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Coastal marine habitats harbor novel early-diverging fungal diversity

Kathryn T. Picard*

Department of Biology, Duke University, Durham, NC, 27708, USA

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ABSTRACT

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

1. Introduction

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

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

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

* Box 90338, Duke University, Durham, NC, 27708, USA.
E-mail address: kathryn.picard@gmail.com.

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

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

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

2. Materials and methods

2.1. Study sites and sampling regime

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

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

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

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

2.2. DNA extraction and sequence data generation

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

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