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ORIGINAL ARTICLE

Patterns of fungal diversity and composition along a salinity gradient

Devon J Mohamed[1,2] and Jennifer BH Martiny[2]
[1]*Department of Ecology and Evolutionary Biology, Brown University, Providence, RI, USA and* [2]*Department of Ecology and Evolutionary Biology, University of California, Irvine, CA, USA*

Estuarine salinity gradients are known to influence plant, bacterial and archaeal community structure. We sequenced 18S rRNA genes to investigate patterns in sediment fungal diversity (richness and evenness of taxa) and composition (taxonomic and phylogenetic) along an estuarine salinity gradient. We sampled three marshes—a salt, brackish and freshwater marsh—in Rhode Island. To compare the relative effect of the salinity gradient with that of plants, we sampled fungi in plots with *Spartina patens* and in plots from which plants were removed 2 years prior to sampling. The fungal sediment community was unique compared with previously sampled fungal communities; we detected more Ascomycota (78%), fewer Basidiomycota (6%) and more fungi from basal lineages (16%) (Chytridiomycota, Glomeromycota and four additional groups) than typically found in soil. Across marshes, fungal composition changed substantially, whereas fungal diversity differed only at the finest level of genetic resolution, and was highest in the intermediate, brackish marsh. In contrast, the presence of plants had a highly significant effect on fungal diversity at all levels of genetic resolution, but less of an effect on fungal composition. These results suggest that salinity (or other covarying parameters) selects for a distinctive fungal composition, and plants provide additional niches upon which taxa within these communities can specialize and coexist. Given the number of sequences from basal fungal lineages, the study also suggests that further sampling of estuarine sediments may help in understanding early fungal evolution.
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Introduction

The distribution of biodiversity along environmental gradients such as latitude, altitude, productivity and salinity has been of long-standing interest to ecologists (Fisher, 1961; Rosenzweig, 1995). Most of what is known about how biodiversity varies along environmental gradients stems from research on plants and animals, although recent studies suggest that microorganisms may exhibit similar gradient patterns. Within the bacteria, for instance, diversity and composition appear to vary along gradients of primary productivity (Horner-Devine *et al.*, 2003), latitude (Blum *et al.*, 2004; Yergeau *et al.*, 2007), disturbance (Johnsen *et al.*, 2001; Muller *et al.*, 2001), climate (Yergeau *et al.*, 2007) and salinity (Blum *et al.*, 2004; Crump *et al.*, 2004; Bernhard *et al.*, 2005, 2007). However, even less is known about the variation of fungal diversity and composition along similar gradients.

Correspondence: JBH Martiny, Department of Ecology and Evolutionary Biology, University of California, 321 Steinhaus, Irvine, CA 92697-2525, USA.
E-mail: jmartiny@uci.edu
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Fungi are ubiquitous and diverse; estimates of global fungal diversity range upward of 1.5 million species (Hawksworth, 2001, 2004). At a local scale, fungal diversity has important consequences for plant communities and ecosystems (van der Heijden *et al.*, 2008). For example, higher saprotrophic fungal diversity increases decomposition rates (Setala and McLean, 2004; Tiunov and Scheu, 2005), and higher mycorrhizal richness increases plant diversity, ecosystem productivity and nutrient capture (van der Heijden *et al.*, 1998). Despite their importance for ecosystems, few studies have considered which factors generate and maintain fungal diversity. In general, fungal diversity or composition is thought to be influenced by nitrogen availability (Allison *et al.*, 2007), resource supply (Waldrop *et al.*, 2006), atmospheric CO_2 concentration (Klamer *et al.*, 2002) and soil depth (O'Brien *et al.*, 2005).

