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Deciphering the presence and activity of fungal communities in marine sediments using a model estuarine system

Gaëtan Burgaud[1,*], Sarah Woehlke[2], Vanessa Rédou[1], William Orsi[3], David Beaudoin[3], Georges Barbier[1], Jennifer F. Biddle[2], Virginia P. Edgcomb[3]

[1]Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA3882), IFR 148, Université Européenne de Bretagne, Université de Brest, ESIAB, Technopole Brest-Iroise, Parvis Blaise Pascal, Plouzané 29280, France

[2]College of Earth, Ocean and the Environment, University of Delaware, Lewes, Delaware 19958, USA

[3]Geology and Geophysics Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

ABSTRACT: Fungi are known to play key roles in ecologically important biogeochemical cycles and food webs. Most knowledge of environmental groups of fungi comes from terrestrial environments, and little is known about the potential for terrestrial fungi to colonize marine environments. We investigated the Delaware River estuary and bay as a model estuarine system to study the fungal community changes occurring along a transect from terrestrially influenced waters and sediments to a higher salinity, truly marine system. DNA-based clone libraries and a culture collection built using subseafloor sediment samples revealed that *Ascomycota* dominated the detected diversity ahead of *Basidiomycota* and *Chytridiomycota*. A clear transition in fungal communities from terrestrially influenced and low salinity environments to marine environments was visualized. A complementary RNA-based analysis coupled with fluorescence *in situ* hybridization of sediments indicated that only few fungi were metabolically active in marine sediments. Cultivation of pelagic and sedimentary fungi allowed clear identification and physiology testing of fungal communities of the Delaware Bay. Most isolates were affiliated to *Ascomycota* and *Basidiomycota*, and their growth was analyzed under different concentrations of salinity to test for habitat preference and degree of adaptability. Interestingly, most of the fungi isolated were halotolerant. The present study informs us on the source and fate of fungi that may be buried in the deep marine subsurface and is informative for future investigations of this environment.

KEY WORDS: Fungi · Subsurface sediments · Delaware Bay · DNA · cDNA · Cultivation

INTRODUCTION

Fungi are known to play key roles in ecologically important symbioses, to represent a significant portion of the biomass in terrestrial systems and to play important roles in biogeochemical cycles and food webs (Bass et al. 2007, Gadd 2007). Despite the high number (~100 000) of fungal species described to date, this number may represent only ~13% of fungal species on Earth (Hawksworth 2009). Recent studies have allowed increases in the understanding of deep marine environments that represent poorly described ecological niches for fungal communities (Edgcomb et al. 2002, López-García et al. 2003, 2007, Bass et al. 2007, Alexander et al. 2009, Burgaud et al. 2009, 2010, Edgcomb et al. 2011). The biogeochemical activities, composition and temporal and spatial dynamics of marine subsurface communities are an emerging topic in marine sciences and biogeochemistry, especially with the recognition that subsurface microbial communities may rival terrestrial ecosystems, both in terms of diversity or complexity. Cover-

*Email: gaetan.burgaud@univ-brest.fr

ing more than two-thirds of the Earth's surface, marine sediments could include between 5 and 15% of the Earth's active microbial biomass (Kallmeyer et al. 2009) and may represent a major reservoir of life, although this estimate was recently reduced (Kallmeyer et al. 2012). While some studies have focused on marine fungi in sediments (Raghukumar et al. 2004, Biddle et al. 2005, 2008, Damare et al. 2006, Bass et al. 2007, Lai et al. 2007, Nagano et al. 2010, Singh et al. 2010, 2011, Edgcomb et al. 2011), subsurface eukaryotic communities still remained largely underexplored. Initial investigations of subseafloor communities revealed low fungal diversity, yet fungi have been identified as likely dominant among eukaryotic microbial populations in deep-sea sediment cores of the Peru Margin and the Peru Trench (Edgcomb et al. 2011). Based on 1 to 35 m below seafloor (mbsf) sediment samples, Edgcomb et al. (2011) revealed a dominance of fungal sequences affiliated to *Basidiomycota* in clone libraries constructed from DNA and cDNA. The recovery of ribosomal RNA at these depths suggests that the microorganisms revealed were living cells rather than inactive spores or dead mycelia (Edgcomb et al. 2011), which may have global implications for ocean carbon and nitrogen cycling, given the extent of the deep subsurface biosphere (Oger & Jebbar 2010). Some fungal sequences retrieved from the Peru Margin represented potentially novel taxa, supporting the fact that marine sediments harbor a large fraction of undescribed fungi (Nagahama et al. 1999, 2003, 2006, 2008). Fungal diversity retrieved by culture-dependent methods in the deep-sea (Mouton et al. 2012, Singh et al. 2010, Edgcomb et al. 2011) also revealed that many of the fungal sequences detected were phylogenetically related to terrestrial fungi, raising ecological questions regarding the abilities of terrestrial fungi to adapt to deep subsurface conditions (low temperatures, high hydrostatic and lithostatic pressures and relative oligotrophy).

The boundaries between terrestrial and marine fungi are currently not clear, with the most widely accepted definition of a marine fungus as being those that grow and sporulate exclusively in a marine or estuarine habitat (Kohlmeyer & Kohlmeyer 1979). Since fungi are increasingly found in deep-sea environments using both culture-dependent and culture-independent methods, a clear differentiation between marine and terrestrial fungi has been lost (Edgcomb & Biddle 2012). There is some evidence that terrestrial/surface-dwelling fungi may be capable of colonizing deep-sea habitats due to their ability to alter their membrane composition to accommodate high hydrostatic pressure (Fernandes et al. 2004). The examination of marine fungi at this point is in need of a detailed biodiversity study to determine, using molecular, microscopic and cultivation approaches, what constitutes a marine fungus and whether there are members of the fungi that are active in the subsurface or if those organisms are merely preserved (either dead or inactive). As future microbiology expeditions targeting marine subsurface sediments and the deep biosphere are planned, it is important to be able to accurately interpret the potential significance of finding particular fungal groups in diverse marine subsurface samples. Additionally, the potential adaptation to, and survival of, terrestrial fungi in the marine subsurface needs to be explored. We chose the Delaware River estuary as a model estuarine system to study the fungal community changes occurring along a transect from terrestrially influenced water and sediments to a higher salinity, truly marine system. Our aim was to determine, using molecular, microscopic and cultivation-based techniques, what portion of detected fungi represents truly marine forms vs. inactive or active and highly adaptable, opportunistic terrestrial fungi and to gain insights into their levels of activity.

MATERIALS AND METHODS

Site description

The Delaware Bay was chosen as a model estuarine system since 3 major ecological zones could be distinguished easily: (1) an upper zone (Stn 23) characterized by fresh water under tidal influence, (2) a transition zone (Stn 11) with a wide range of salinity, high turbidity and low primary biological productivity and (3) a lower bay zone (Stn 1), extending to the Atlantic Ocean, with higher salinity and the highest levels of primary biological productivity (Santoro 2004) (Fig. 1).

Water and sediment sampling

Samples were collected using the RV 'Hugh Sharp' (Table 1). Water samples were collected using a submersible pump 1 m below the surface at 3 different stations representing the 3 major salinity zones of the Delaware Bay (Table 1).

Water samples were used for cultivation-based approaches and *in situ* hybridization. Regarding cultivation, water samples were stored at 4°C for a few

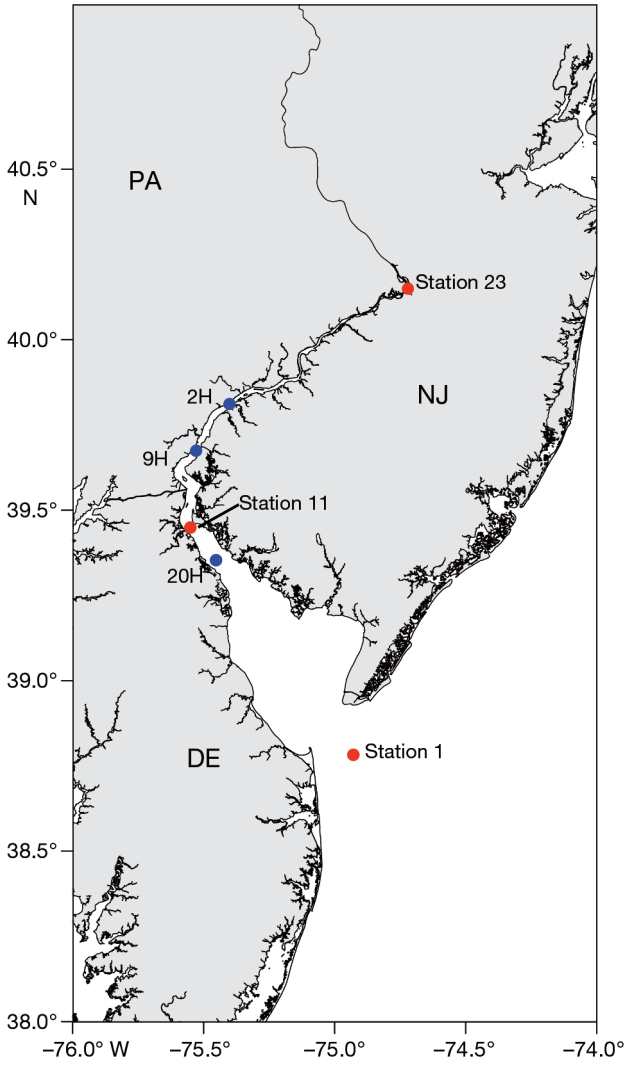


Fig. 1. Overview of sample collections with positions of the 3 water-sampling stations (1, 11 and 23) and the 3 sediment-sampling sites (2H, 9H and 20H). PA: Pennsylvania; NJ: New Jersey; DE: Delaware

