly, recent research suggests that chytrids may also play an important role in aquatic food webs by transferring nutrients between phytoplankton and zooplankton via grazing (36, 68). Similar to patterns found in the Western Pacific Warm Pool (13), at PICO, half of the phytoplankton OTUs were negatively connected with fungi, with more than 20 edges linked to *Synechococcus*. Phytoplankton undoubtedly largely support both bacterial and fungal heterotrophic communities, driving increases in abundance and seasonal changes in composition. This finding and others from recent studies necessitate revisiting the classical thinking that coastal fungi are primarily supported by allochthonous organic matter from adjacent terrestrial ecosystems (47). Overall, most predicted interactions between fungi and phytoplankton at PICO were competition/mutual exclusion, but positive cooccurrences were also observed, which could represent specific fungi acting as parasites of phytoplankton (16). Nevertheless, depending on the health of the host and environmental conditions, the relationship between a fungus and its host is delicately balanced and can switch between commensal, mutualist, and saprotroph or parasite (46).

Conclusions. This high-resolution time series study reveals novel insights into the seasonal dynamics of planktonic fungi and their associated environmental drivers in the temperate coastal ocean. Prominent seasonal patterns in the abundance and diversity of the mycoplankton community were driven by multiple environmental factors, including temperature, insolation, pH, and dissolved inorganic carbon. While some of the key variables also structure the bacterial community (e.g., temperature and light), the timing of diversity peaks and taxon transitions differed between the two microbial groups. Additionally, although cooccurrence networks cannot be used to determine interactions, they did identify a number of negative correlations between specific

mycoplankton and phytoplankton, consistent with fungal diatom parasitism (69). Overall, the findings of this study highlight the intricate dynamic relationship of mycoplankton with environmental factors and other plankton communities (i.e., bacterioplankton and phytoplankton) in the coastal marine system.

MATERIALS AND METHODS

Seawater sampling and environmental metadata. A single seawater sample was taken for each time point as part of the Pivers Island Coastal Observatory (PICO) time series, located in Beaufort, NC, USA (34.7181°N, 76.6707°W), weekly between 2011 and 2013. About 1 liter of near-surface seawater (1 m) was filtered through 0.22-μm Sterivex filter units (Millipore), and the resulting filters stored at −80°C until DNA extraction. The environmental metadata were collected at the same time as the seawater samples for DNA extraction. Methods for determination of surface water temperature, pH, salinity, dissolved inorganic nutrient concentrations, chlorophyll *a* concentration, dissolved inorganic carbon, tidal height (mean lower low water), insolation, and oxygen saturation were as described previously (28, 30).

DNA extraction, PCR, and sequencing analysis. Nucleic acids were extracted as described previously (28, 70), using physical lysis with bead beating coupled with phenol-chloroform extraction and treatment with RNase, followed by isopropanol precipitation and PCR inhibitor removal (Zymo). The fungal internal transcribed spacer (ITS) region was amplified using the PCR primers ITS1-F (5′-CTTGGT CATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (71) with added bar codes and Illumina adapters. The 25-μl PCR mixtures contained 0.625 U of JumpStart *Taq* (Sigma), 2.5 μM each primer, and ~20 ng of DNA template. The PCR mixture was cycled at 94°C for 4 min and then 30 cycles at 94°C for 30 s, 56°C for 40 s, and 72°C for 60 s, followed by a final extension of 5 min at 72°C. PCR products were verified by agarose gel electrophoresis. Thereafter, the products of triplicate PCRs per sample were pooled and purified using the QIAquick PCR purification kit (Qiagen). The resulting PCR products were quantified using a Qubit (Invitrogen). Finally, 10 ng of each library (171 libraries) was pooled and then purified using Agencourt AMPure XP beads (Beckman Coulter). MiSeq (Illumina) sequencing with a read length of 2 × 250 bp was conducted at Duke's Genome Sequencing and Analysis Core Facility.

Fungal ITS sequence analysis. The resulting sequences were demultiplexed and assigned to corresponding samples based on their barcode sequence using CASAVA software (Illumina). Further sequence analyses were conducted using USEARCH version 7 and version 8 software (72). To maintain the highest sequence quality, only the forward read was used in this analysis. Sequences were further processed as follows: all the sequences were first truncated to 200 bp; a total quality score threshold was applied to filter reads, with expected errors at >1 or a length of <200 bp; singleton sequences were excluded from further analyses; the remaining sequences were assigned to OTUs of 98% pairwise identity using the centroid-based clustering UPARSE-OTU algorithm (73), resulting in OTUs of at least 96% similarity; chimeras were then removed using UCHIME (74); and OTUs occurring fewer than five times in the data set were removed.