Salinity, in particular, has been implicated as a major factor regulating bacterial composition and diversity across many different habitats (Bernhard *et al.*, 2005; Lozupone and Knight, 2007), and therefore a salinity gradient is likely to influence fungal diversity. In estuaries, the mixing of marine and fresh waters results in a striking gradient of salinity that varies, often in complex, nonlinear ways (Ewing, 1986), with organic matter,

plant biomass, sulfide, oxygen and other abiotic parameters (Odum, 1988). Salinity is correlated with changes in bacterial (Crump *et al.*, 2004; Bernhard *et al.*, 2005) and archaeal community composition (Walsh *et al.*, 2005). In addition, marsh plant composition shifts dramatically over the estuarine gradient, and overall plant diversity increases with decreasing salinity (Crain *et al.*, 2004). Generally, plants are thought to strongly influence soil microorganisms through the quantity and quality of resources they return to soils and sediments. In particular, fungal abundance often increases with plant diversity (Kowalchuk *et al.*, 2002; Zak *et al.*, 2003; van der Heijden *et al.*, 2008). Greater plant diversity may also increase fungal diversity by increasing microclimate variability, habitat complexity and organic substrate diversity, but experiments that seek to link plant diversity with fungal diversity report inconsistent findings (Kowalchuk *et al.*, 2002; Brodie *et al.*, 2003; Waldrop *et al.*, 2006; Artz *et al.*, 2007).

Previous studies on marsh fungi focus on aboveground communities, which remineralize and transform decaying leaf litter and standing-dead plant material (Buchan *et al.*, 2003). Generally, it is assumed that fungi are less active in anoxic sediments; however, studies document their presence in these habitats and indicate that they can account for up to 10% of the microbial biomass in surface sediments (Padgett and Celio, 1990; Cordova-Kreylos *et al.*, 2006; Ipsilantis and Sylvia, 2007). In addition, mycorrhizal fungi have recently been shown to interact with *Spartina patens* roots in salt marshes (Burke *et al.*, 2003) and to influence plant zonation (Daleo *et al.*, 2008).

In this study, we investigate whether the diversity and composition of sediment fungal communities varies along a salinity gradient. We sampled three marshes—a salt, brackish and freshwater marsh—spanning Narragansett Bay, RI and the Barrington River estuary. We also compared the relative effect of abiotic factors that covary along the salinity gradient with the presence of plants. To do this, we sampled fungal communities in plots with plants (holding plant composition constant across the gradient) and in plots in which plants were experimentally removed 2 years prior to sampling. We hypothesized that fungal diversity (both richness and evenness) would decrease in marshes of increasing salinity, regardless of the presence or absence of plants. Alternatively, fungal diversity might be highest in the salt marsh with plants present, as the prominence of positive interactions, such as those between mychorrizal fungi and plants, typically increases with the severity of the abiotic environment (Bertness and Callaway, 1994). Indeed, arbuscular mychorrizae are abundant in salt marshes and can alter plant composition (Burke *et al.*, 2003; Daleo *et al.*, 2008). Finally, fungal diversity might peak at an environment of intermediate salinity, in the brackish marsh, because of the mixing of specialized freshwater and marine communities (Crump *et al.*, 2004). Below we interpret differences in the fungal communities between the salt, brackish and freshwater marshes as due to salinity or factors that closely covary with salinity. It is important to note, however, that these differences could also be due to other intrinsic differences between these three particular marshes. Ideally, one would carry out the same experiments in multiple, independent estuaries, although this is rarely possible in estuarine studies because of limited resources. Thus, future studies along salinity gradients are needed to corroborate the evidence presented here.