hours before filtration and plating. For *in situ* hybridization, 200 ml of water samples were directly fixed with paraformaldehyde (2% final concentration) for 2 h at 4°C in the dark before filtration on polycarbonate filters (0.8 µm, Millipore), with a pre-filtration through 200 µm mesh (Pernthaler et al. 2001).

Bottom sediment samples were obtained on the same cruises using a KC Denmark HAPS corer, which recovers undisturbed samples of the sediment-water interface. The cores were extruded vertically aboard the ship and sectioned in 2 cm intervals. The sediment was placed in Whirl-pak sterile bags and frozen at −80°C. Samples were processed from the 2 to 4 cm below seafloor (cmbsf) depth horizon to avoid seawater contamination.

Sediment samples were used for cultivation-based approaches, *in situ* hybridization and calcofluor staining as well as analyses of small subunit ribosomal RNA (SSU rRNA) and internal transcribed spacer region 1 (ITS1) sequences. For *in situ* hybridization, multiple aliquots of 100 mg sediment samples were fixed in 2% paraformaldehyde (final concentration) for at least 2 h at 4°C in the dark. After fixation, sediment samples were washed 2 times with sterile 1× PBS and stored at −20°C in 50:50 1× PBS:96% ethanol (Pernthaler et al. 2001). Multiple 2 g aliquots were directly frozen at −80°C for extraction of nucleic acids. A summary of samples collected and methods applied to each sample can be found in Table 1.

Fungal isolation from sediment and water samples and salinity tolerance assays

Cultures were prepared aerobically at 25°C and atmospheric pressure using GYPS0, GYPS3, MEA0

Table 1. Delaware estuary sample collection metadata

Station	Sample	Date sampled	Latitude (N)	Longitude (W)	Salinity (PSU)	Study performed
23	Water	11 Mar 10	40° 08.96′	74° 43.31′	0.1	Microscopy
	Water	4 Jun 11			0.08	Culturing, salinity assay
11	Water	11 Mar 10	39° 26.916′	75° 33.125′	6.9	Microscopy
	Water	3 Jun 11			2.58	Culturing, salinity assay
1	Water	10 Mar 10	38° 46.94′	74° 55.76′	31.5	Microscopy
	Water	3 Jun 11			29.23	Culturing, salinity assay
2H	Sediment	12 Mar 10	39° 48.714′	75° 24.167′	0.19	Culturing, microscopy, DNA/cDNA, environmental cloning
9H	Sediment	12 Mar 10	39° 40.442′	75° 31.774′	1.5	Culturing, microscopy, DNA/cDNA, environmental cloning
20H	Sediment	11 Mar 10	39° 21.143′	75° 27.175′	9.2	Culturing, microscopy, DNA/cDNA, environmental cloning

and MEA3 culture media and a laminar flow hood. The GYPS medium contained the following components per liter: glucose 1 g, yeast extract 1 g, peptone 1 g, starch 1 g and agar 30 g. Sea salts (3%) were added to GYPS3. The MEA medium contained the following components per liter: maltose 12.75 g, dextrin 2.75 g, glycerol 2.35 g, peptone 0.78 g and agar 30 g. Sea salts (3%) were added to MEA3. All the culture media were supplemented with 500 mg chloramphenicol and 250 mg vancomycin per liter. For water samples, 20 ml of the 3 different suspensions were filtered on sterile 0.45 µm cellulose acetate filters and then deposited on the culture media. For sediment samples, 3 suspensions were processed (no dilution, 1:10 and 1:100) with sterile water amended with different sea salt concentrations depending on the salinity of the site sampled. Aliquots of 0.2 ml of each suspension were plated on each culture medium and spread with sterile flat rakes. Duplicates were processed for each condition. Pure cultures were obtained by streaking yeasts and central picking filamentous fungi on their respective enrichment media, and these pure cultures were kept in our culture collection for physiological analysis, morphological characterization and molecular identification. Nutrient plates exposed within a laminar flow hood during our isolation procedure were processed as a control. No fungal colonies appeared on our control plates, indicating no occurrence of aerial contaminations during isolation.

The filamentous fungal strains isolated were grown on solid GYPS medium. The effect of salinity on growth was assessed by modifying sea salt concentrations in culture media (0, 1.5 and 3%). Colony diameters were measured each 2 d for a maximum of 10 d to characterize fungal growth. All experiments were processed in triplicate. Regarding yeasts, the same procedure was established except that growth rate was visually estimated.

Nucleic acid extraction and PCR from sediment samples and fungal isolates

Sediment cores

RNA extractions were processed on frozen sediment cores (–80°C) with a sterile metal spatula. To avoid any seawater contamination, scrapings were taken from the central part of each different core (House et al. 2003). RNA was isolated from 2 g samples for each sediment core (4 RNA extractions of 0.5 g sediment) using the Fast RNA Pro Soil-Direct kit (MP Biomedicals) according to the manufacturer's instructions with few modifications, i.e. a treatment with 0.02 U Turbo DNAse with 1× Turbo DNAse Buffer (Invitrogen) and 15 mM EDTA for 30 min at 37°C followed by 10 min denaturation at 75°C. The Super Script III One-Step RT-PCR System (Invitrogen) was then used to reverse-transcribe total RNA to cDNA (following the manufacturer's instructions), and this step incorporated the first PCR amplification with f-ITS1F/r-ITS4 (for ITS locus amplification) or with f-EF4/r-EF3 (for SSU rRNA gene amplification). As fungal biomass was low, we processed a second nested PCR reaction. The GoTaq kit was used with f-ITS1F/r-ITS2 or f-EF4/r-Fung5. RNA samples were tested for DNA contamination by PCR amplification prior to reverse-transcription to control for complete elimination of residual DNA. All sediment-core RNA samples passed this test (no PCR amplification was detectable after 40 PCR cycles) and were used to prepare cDNA libraries. RNA yield was quantified using a Qubit fluorometer (Life Technologies), and cDNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). ITS rRNA gene sequences were obtained using primers designed by White et al. (1990): ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC-3′), followed by a second nested PCR reaction with ITS1F and ITS2 (5′- GCT GCG TTC TTC ATC GAT GC-3′). For 18S rRNA gene sequences, we used a primer set described by Smit et al. (1999), EF3 (5′-TCC TCT AAA TGA CCA GTT TG-3′) and EF4 (5′-GGA AGG GNT GTA TTT ATT AGA T-3′), followed by a second nested PCR reaction with EF4 and Fung5 (5′-GTA AAA GTC CTG GTT CCC C-3′). All PCR reactions were performed in 25 µl reaction volumes containing 24 µl of 1× PCR buffer (Promega), 2 mM of $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.6 mM of primers (forward and reverse), 1.25 U of GoTaq Polymerase (Promega) and 1 µl of DNA. The cycling parameters were as follows: after the cDNA synthesis step (55°C for 30 min), 94°C for 2 min, followed by 39 cycles of 95°C for 20 s, 55°C for 30 s and 68°C for 40 s, and a final 5 min at 68°C. Regarding the second nested PCR reaction, we used the following conditions: 94°C for 5 min, followed by 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 2 min and a final 7 min at 72°C. PCR amplifications from DNA prepared from sediment samples were conducted using the same procedure but only on ITS rRNA gene sequences.