After quality filtering, a total of 22,787,772 sequences were obtained from the 171 fungal ITS gene libraries and classified into 16,356 OTUs. Taxonomy was assigned to OTUs using BLAST against the NT database (17 February 2016) using the top nonenvironmental hit. Of these sequences, 1,569,959 (6.89%) had top matches with cultured fungi and 4,255,894 (18.68%) with uncultured fungi (but the closest taxonomy being fungi), 11,293,518 (49.56%) had ITS sequences from other eukaryotes, and the remaining 5,668,401 (24.88%), which were not associated with any sequences in the database, represent potential fungal OTUs. In the subsequent analysis, we excluded sequences identified as other eukaryotes but included both known and potential fungal sequences; this likely overestimates the true fungi, but as these are fungal primers, we erred on the side of including these putative new fungal sequences rather than excluding poorly described diversity in the fungi (31). The temporal patterns and statistical associations were similar, though not identical, when using only the known fungal sequences. The final OTU table was rarefied to 5,000 sequences per library for downstream analysis.

OTU richness was calculated using QIIME 1.9 (75). Shannon's diversity index was computed using the *vegan* R package (76). The redundancy analysis (RDA), including the 100 most abundant fungal OTUs and environmental variables, was performed and visualized in R. The OTU table was log transformed [e.g., $\log(x + 1)$] to reduce the distortion due to sparse matrices. The environmental variables with statistical significance ($P < 0.05$) were preselected as assessed by the marginal effects of terms. The cooccurrence network was constructed with CoNET (77), a plugin in Cytoscape software (78), as described previously. Pearson, Spearman, mutual information, Bray Curtis, and Kullback Leibler were selected as measures, and the thresholds were set automatically with the quantile (edge selection parameter) set to 0.01. Finally, the Benjamini-Hochberg method was used to correct for multiple hypothesis testing, with the *P* value threshold set to 0.05. This Targeted Locus Study project has been deposited at DDBJ/EMBL/GenBank as Bioproject KBPY00000000.

Phytoplankton diversity. In order to investigate the relationship between phytoplankton and fungi, we obtained the relative abundances of phytoplankton from a published bacterial 16S rRNA amplicon library data set constructed from the same samples (28). Briefly, we used RDP Classifier to identify OTUs (97% similarity) corresponding to cyanobacteria and eukaryotic chloroplasts. The resulting phytoplankton OTU table was then subsampled to 5,000 sequences per sample and used to construct the cooccurrence network of fungi and phytoplankton.

Quantitative PCR of the fungal 18S rRNA gene. Quantitative PCR (Q-PCR) was used to assess the total abundance of the fungal 18S rRNA genes. Primers FR1 (5′-AICCATTCAATCGGTAIT-3′) and FF390

(3′-CGATAACGAACGAGACCT-5′) (18, 79) were used with the SYBR premix *Ex Taq* (TaKaRa, Japan). The 10-μl reaction mixture contained 1× SYBR premix *Ex Taq*, 0.25 μM each primer, and ~20 ng of DNA template. The Q-PCR was performed on a DNAEngine Peltier thermal cycler with a Chromo4 real-time PCR detector (Bio-Rad, USA). The reaction mixtures were amplified with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, annealing at 46°C for 30 s, and elongation at 72°C for 30 s. Standard curves were constructed using known amounts of standard linearized plasmid, a combination of the pTOPO-TA vector (Gene-better, Beijing, China) and the target gene derived from genomic DNA of *Rhodosporidium diobovatum*.

Accession number(s). Newly determined sequence data have been deposited in GenBank under accession number KBPY00000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00967-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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The authors declare no conflict of interest.

Z.I.J., G.W., and Y.D. conceived and designed the experiments. Y.D., Z.I.J., and N.X. performed the experiments. Y.D., Z.I.J., G.W., D.E.H., N.X., Z.S., C.S.W., and C.-M.Y. analyzed the data. G.W., Y.D., Z.I.J., and D.E.H. wrote the manuscript.

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