Materials and methods

Study sites and experimental manipulation

Sediment samples were collected from three tidal marshes along a salinity gradient in Rhode Island, USA. These marshes were chosen because, for their given salinities, their plant composition is representative of southern New England marshes (for further site descriptions, see Crain *et al.*, 2004). Further, our study takes advantage of experimental plots that were initiated for a plant succession study (Crain *et al.*, 2008). The salt marsh is located on Prudence Island in the Narragansett Bay National Estuarine Research Reserve exposed to full-strength seawater (sediment salinities range from 27 to 33 p.p.t. throughout the plant growing season). The brackish marsh is 16.1 km from the salt marsh along the Barrington River, which empties into Narragansett Bay, with sediment salinity ranging from 15 to 25 p.p.t. (Crain *et al.*, 2004). The oligohaline—or for simplicity, what we term 'freshwater'—marsh (0–10 p.p.t.) is 1.25 km upriver of the brackish marsh (1.1 km as the crow flies), in the uppermost tidal reaches of the Barrington River. The plant removal experiment was conducted in the *S. patens* zone of each of these marshes (Crain *et al.*, 2008). *S. patens* is common to the mid-tidal zone of marshes of all salinities in southern New England. In the salt and brackish marshes, more than 95% of the plant cover in this zone was *S. patens*. *Salicornia maritima* and *Distichlis spicata* were also present in this zone in the salt marsh, and *D. spicata*, *Pluchea odorate* and *Schoenoplectus robustus* were present in this zone in the brackish marsh. The freshwater marsh was more diverse, with *Agrostis stolonifera*, *Solidago sempervirens, Eleocharis* spp. and *Argentina anserina* growing in the *S. patens* zone.

In each marsh, three plant removal plots (1 m^2) were established at least 2 m apart from one another in the *S. patens* zone as described in Crain *et al.* (2008). In August 2003, the systemic herbicide Roundup (ScottsMiracle-Gro, Marysville, OH, USA) was applied at low tide to kill above- and belowground plant material. The herbicide was applied during peak growing season and effectively

killed both above- and belowground plant tissue. In October 2003, plots were cleared of all standing plant matter using a string trimmer. The aboveground plant material was removed from the plots, whereas the belowground material was left intact. In early spring 2004, black weed cloth was pinned with garden staples into the marsh surface to prevent regrowth in the plots.

In summer 2005, we sampled sediment from the nine plant removal plots (three replicates in three marshes) and adjacent monotypic stands of *S. patens* at each marsh, for a total of 18 samples. At each plot, we aseptically collected a 1-cm-diameter sediment core to a depth of 1 cm with the top half of a cut 5-ml sterile pipette tip. All cores were stored on ice in the field and returned to the lab for immediate storage at −80 °C.

DNA extraction and PCR amplification

Whole community DNA was isolated from the uppermost 0.5 g of each sediment core using the FastDNA spin kit for soils (Qbiogene, Inc., Montreal, Canada), according to the manufacturer's instructions. An approximately 760-bp region of the 18S ribosomal gene was amplified from these extracts using the general fungal primers nu-SSU-0817-5′ (5′-TTAGCATGGAATAATRRAATAGGA-3′) and nu-SSU-1536-3′ (5′-ATTGCAATGCYCTATCCCCA-3′) (Borneman and Hartin, 2000). We targeted the relatively conserved 18S so that we could examine the phylogenetic placement of the sequences from these understudied sediment communities. However, this region masks finer-scale genetic diversity that would be detected using a less conserved marker such as the internal transcribed spacer (ITS).

PCR amplification was performed in triplicate 25 μl reaction volumes containing a solution of 15 μl Premix F (Epicentre Biotechnologies, Madison, WI, USA), 14 μl sterile water, 0.5 μl (25 pmol) of both the forward and reverse primers, 0.25 μl of Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 1.25 μl of template DNA. PCR was carried out on an iCycler thermocycler (Bio-Rad, Hercules, CA, USA) using the following protocol: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (1 min at 94 °C), primer annealing (1 min at 56 °C) and extension (1 min at 72 °C), with a final extension of 10 min at 72 °C. Aliquots of PCR products were checked by electrophoresis on 1% agarose gels.