Fungal isolates

DNA of fungal isolates was extracted with a Fast DNA Spin Kit (MP Biomedicals) according to the manufacturer's instructions. For filamentous fungi, the 18S rRNA gene and ITS were amplified and sequenced. SSU rRNA gene sequences were amplified with NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′), NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′) and ITS5R (5′-CCT TGT TAC GAC TTT TAC TTC C-3′) primers (White et al. 1990). ITS were amplified using ITS1F and ITS4 primers. For yeasts, amplifications of the D1/D2 region of the 26S rRNA gene were carried out with rRNA gene primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG-3′), LR6 (5′-CGC CAG TTC TGC TTA CC-3′), NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) as described by Gadanho & Sampaio (2005). All PCR reactions were performed in 20 µl reaction volumes containing 1× GoTaq Buffer (Promega), 2 mM of each of the 4 dNTPs (Promega), 0.08 mM of each primer (Proligo), 1 U of GoTaq DNA polymerase (Promega) and 1 µl of genomic DNA. PCR reactions used a PTC-200 thermal cycler (MJ Research). The PCR temperature profile consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C and 2 min at 72°C and a final extension step of 2 min at 72°C before a conservation at 4°C. A negative control in which DNA was replaced by sterile water was included. PCR products were evaluated by electrophoresis in 0.8% (w/v) agarose gel (Promega) in 0.5× Tris-borate EDTA (TBE) buffer at 90 V for 90 min and stained with ethidium bromide. The molecular size markers were Lambda DNA/EcoR1 + HindIII (Promega). DNA banding patterns were visualized under UV transillumination, and picture files were generated using Gel-Doc 2000 (Bio-Rad).

Environmental cloning of sediment samples and sequencing of fungal isolates

Sediment cores

The amplified SSU and ITS rRNA gene fragments obtained from cDNA prepared from sediment samples were cloned into the TOPO TA cloning vector pCR2.1 and transformed into TOP10 chemically competent *Escherichia coli* according to the manufacturer's instructions (Invitrogen). Transformants were selected by blue-white selection on Luria-Bertani agar plates containing ampicillin (100 µg ml^{-1}). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F and M13R. Clones were then sequenced by Genewiz.

Fungal isolates

Sequences obtained by Big Dye Terminator technology (Applied Biosystems) at the 'Ouest Génopole' sequencing facility in the 'Station Biologique de Roscoff' were edited and processed for quality control using SEQUENCHER v. 4.8 (Gene Codes). Sequences were then imported to MEGA 4.0 software (Tamura et al. 2007). Each sequence was analyzed to find GenBank sequences with closest BLASTn hits (Altschul et al. 1990). Similarities between sequences were assessed using pairwise distance calculation with MEGA 4.0 for the purpose of taxonomic assignment and calculating OTUs.

Phylogenetic analyses

Sequences were trimmed to ensure that all had the same start and end point and checked for chimeras using Bellerophon Chimera Check and the Check_Chimera utilities (Ribosomal Database Project) (Cole et al. 2003). All SSU and ITS rRNA gene sequences were aligned using CLUSTALW v. 1.83 (Thompson et al. 1994). After visual inspection and manual correction, the alignments were analyzed using MODELTEST v. 3.7 (Posada & Crandall 1998) to obtain the optimal evolutionary model for subsequent phylogenetic analyses.

The phylogeny of sediment SSU rRNA sequences obtained from cDNA was evaluated using maximum likelihood. For this approach, we used RAxML version 7.0 as implemented on the CIPRES Portal (www.phylo.org). The tree was constructed using an alignment of 503 unambiguous positions under the General Time Reversible (GTR)+I+Gamma model of sequence evolution. The tree contains the sequences of the most closely affiliated cultured representatives of each sequence based on BLASTn analyses against GenBank's nr database. The phylogenetic analysis included 47 sequences from Stn 2H, 56 from Stn 9H and 57 from Stn 20H. Bootstrapping and determination of the best estimate of the maximum likelihood (ML) tree topology for this data set was conducted with the Rapid Bootstrapping algorithm of RAxML. Rarefaction analysis was performed using the alpha_rarefaction.py command in QIIME (Caporaso et al. 2010).

Fluorescence *in situ* hybridization (FISH) and calcofluor staining for water and sediment samples

For sediment samples, 100 µl of fixed sediments were first treated to separate microbial cells from the sediment matrix as described by Kallmeyer et al. (2008). Briefly, cells were detached using a detergent mix (EDTA, Tween 80, sodium-pyrophosphate and methanol) and an ultrasonic treatment (Aquasonic model #75HT, 20 s), followed by density centrifugation through a cushion of Nycodenz (3000 × *g* for 10 min). Supernatants (~10 ml) were then filtered on 0.2 µm white polycarbonate filters (Millipore) using a gentle vacuum (<0.2 bar) before hybridization of the eukaryotic probe. For water samples, 20 ml of fixed water were filtered onto white polycarbonate filters using gentle vacuum (<0.2 bar).

Pieces of polycarbonate filters (3 per sediment and water sample) were cut in squares and pasted onto slides with one drop of 0.2% low-gelling point agarose (35 to 40°C) (Menzel-Glaser). All slides were then dipped in 0.2% agarose and air-dried. Samples were then subjected to dehydration with increasing concentrations of ethanol (50, 80 and 96%, for 3 min each). The concentration of working solutions of the eukaryotic probe Euk516-Cy3 (5′-ACC AGA CTT GCC CTC C-3′, Amann et al. 1995) was 30 ng l^{-1}. The hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.03% SDS, 20% formamide and the fluorescent probes were gently mixed in a ratio of 10:1 (v/v) to obtain a final oligonucleotide concentration of 3 ng l^{-1}. For hybridization, slides were placed in sampling tubes and incubated at 46°C in the dark for 3 h. Following hybridization, the slides were washed with pre-warmed washing buffer (20 mM Tris/HCl, 5 mM EDTA [pH 8.0] and 215 mM NaCl) for 20 min at 48°C. Slides were rinsed with double-distilled water, air dried, DAPI stained (final concentration 1 µg ml^{-1}) and mounted with the antifading reagent Citifluor AF 2 (Citifluor) before observation under a fluorescent microscope.

Calcofluor white staining was also used to examine fungal cells on pieces of the same polycarbonate filters described above. Slides were stained with an 0.5 mM solution of calcofluor white M2R (Sigma Aldrich) (4,4′-bis[4-anilino-6-bis{2-ethyl}amino-s-triazin-2-ylamino]-2,2′-disulfonic acid). The targets of calcofluor white M2R are chitin, cellulose and carboxylated polysaccharides. Following incubation in the dark for 5 min, the slides were washed with sterile water and observed using epifluorescence microscopy (with a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss AxioCam camera).

Sequence accession numbers

Nucleotide sequences obtained in the present study were deposited in GenBank under accession numbers JX846650 to JX846808 (SSU rRNA sequences), JX967357 to JX967404 (ITS1 from DNA-based approach) and JX967405 to JX967502 (ITS1 from RNA-based approach). Sequences from cultured fungi (18S, ITS1 and 26S rRNA gene) were also deposited in GenBank under accession numbers JX967503 to JX967533.

RESULTS

Fungal isolation from sediment and water samples

A total of 141 fungal strains were isolated from the water of the different stations (Fig. 1, Table 1) using the 4 different culture media chosen in the present study. The GYPS base medium allowed us to cultivate 64 strains as well as the MEA base medium that produced 77 strains. The isolation ratio between salted and unsalted media gave different results depending on the isolation medium used; 28 strains were isolated from GYPS3 and 36 from GYPS0. For MEA, 51 strains were isolated from MEA3 and 26 strains from MEA0. More filamentous fungi were isolated from the Delaware Bay compared to unicellular yeasts: 84 filamentous fungi (60%) were retrieved compared to 57 yeasts (40%). The isolation ratio was dependent on the station sites since a pattern of distribution was observed. An increasing number of strains were retrieved as we transitioned from marine to more freshwater sites: 12 were obtained from Stn 1, 63 from Stn 11 and 66 from Stn 23, representing 8.5, 44.5 and 47% of all fungal strains isolated, respectively. Filamentous fungi and yeasts were mostly isolated from the almost freshwater Stn 23 and the brackish Stn 11. Only a few species were retrieved from the truly marine Stn 1, i.e. 7 yeasts and 5 filamentous fungi. We did not succeed in isolating fungi from sediment samples.