Clone library construction

We constructed clone libraries from each of the 18 sediment samples for the analysis of small-subunit rRNA genes. For each sample, amplified products from three replicate PCRs were pooled together and purified using the QIAQuick PCR purification kit (Qiagen Inc., Valencia, CA, USA). Purified amplicons were cloned into *Escherichia coli* using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Two steps of the manufacturer's protocol were modified: 6 μl of the purified PCR product was ligated and the transformation was incubated for 90 min. Sequencing of 96 clones per library was performed by Cogenics (Houston, TX, USA) using M13F primers.

Sequence editing

The 18 clone libraries yielded a total of 1728 sequences. Vector contamination was removed from all sequences using the automated vector trimming function in Sequencher (Gene Codes, Ann Arbor, MI, USA). We identified and excluded from analyses 110 chimeric sequences by using the Chimera Check Program on the Ribosomal Database Project (Cole *et al.*, 2005). We also removed 158 short sequences, 231 sequences that contained unalignable inserts and 132 poor-quality sequences from further analysis. The remaining 1097 sequences were aligned and manually edited in ARB (Ludwig *et al.*, 2004). These sequences were submitted to the GenBank under accession numbers FJ482264–FJ483360.

Operational taxonomic unit diversity and composition analyses

A distance matrix of the aligned sequences was generated with Dnadist using the default F84 evolutionary model that allows for different rates of transition and transversion (Felsenstein, 2005). We used the furthest-neighbor algorithm in DOTUR (Schloss and Handelsman, 2005) to designate operational taxonomic units (OTUs) at four genetic resolutions: ⩾90%, 95%, 97% and 99% sequence similarity. We then used EstimateS (version 8; RK Colwell, http://purl.oclc.org/estimates) to calculate several diversity indices and estimators for each OTU cutoff (Shannon's diversity index, Simpson's diversity index, Jack1 richness estimator, Chao1 richness estimator and number of singletons (Colwell and Coddington, 1994). For the Shannon and Simpson indices and observed (Coleman) richness, we report 'rarified' values at the lowest common sample size of 38 sequences. For samples with more than 38 sequences, this value is an average of the random selection of 38 sequences from the larger pool of sequences over 100 runs. We tested the influence of marsh type and plant treatment on richness and evenness with two-way analysis of variance (ANOVA) tests in JMP (SAS, Cary, NC, USA).

To compare the taxonomic composition of the 18 samples, we calculated the Bray–Curtis similarity index (Magurran, 1988) between all pairwise samples for each of the four sequence similarity cutoffs. We tested the significance of these patterns with an analysis of similarity test (PRIMER v5, PRIMER-E, Ivybridge, UK; Clarke, 2001). The analysis of similarity *R*-value represents the difference between two or more groups of samples on a scale

from 0 (indistinguishable) to 1 (all similarities within groups are less than any similarity between groups). We used *post-hoc* comparison tests to determine which treatments drove the overall differences.

Phylogenetic analyses

A representative sequence from each OTU defined at $\geqslant$95% similarity was randomly selected and compared using the BLASTn algorithm with known organisms in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). This OTU cutoff was chosen to reduce the number of sequences in the phylogenetic tree, while still capturing the range of diversity observed. We recorded the phylum of the cultured isolate that had the highest sequence similarity to each query.

To further explore the taxonomic identity of the sequences, we constructed a phylogenetic tree with the OTU representatives and the AFTOL 18S rDNA sequences (James *et al.*, 2006b). Our sequences aligned to positions 2657–7766 of this database, and we removed two AFTOL sequences with poor coverage in this region (leaving 219 AFTOL sequences). We then used the RAxML algorithm and Web servers (Stamatakis *et al.*, 2008) to find the best-scoring maximum likelihood tree with bootstrap values. We displayed the results using the Interactive Tree of Life (Letunic and Bork, 2007).