Salinity tolerance assays

To visualize a pattern of distribution of filamentous fungi along a salinity gradient, diameters of growth of all strains after 3 d at 0 and 3% sea salt concentrations were plotted on a simple graph (Fig. 2A). A first clear clustering can be visualized between slow-growing strains (diameters <2 cm) and fast-growing strains

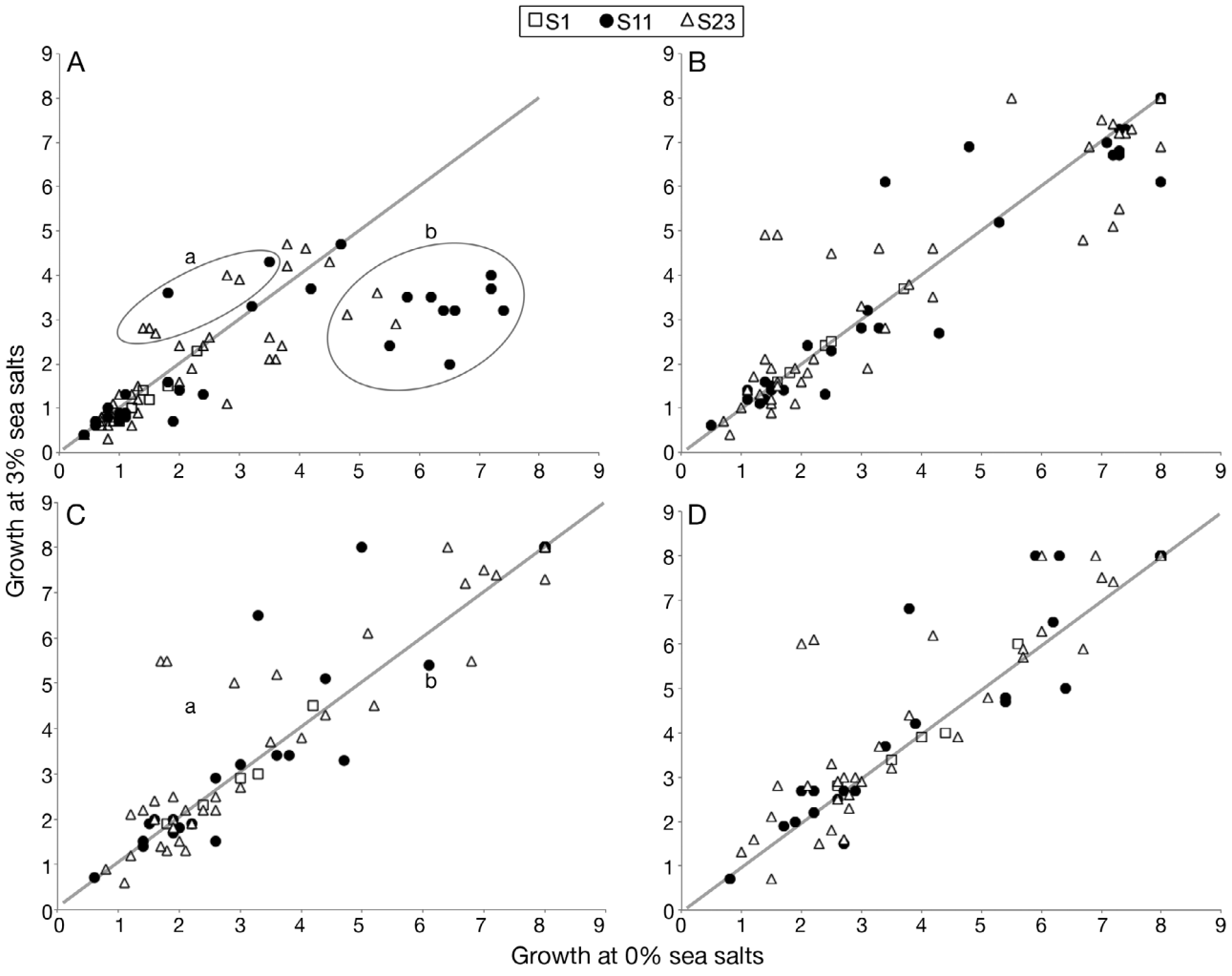


Fig. 2. Growth rate of filamentous fungal strains at 0 and 3% sea salts depending on the geographic origin (Stns 1, 11 and 23; see Fig. 1). Mycelial colony diameters (cm) were measured after (A) 3, (B) 5, (C) 7, and (D) 10 d. Cluster a represents halophiles (7 strains) and Cluster b represents non-halophiles (12 strains)

along a straight line that characterizes 58 halotolerant strains. We were able to extract a pattern of response for halophiles (7 strains in Cluster a) and non-halophiles (12 strains in Cluster b). Regarding halophiles, the same pattern was obtained after 5, 7 and 10 d of growth (Fig. 2B,C,D) although the growth difference between 0 and 3% sea salts was minimal. For non-halophiles, it appears that those strains were able to further adapt to higher salinity since after 5, 7 and 10 d of growth, Cluster b was not visible anymore, and the strains appeared halotolerant.

Regarding yeasts, only qualitative/semi-quantitative information was gathered depending on growth in culture media at different sea salt concentrations. All data were compiled in Table 2, and we were able to process a comprehensive analysis of the profile obtained and to form clusters with halophilic (9), slightly halophilic (4), halotolerant (21) and non-halophilic (23) yeast strains.

The isolation rate of yeasts depending on (1) sampling sites and (2) tolerance to sea salts indicated that, as shown for filamentous fungi, a pattern of distribution was clearly apparent and appears linked with salinity concentrations at the 3 different sites. Indeed, halophilic and slightly halophilic yeasts were mostly isolated from Stns 1 (marine) and 11 (brackish). More halotolerant yeasts were isolated from Stn 11 than Stn 23 (freshwater), and more non-halophiles were isolated from Stn 23 than Stn 11. Regarding filamentous fungi, non-halophiles were mostly isolated from Stns 11 and 23 (Fig. 2), but unexpectedly, the rare halophiles isolated were harvested more frequently from freshwater and brackish stations and not from the fully marine station.

Taxonomic assignments of fungal cultures were made on the basis of BLASTn analyses using the GenBank nr database (SSU, ITS and/or 26S rRNA genes) as detailed in Appendix 1, and assignments

Table 2. Physiological analysis of yeast strains. Growth was visually estimated on broth media at different sea salt concentrations (0, 1.5 and 3 %) and is shown as 3-part bars representing small, middle and maximum growth

Yeast strain	Station	Salinity 3 - 1.5 - 0	Physiology
Y1	11		Non halophile
Y2	11		Halophile
Y3	11		Non halophile
Y4	11		Slightly halophile
Y5	11		Halotolerant
Y6	11		Halophile
Y7	11		Non halophile
Y8	11		Halotolerant
Y9	11		Halotolerant
Y10	23		Halotolerant
Y11	23		Halotolerant
Y12	1		Slightly halophile
Y13	11		Non halophile
Y14	11		Halophile
Y15	11		Non halophile
Y16	11		Halotolerant
Y17	23		Halotolerant
Y18	23		Non halophile
Y19	11		Non halophile
Y20	11		Halotolerant
Y21	1		Halophile
Y22	1		Non halophile
Y23	11		Halotolerant
Y24	11		Slightly halophile
Y25	23		Non halophile
Y26	23		Non halophile
Y27	23		Non halophile
Y28	23		Non halophile

Yeast strain	Station	Salinity 3 - 1.5 - 0	Physiology
Y29	1		Non halophile
Y30	1		Non halophile
Y31	11		Non halophile
Y32	11		Halotolerant
Y33	11		Halotolerant
Y34	11		Slightly halophile
Y35	11		Halophile
Y36	11		Halotolerant
Y37	11		Halotolerant
Y38	11		Non halophile
Y39	11		Halophile
Y40	23		Non halophile
Y41	23		Halotolerant
Y42	23		Halotolerant
Y43	23		Halotolerant
Y44	23		Non halophile
Y45	23		Halotolerant
Y46	11		Non halophile
Y47	11		Halophile
Y48	11		Halotolerant
Y49	11		Halophile
Y50	23		Non halophile
Y51	11		Non halophile
Y52	23		Non halophile
Y53	23		Halotolerant
Y54	1		Halotolerant
Y55	1		Non halophile
Y56	11		Halotolerant
Y57	23		Halophile

were confirmed by observations of morphology. Table 3 presents the identity of each cultured isolate and the salinity of the waters from which it was isolated. In most cases, we did not recover the same isolates from waters of different salinity. Exceptions were *Fusarium oxysporum, Leptosphaeria maculans* and *Cadophora luteo-olivacea*, which were isolated from both 0.1–0.08 and 2.3–6.9 PSU salinity, and *Acremonium* sp., which was isolated from both 2.3–6.9 and 29.3–31.5 PSU salinity.