We used UniFrac to test for differences in phylogenetic representation of fungi between marshes and plant treatments (Lozupone and Knight, 2005; Lozupone *et al.*, 2006). UniFrac calculates a metric comparing the sum total branch length for all the sequences from each sample with the branch length shared by each sample pair. This metric represents the degree of divergence between sequences present in each sample, without the assignment of sequences to OTUs. To test whether phylogenetic composition is significantly different among the three marshes and plant treatments, the metric value was compared with that calculated from 100 random permutations of the original phylogenetic tree.

Finally, we used principal coordinate analysis to visualize the differences in phylogenetic composition between the various treatments. Principal coordinate analysis reduces the dimensionality of the phylogenetic distance matrix (representing the compositional distance between each pair of samples) into a smaller number of uncorrelated variables (principal coordinates). We graphed our samples in a two-dimensional space, in which the axes are the first and second principal coordinates, the two variables that account for the highest amount of variation in the distance matrix.

Results

To examine the phylogenetic breadth of the fungal sediment communities, the 1097 sequences were classified into 104 OTUs (defined at 95% sequence similarity). The basal regions of the fungal tree of life (that is, below the Ascomycota and Basidiomycota) remain poorly resolved outside of the Glomeromycota and core Chytridiomycota (the euchytrids, sensu James *et al.*, 2006b). Comparison with the AFTOL sequences (James *et al.*, 2006b) suggested that the sequences can be classified into Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota and three other basal fungal lineages (BFL1–3) (Figure 1; Supplementary Figure 1). (The phylogenetic analysis also revealed that two sequences assigned to two different OTUs were not fungi, and these were removed from further analyses.)

The majority of the fungal sequences (78%) cluster within the phylum Ascomycota and, specifically, the subphylum Pezizomycotina (Figure 1). Only one of these 853 sequences falls with the subphylum Saccharomycotina. The second most abundant group, including 9% of the sequences, is BFL1 (albeit with low bootstrap support). Only two AFTOL sequences cluster with this group, *Rozella allomycis* (Chytridiomycota) and *Smittium culisetae* (Zygomycota). However, a previous study including five genes in addition to 18S rDNA demonstrates that these organisms are likely not closely related (James *et al.*, 2006b). The 28 OTUs in this unidentified group share only 86.9–93.2% sequence similarity to known cultured isolates representing a variety of phyla (Supplementary Table 1), including those from two newly established phyla, Blastocladiomycota (James *et al.*, 2006a) and Neocallimastigomycota (Hibbett *et al.*, 2007).

The remaining groups, Basidiomycota, Glomeromycota, Chytridiomycota and BSL2/BSL3, include 6%, 4%, 4% and 0.5% of the sequences, respectively (Figure 1). The vast majority (59 out of 62) of the Basidiomycota sequences appear to fall into the subphylum Agaricomycotina, with three additional sequences grouping mostly closely with representatives from the Pucciniomycotina.

The distribution of sequences among these taxonomic groups was relatively similar between the freshwater, brackish and salt marshes (Figure 2). Notably, however, the highest proportion of Chytridiomycota sequences was detected in the salt marsh samples (7% vs 3% and 1% in the freshwater and brackish marshes, respectively). In addition, the freshwater marsh samples contained a higher proportion of sequences from the BFL1 than the brackish or salt marshes (12% vs 8% and 5%, respectively).

OTU diversity

Across marshes (lumping sequences from the plant and plant removal plots), observed fungal richness for OTUs $\geqslant$99% similar was highest in the brackish marsh; 113 OTUs were observed in the brackish marsh compared with 79 and 83 OTUs in the salt and freshwater marsh, respectively. Rarefaction