Environmental cloning of sequences from sediment samples

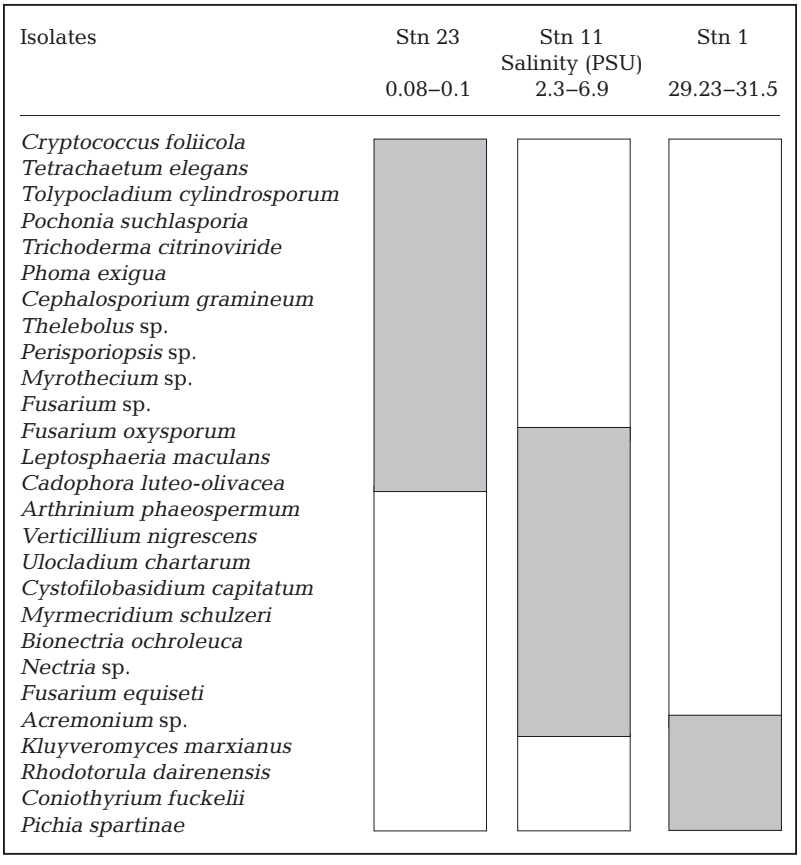
ITS from cDNA and DNA

We first utilized the ITS1 as a universal fungal barcode to reveal the fungal communities present in different sediment samples using DNA. Then, the active fraction of the fungal community was targeted by using cDNA that was reverse-transcribed from the total RNA extracted from each sediment sample.

For the DNA-based analysis, 192 cloned ITS1 sequences were analyzed for Stns 2H, 9H and 20H (equal sequencing effort for each station). After removal of 148 sequences that were either suspected chimeras, poor quality sequences or highly divergent sequences matching only 1 end of the SSU rRNA gene flanking the ITS1 sequence (with the remaining sequence not matching anything in GenBank), we only analyzed 10 clones from Stn 2H, 14 from Stn 9H and 24 from Stn 20H. These clustered into 8, 3 and 5 different taxonomic groups at 97% sequence similarity, respectively (Table 4).

For the cDNA-based analysis of ITS1, 192 clones were also processed for all stations. After the removal of 83 poor-quality sequences (see above) and 11 sequences affiliating with choanoflagellates, we were able to analyze 2 clones from Stn 2H, 65 from Stn 9H and 31 for Stn 20H. Diversity obtained using the RNA-based analysis was clearly lower than for the DNA-based approach, as clones from Stns 2H, 9H and 20H clustered into only 3 OTUs at 97% sequence similarity. Clones from 9H and 20H produced only 1 identical OTU (Table 5).

Table 3: Presence (grey) and absence (white) map of cultured fungal isolates and their isolation sites



SSU rRNA gene from cDNA

The active fraction of the fungal community in our sediment samples was also targeted by using cDNA prepared as above to amplify the eukaryotic SSU rRNA genes present in each sample. A total of 192 clones were sequenced for these analyses (equal sequencing effort for each of the 3 stations), producing 155 sequences after removal of poor-quality sequences or suspected chimeras (as above). A total of 57 sequences were retained for downstream analysis from Stn 20H, 56 from Stn 9H, and 47 from Stn 2H.

Phylogenetic analysis

A total of 59 non-redundant sequences obtained after OTU clustering recovered from analysis of SSU rRNA genes based on cDNA prepared from sediment samples (Fig. 3A) affiliated with members of the *Basidiomycota* and *Chytridiomycota*: 3 from Stn 2H (0.19 PSU salinity) and 55 from Stn 9H

Table 4. Sequence-based identification of OTUs (DNA-based analysis)

Station	Name / No. of clones	Close neighbor	%ID	Environments	Accession no.	Source
2H	OTU1 / 1	Uncultured fungus clone	93.9	Phyllosphere	FJ758448	Jumpponen & Jones (2009)
2H	OTU2 / 2	*Cochliobolus lunatus*	97.3	Plants	JN107740	H.Q. Hu & Y.L. Liu (unpubl.)
2H	OTU3 / 1	*Cryptococcus aquaticus*	98.0	Lakes, cold ecosystems	AF410469	Scorzetti et al. (2002)
2H	OTU4 / 2	Uncultured fungus clone	98.4	Rhizosphere	AJ920023	Paradi & Baar (2007)
2H	OTU5 / 1	*Scutellospora heterogama*	81.0	Plant rhizosphere	FM876839	Krüger et al. (2009)
2H	OTU6 / 1	Uncultured fungus clone	83.3	Mangrove sediments, dead leaves	JQ038341	Fasanella et al. (2012)
2H	OTU7 / 1	*Rhizophydium* sp.	97.5	Soil	DQ485685	Letcher et al. (2006)
2H	OTU8 / 1	Uncultured fungus clone	100	Phyllosphere	JN905553	Cordier et al. (2012)
9H	OTU1 / 5	*Fibulochlamys* sp.	100	Soil	FM955450	Madrid et al. (2010)
9H	OTU2 / 7	Uncultured fungus clone	98.4	Deep-sea sediments	DQ279844	Lai et al. (2007)
9H	OTU3 / 2	Uncultured fungus clone	83.9	Rhizosphere	GU366680	Yarwood et al. (2010)
20H	OTU1 / 5	*Pestalotiopsis maculiformans*	98.7	Dead leaves, leaf litter	EU552147	Marincowitz et al. (2008)
20H	OTU2 / 4	*Lycoperdon pyriforme*	100	Decaying logs	AY854075	P.B. Matheny & D.S. Hibbett (unpubl.)
20H	OTU3 / 3	Uncultured *Chytridiomycota*	79.2	Lakes	HQ191408	Monchy et al. (2011)
20H	OTU4 / 5	Uncultured fungus clone	92.5	Mangrove sediments, dead leaves	JQ038342	Fasanella et al. (2012)
20H	OTU5 / 7	*Phaeosolania densa*	89.9	Decaying wood	AY571056	Bodensteiner et al. (2004)

Table 5. Sequence-based identification of OTUs (cDNA-based analysis)

Station	Name/ No. of clones	Close neighbor	%ID	Environments	Accession no.	Source
2H	OTU1 / 2	Uncultured fungus clone	96.5	Phyllosphere	JN904921	Cordier et al. (2012)
9H	OTU1 / 65	*Rhodotorula glutinis*	99.3	Deep-sea environments	AB025993	Nagahama et al. (2001)
20H	OTU1 / 31	*Rhodotorula glutinis*	99.3	Deep-sea environments	AB025993	Nagahama et al. (2001)

(1.5 PSU salinity). The nearest cultured relatives of these sequences were *Nematoloma* and *Cortinarius*. One sequence from Stn 2H was affiliated to *Chytridiomycota* (nearest relative an uncultured *Spizellomyces*).

The recovered sequences that affiliated with the *Ascomycota* were broadly distributed over several *Ascomycota* classes. Eight sequences from Stn 2H (0.19 PSU salinity) affiliated with relatives of *Preussia, Phoma, Westerdykella* and *Eremodothis*. Five sequences from Stn 2H affiliated with *Dothideomycete* sp., and 10 affiliated with members of *Penicillium*, *Thysanophora* and *Aspergillus*. A total of 57 sequences from Stn 20H (9.2 PSU salinity) and 1 sequence from Stn 9H (1.5 PSU salinity) also affiliated with *Aspergillus*. Ten sequences from Stn 2H affiliated with species of *Capronia* and *Phialophora* and with an uncultured fungus from soil. Four sequences from Stn 2H affiliated with species of *Trichoderma*, and 4 affiliated with *Phialophora*. However, diversity was only partially described based on the fact that the accumulation curves did not reach saturation (Fig. 3B).

FISH and calcofluor staining for water and sediment samples

Calcofluor-stained fungal cells were visualized from water samples collected from Stns 11 and 23. No fungal cell was ever visualized from Stn 1. Multicellular conidia with a short conical beak were retrieved from Stn 11 (Fig. 4A). Fungal spores showing a blastic conidiogenesis are typical members of the *Ascomycota*. The conidium in Fig. 4A appears morphologically similar to *Alternaria*, an ubiquitous dematiaceous fungus. One tetraradiate conidium with long arms of equal length (40 µm) and showing phialidic conidiogenesis was visualized from water collected at Stn 23 (Fig. 4B) that is typical of *Lemonniera* sp. Stained fungal spores were also visualized from sediment samples of Stns 2H and 9H (Fig. 4C–F), although their presence was sporadic. No fungal cells were visualized from Stn 20H, despite multiple attempts.

FISH coupled with calcofluor staining also allowed us to detect active fungal cells in sediment samples from Stn 2H (0–2 and 2–4 cmbsf), representing the

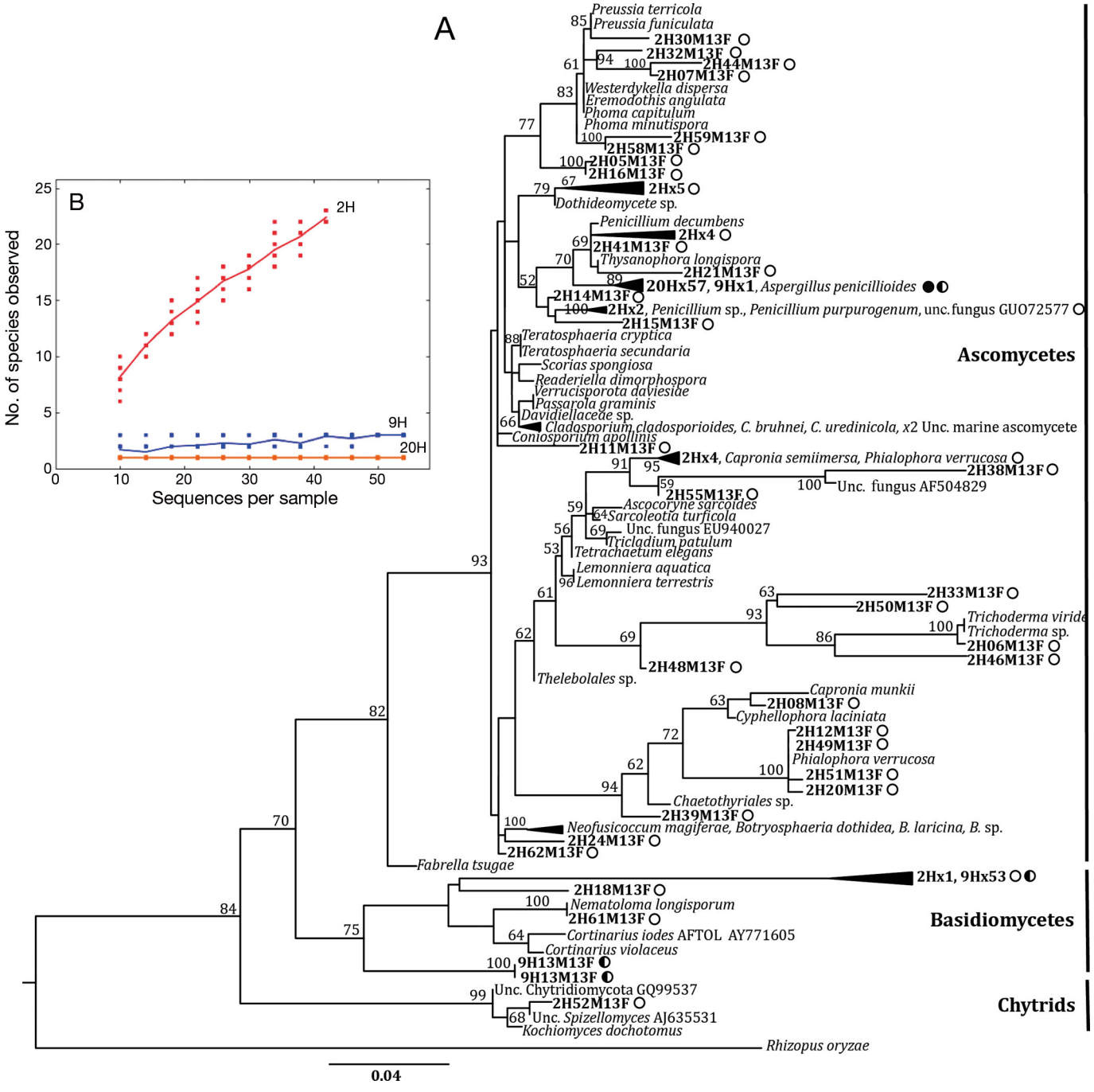


Fig. 3. SSU rRNA-based phylogeny (maximum likelihood) of sediment fungal sequences obtained from cDNA-based analyses. Numbers at nodes are bootstrap values ≥50% under (A) maximum likelihood and (B) rarefaction curves. Environmental sequences are marked according to geographic origin: (white circles) Stn 2H, (black and white circles) Stn 9H and (black circles) Stn 20H. Unc.: uncultured

nearly freshwater station. Fungal cells appeared red/pink with blue cell walls since we used a Cy3 eukaryotic probe and calcofluor (Fig. 4D,G). Few fungal cells were observed in samples prepared from marine sites.

DISCUSSION

Our research objectives were to study the fungal community changes occurring along a transect from the freshwater zone to marine waters utilizing metho-