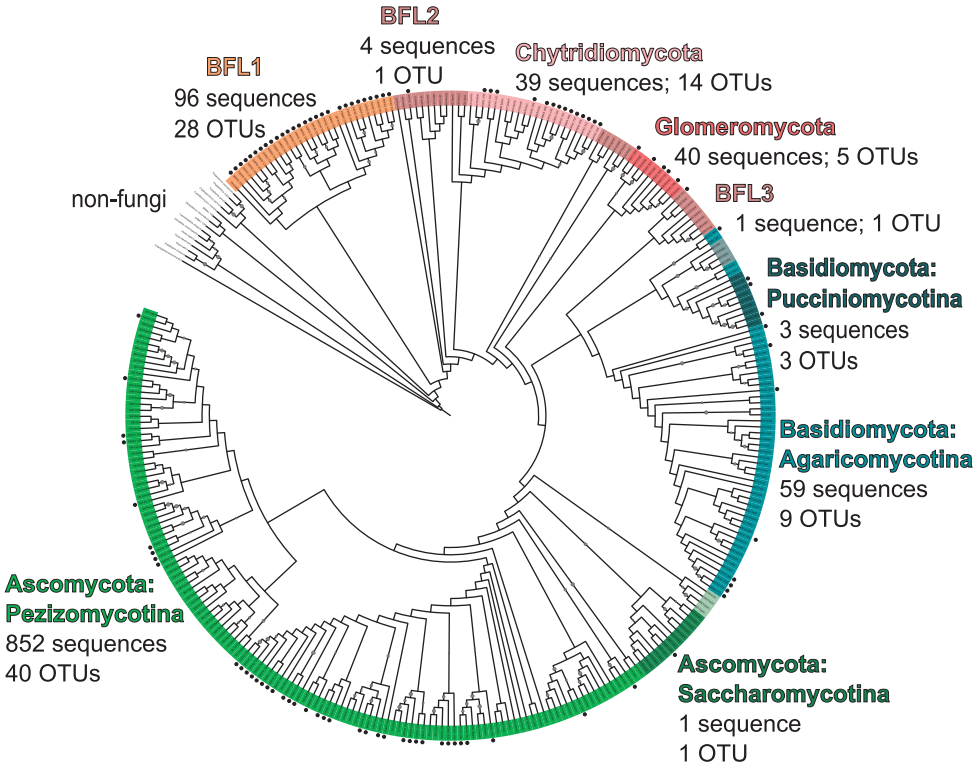

Figure 1 The branching order of the best scoring maximum likelihood tree, including the 104 OTU representatives from this study plus 219 AFTOL sequences. The major taxonomic groups found in this study are labeled, and the number of sequences and OTUs within each group is noted. The black dots at the tips denote OTU representatives from this study. Supplementary Figure 1 is an enlarged version of the tree.

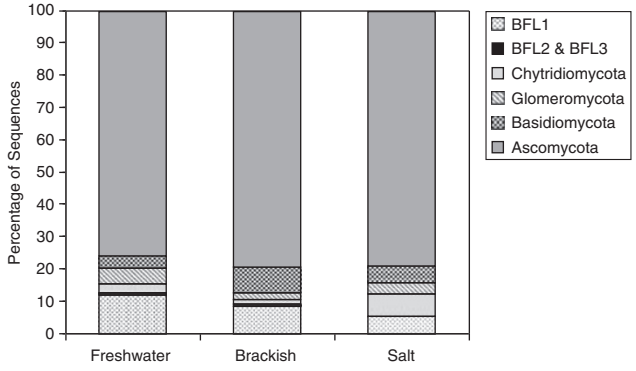

Figure 2 The percentage of sequences recovered from the five major groups in the freshwater, brackish and salt marshes.

curves suggest that this difference is significant (that is, the confidence intervals do not overlap; Figure 3a). The rarefaction curves did not reach an asymptote, indicating that further sampling would have revealed additional diversity at this genetic resolution. In contrast, the observed richness of broader fungal taxa (defined at $\geqslant$97%, $\geqslant$95% or $\geqslant$90% similar) did not vary significantly between the marshes (Figure 3b). Further, the 95% OTU rarefaction curves appear to level off, suggesting that we sampled most fungal taxa at this broader scale.