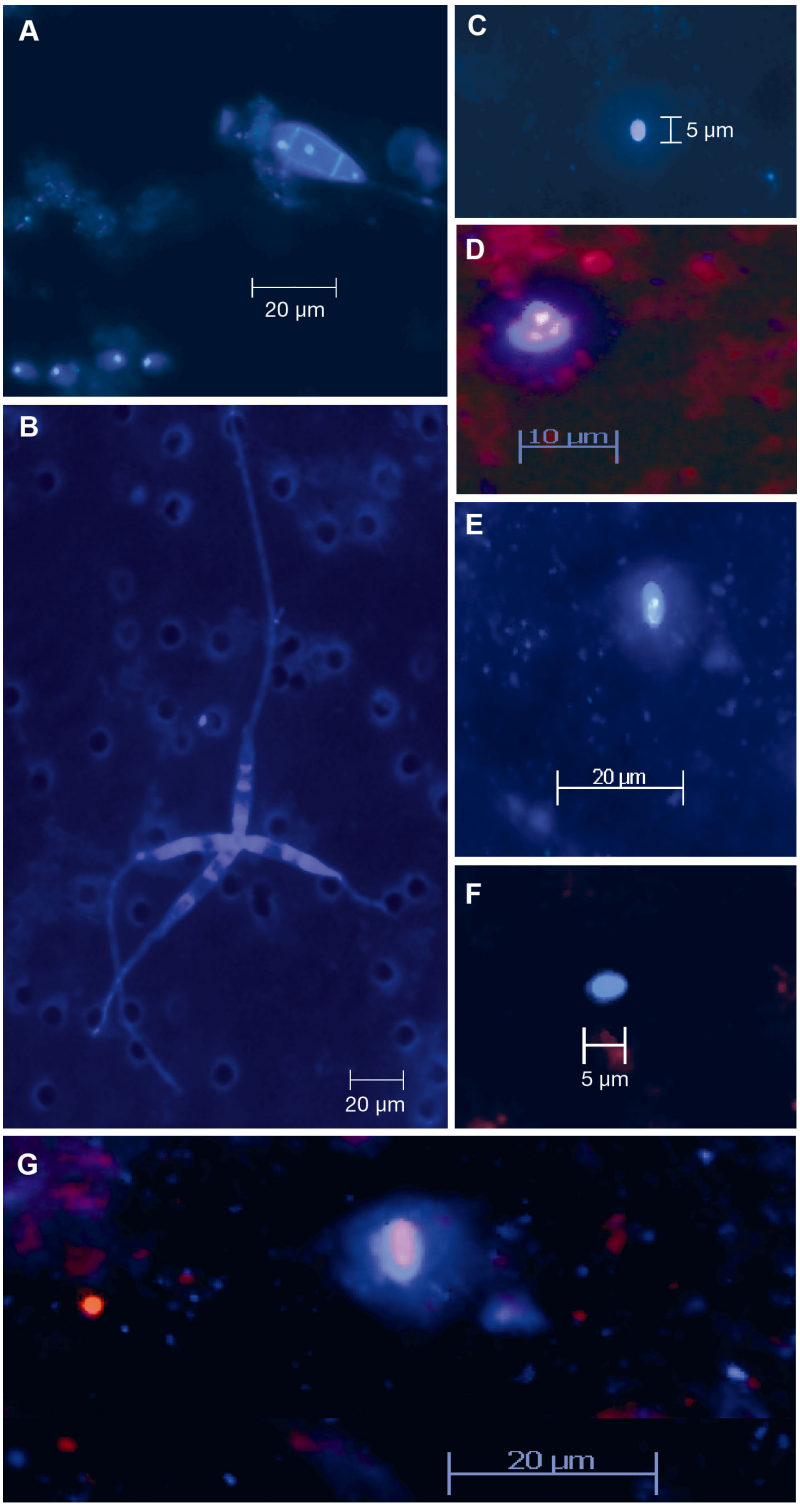


Fig. 4. Observation of fungal cells using calcofluor/DAPI staining and fluorescence *in situ* hybridization (FISH) from filtered waters and sediments. (A) Filtered water from Stn 11: 20 ml through 0.8 µm; stained with calcofluor and DAPI; bar = 20 µm. (B) Filtered water from Stn 23: 20 ml through 0.8 µm; calcofluor; bar = 20 µm. (C) Sediments from Stn 2H (0–2 cmbsf); calcofluor; bar = 5 µm. (D) Sediments from Stn 2H (0–2 cmbsf); calcofluor and FISH; bar = 10 µm. (E) Sediments from Stn 2H (2–4 cmbsf); calcofluor and DAPI; bar = 20 µm. (F) Sediments from Stn 9H (0–2 cmbsf); calcofluor; bar = 5 µm. (G) Sediments 2H (2–4 cmbsf); calcofluor and FISH; bar = 20 µm

dologies to segregate between the active and inactive fraction of fungi. The main aim was to better interpret the significance of fungal signatures being increasingly found in subsurface environments using both culture-dependent and culture-independent methods.

Evidence for a distribution pattern

Success in fungal isolation was dependent on the salinity of the different sites since a decreasing number of fungal strains, both yeasts and filamentous fungi, were harvested as we transitioned from freshwater to marine sites. Another correlation was observed when comparing isolation sources and physiology (Fig. 2, Table 2), showing that the yeasts from marine sites exhibited growth consistent with a strong preference for higher salinity, while the isolates from brackish sites were haloterant, and the isolates from fresh waters were clearly non-halophiles. Indeed, based on the culturing results, each site/salinity condition harbored a mostly distinct fungal community (Table 3). Interpretation appeared more complex for filamentous fungi and showed that those organisms are more able to adapt to different salinities.

Few fungi were retrieved in culture at >1 site, despite using the same enrichment media. While it is possible that expanded culturing efforts using additional filtered water samples as inocula would have produced a greater number of overlapping cultures among sites, we think the observed pattern of culture recovery suggests very different active fungal populations in waters of significantly different salinity. This is particularly striking when one compares the successful isolates from Stn 23 (0.08 to 0.1 PSU) to those from Stn 1 (29.2 to 31.5 PSU) (Table 3). If our cultures were being seeded primarily by inactive spores, it is likely that we would observe a greater overlap in our culturing results for the different stations.

Molecular results from SSU rRNA and ITS1 region analyses support the idea of a transition in fungal community structure along the salinity gradient since a much narrower spectrum of taxonomic signatures was recovered from the fully marine sediments.

The same pattern was also confirmed using FISH and calcofluor staining since most of the fungal cells visualized were from brackish and freshwater sediment samples (Fig. 4). The observed conidia indicated the presence of fungi, although the fungal biomass appeared insignificant. Abundant visible aggregations of organic particles in our water samples resulted in a strong underestimation of fungal biomass using microscopy-based approaches since many cells were trapped in these micro- and macroaggregates. Moreover, many unstained dead/broken hyphae were also visualized in all sediment samples studied, which seems to indicate that some fungal populations settle from the water column into the sediments but have no ecological role in such environments. These results influenced our choice to work primarily with cDNA to reveal the molecular diversity of fungal communities in sediments since RNA-based approaches minimize the influence of dead cells.

Fungal abundance and diversity appeared to be inversely correlated with increasing salinity, as revealed recently in a study of saline, freshwater and brackish marshes, where fungal diversity and composition were influenced by both salinity, shaping the fungal composition, and the presence of plants, strengthening the fungal diversity (Mohamed & Martiny 2011). There are likely other factors that also influence fungal diversity and abundance, such as bacterioplankton (Grubisic et al. 2012) and eukaryotic photosynthetic producer communities (Gutiérrez et al. 2011) that are known to shape heterotrophic fungal communities in freshwater, brackish and marine sediment ecosystems.

Diversity of culturable fungi and tolerance to salinity

The decline in fungal diversity from more freshwater to fully marine samples is also reflected by the results of the culture-based studies. Isolations from the fresh waters at Stn 23 produced 14 different isolates, the brackish waters at Stn 11 produced 12, and the fully marine Stn 1 produced only 5 (Table 3). Three of the isolates overlapped between Stns 23 and 11 (*Fusarium oxysporum, Leptosphaeria maculans* and *Cadophora luteo-olivacea*), and only 1 isolate was recovered from water from Stns 11 and 1 (*Acremonium* sp.). Those overlapping isolates are congruent with their ecology since *F. oxysporum* and *L. maculans* are plant pathogens (Diener 2012, Pedras & Sarma-Mamillapalle 2012). *C. luteo-olivacea* has been described as a wood-destroying soft-rot fungus (Blanchette et al. 2004) and has also been isolated from lakes (Gonçalves et al. 2012). *Acremonium* represents a genus found in several marine environments, including deep-sea sediments (Singh et al. 2010) and seaweed (Zuccaro et al. 2008). Together with molecular results and the discussion above, this suggests that the active fungal community transitions in composition along this salinity gradient and that many fungi found in fresh waters are not adapted for survival in the fully marine environment. This is consistent with previous findings of a relatively simple fungal community composition in marine systems (based on fungal-specific clone library analyses), with relatively few phylotypes in total relative to terrestrial systems (Bass et al. 2007, Cathrine & Raghukumar 2009, Le Calvez et al. 2009, Edgcomb et al. 2011).

An increasing number of fungal isolates, filamentous fungi and yeasts were recovered from more fresh waters. Only 12 fungal strains were recovered from the fully marine Stn 1, while 63 and 66 strains were recovered from Stns 11 and 23, respectively. In agreement with Richards et al. (2012), this suggests that fewer fungi are adapted to life in fully marine waters and that a greater diversity exists in brackish and fresher waters. Our results support the notion of a gradient in fungal populations along this transect, with fungi that are adapted to marine conditions isolated from the fully marine site, non-halophiles isolated from the almost freshwater site, and some strains with modest abilities to tolerate saline conditions isolated from brackish conditions.

The discovery of halotolerant fungi of likely terrestrial origin is consistent with other studies of deep-sea and subsurface marine sediments where many signatures affiliated with clades of known terrestrial fungi (Edgcomb et al. 2011, Takishita et al. 2006). Some yeasts are known to be capable of altering their membrane composition to accommodate high hydrostatic pressure under short-term experimental conditions (Fernandes et al. 2004). Such an adaptation would be required of any terrestrial fungi dispersed to the marine environment and subsequently deposited in deep marine sediments. The chitin-rich cell walls of fungi likely confer an advantage when it comes to adapting to higher osmotic pressure and the stresses of osmotrophic feeding (reviewed by Richards et al. 2012).

Molecular diversity in sediments

The SSU rRNA analysis indicates 3 very different communities along this salinity gradient, but a community dominated in all cases by Dikarya (the vast majority of sequences affiliated with ascomycetes, followed by basidiomycetes) (Fig. 3). This is not surprising since the dominance of Dikarya has already been observed in deep oceans, deep-sea sediments and anoxic sediments (Bass et al. 2007, Edgcomb et al. 2011, Cathrine & Raghukumar 2009, Jebaraj et al. 2010). Sediment samples from Stn 2H (0.19 PSU salinity) produced the greatest diversity of SSU rRNA signatures based on cDNA, reflecting the presence of living *Basidiomycota* and *Ascomycota* (41 of 45 sequences). The 2 more saline sediment samples we analyzed from Stns 9H and 20H were dominated almost entirely by sequences affiliating with a much more restricted group of fungi. The nearest cultured relatives of the rRNA signatures from Stn 2H included different genera that are frequently retrieved from soils, decaying wood, stems and leaves and from leaf litter (Osono & Takeda 2007, Persson et al. 2009, Song et al. 2010). Isolates of *Westerdykella* have also been retrieved from mangrove mud (Stolk 1955), indicating that those ascomycetes are typical terrestrial/aquatic fungi. The fungal communities at Stn 2H seem to have originated from terrestrial sources but survive and grow in the freshwater environment. The dominance of *Ascomycota* in marine sediments was observed previously in the Central Indian Basin (Nagahama et al. 2001, Singh et al. 2011, 2012), contrary to a majority of *Basidiomycota* in deep-sea sediment cores of the Peru Margin and the Peru Trench (Edgcomb et al. 2011). This question of dominance may depend on sediment composition, i.e. the availability of different carbon sources (Edgcomb & Biddle 2012). However, it is quite hard to compare such studies that used different methodologies, i.e. culture-based and culture-independent methods, and also used different primers. Indeed, it has been shown that the described diversity mainly depends on the primers used (Stoeck et al. 2006). At Stn 9H, where the sediments had 1.5 PSU salinity, our clone libraries were dominated almost entirely (55 of 56 signatures) by relatives of *Nematoloma* and *Cortinarius*, basidiomycetes typically found living on dead wood. At Stn 20H, with 9.2 PSU salinity, our clone library included 57 sequences affiliated with only 3 genera: *Penicillium, Thysanophora* and *Aspergillus*. As discussed above, those species are fungi typically of terrestrial origin. At the marine Stn 20H, the fungal diversity appears lower than at Stn 2H. Such a result can be explained by the occurrence of more stringent environmental conditions (lower temperature, higher salinity and hydrostatic pressure) that likely result in fungal communities with decreasing representation from fungi of terrestrial origin.

The ITS1 rRNA analysis (DNA-based approach) supports the RNA-based and culture-based data since 3 very different sets of sequences were obtained (Table 4). Using this less-conserved genetic marker, the overall diversity revealed was lower, but the species composition was different: some basidiomycetous yeasts, 2 chytrids and 1 glomeromycete (*Scutellospora heterogama*) were revealed contrary to the SSU rRNA analysis. The ITS1 sequences we obtained were from the same types of terrestrial origins as many of the SSU rRNA sequences (phyllosphere, rhizosphere, soil, leaf litter and mangrove mud). For nearly 2 decades, eukaryotic phylogeny has been studied using SSU rRNA genes as the most common genetic marker. Recently, the ITS region was proposed as a universal DNA barcode marker for Fungi (Schoch et al. 2012) since the ITS region has the highest probability of successful identification for the broadest range of fungi. However, the suitability of the ITS region as a genetic marker is still debated due to several biases with ITS primers (Bellemain et al. 2010). Indeed, the ITS1 analysis (cDNA-based approach) produced a much more incomplete picture of diversity relative to the SSU rRNA gene in the present study (Table 5).

Fungi in the subsurface sediments seem to be more diverse than previously thought (Edgcomb et al. 2011) and could play a major role in biogeochemical cycles in the buried sediment biosphere, i.e. in denitrification processes (Cathrine & Raghukumar 2009, Mouton et al. 2012), hydrolysis and the metabolism of organic carbon.

Overlap between culture-based and culture-independent methods

Focusing on the degree to which cultured fungi appeared in the ITS1 and SSU rRNA clone libraries indicates a poor overlap between the fungi revealed using molecular vs. culture-based methods; only *Phoma* and *Trichoderma* were detected using both methods. Rarefaction curves (Fig. 3B) indicated that the sequencing effort was clearly insufficient to cover *in situ* diversity, and hence, only a fraction of the molecular diversity was revealed. Moreover, some technical biases are also inherent in culture-based studies, i.e. unculturable fungi, endophytes, cryptic

species, dormant spore germination, fast/slow growing strains, cryoconservation of sediment samples, etc., that explain this lack of overlap. Only 2 culture media were used in the present study, and fungi occurring in macro-aggregates may have difficulties growing in Petri dishes or marine broth (Damare & Raghukumar 2008). As a first investigation into the transitions within occurring and active fungal communities along a salinity gradient, the present study does indicate that fungal communities are not the same along this gradient and does provide evidence of active, truly marine or halotolerant fungi. Coupling a deeper sequencing effort and a more complex culture-based experimental plan in the future will certainly increase the overlap in fungal types detected using culture-based and molecular methods.

CONCLUSION

To determine what portions of fungi represent truly marine forms vs. inactive or active and highly adaptable, opportunistic, terrestrial organisms, we investigated fungi of the Delaware estuary. On the basis of our cultivations, microscopy (using FISH and calcofluor staining) and molecular work, we conclude that living and active fungal communities do exist in the water column and sediments of Delaware Bay, that communities in different salinity regimes are different and that there appear to be a majority of metabolically active marine fungi of terrestrial origin. Fungi may originate from terrestrial sources and be dispersed to fully marine water columns and sediments, where they are capable of surviving. The Delaware estuary community includes relatives of *Penicillium, Thysanophora* and *Aspergillus*, confirming previous studies that also showed these marine invaders in other waters (Raghukumar et al. 2004, Damare et al. 2006, Pindi 2012). In buried marine sediments, a fungal lifestyle may be advantageous, as attachment to larger physical substrates, such as buried organic material, and osmotrophy via secreted enzymes is much easier than in the water column, where needed nutrients and secreted enzymes can be lost more easily by diffusion. The mounting evidence for successful filamentous fungi and yeast forms in marine environments suggests that fungi may play a much more significant role in carbon cycling in the buried marine subsurface than previously thought. Finding a major group of halotolerant fungi in the Delaware estuary also suggests that fungi may be able to act in the marine environment, regardless of their point of origin.

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Appendix 1. Sequence-based identification of fungal strains

Station	Close neighbor	%ID	Environments	Accession no.	Source
23	*Fusarium oxysporum*	99.9	Endophytes	JF807401	Gao et al. (2011)
23	*Cryptococcus foliicola*	100	Plant leaves	AY557599	Wang et al. (2011)
23	*Tetrachaetum elegans*	99.1	Aquatic	AY357281	Belliveau & Bärlocher (2005)
23	*Leptosphaeria maculans*	99.6	Plant	LMU04238	Morales et al. (1995)
23	*Tolypocladium cylindrosporum*	99.5	Insects	AB208110	Yokoyama et al. (2006)
23	*Pochonia suchlasporia*	100	*Hevea brasiliensis*	FJ884150	Gazis & Chaverri (2010)
23	*Trichoderma citrinoviride*	100	Roots, soil, ants	HQ608144	Rodrigues et al. (2011)
23	*Phoma exigua*	100	Decaying wood	GU062320	Arhipova et al. (2011)
23	*Cadophora luteo-olivacea*	97.8	Fruits	GQ214538	Spadaro et al. (2010)
23	*Cephalosporium gramineum*	91.0	Plant	HQ322374	Wafai Baaj & Kondo (2011)
23	*Thelebolus* sp.	100	Polar environments	HQ533859	Gonçalves et al. (2012)
23	*Perisporiopsis* sp.	99.5	Endophytes	HM031458	Chaverri & Gazis (2011)
23	*Myrothecium* sp.	97.1	Plants	HQ631067	Shrestha et al. (2011)
23	*Fusarium* sp.	100	Plants	AY160209	Flowers et al. (2003)
11	*Fusarium oxysporum*	99.9	Endophytes	JF807401	Gao et al. (2011)
11	*Cystofilobasidium capitatum*	100	Soil, sediments	EU287890	Branda et al. (2010)
11	*Myrmecridium schulzeri*	99.5	Wheat straw	EU041774	Arzanlou et al. (2007)
11	*Bionectria ochroleuca*	100	Soil, plants	HQ115731	Gorfer et al. (2011)
11	*Nectria* sp.	100	Soil, rhizosphere	JF311955	Ellouze et al. (2013)
11	*Fusarium equiseti*	100	Soil, seed, dead stem	JQ234964	A. Jia et al. (unpubl.)
11	*Leptosphaeria maculans*	100	Plants	LMU04238	Morales et al. (1995)
11	*Arthrinium phaeospermum*	100	Common reed	AJ279456	Wirsel et al. (2001)
11	*Verticillium nigrescens*	99.5	Plants, rhizosphere	EF543851	Zare et al. (2007)
11	*Ulocladium chartarum*	100	Soil, wood, plants	JN578634	Cueva et al. (2012)
11	*Acremonium* sp.	100	Soil, wood, plants	JN578630	Cueva et al. (2012)
11	*Cadophora luteo-olivacea*	99.3	Fruits	GQ214538	Spadaro et al. (2010)
1	*Pichia spartinae*	99.8	Marine	FJ432595	W. Cui et al. (unpubl.)
1	*Kluyveromyces marxianus*	100	Marine	EU807912	J. Zheng et al. (unpubl.)
1	*Acremonium* sp.	100	Mangrove	JN687975	Cueva et al. (2012)
1	*Rhodotorula dairenensis*	100	Soil	JN246550	Coelho et al. (2011)
1	*Coniothyrium fuckelii*	100	Endophytes, soil	AB665313	F. Tomita et al. (unpubl.)

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