At the 99% cutoff, evenness indices and richness estimators tended to be higher in the brackish marsh and similar in both the salt and freshwater marsh (Table 1 and Figure 4), although none of these measures were significantly different between marshes (Supplementary Table 2). At lower sequence similarity cutoffs, this trend among marshes disappeared (Figure 4 and data not shown).

Fungal diversity (richness and evenness) was significantly higher in plots with plants than without, whether measured by the Chao1 estimator (Figure 4) or the four other richness and evenness measures tested (Supplementary Table 2). Further, this difference in diversity was apparent at all OTU resolutions, although less so at the broadest taxonomic level ($\geqslant$90% sequence similarity). Only the Jack1 estimator was higher in the plots with plants than without (ANOVA: $P<0.02$). Finally, the influence of plants on fungal diversity in the surrounding marsh sediments did not vary between the marshes—that is, there was no interaction between plant presence and marsh salinity (ANOVA: $P\geqslant0.32$ for all five measures at all four similarity cutoffs; Supplementary Table 2).

OTU composition

Fungal composition, as measured by the relative abundance of the OTUs, was significantly different

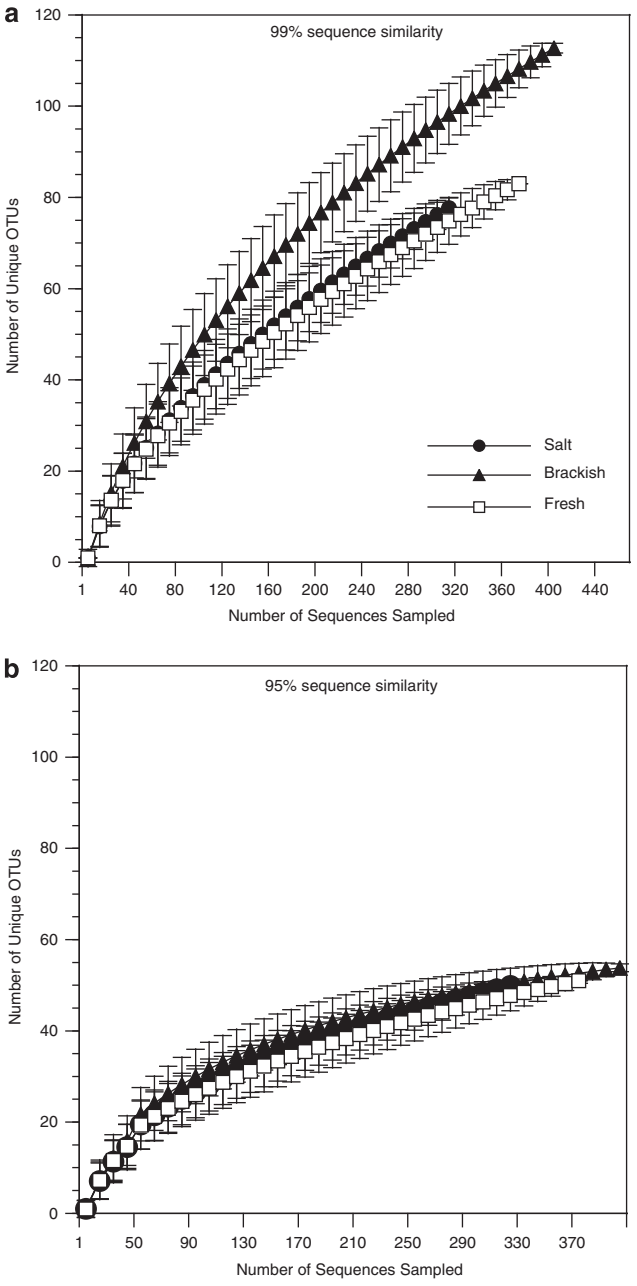

Figure 3 Coleman rarefaction curves for salt, brackish and freshwater marshes (plant and plant removal plots combined) for operational taxonomic units defined by $\geqslant$99% sequence similarity (**a**) and $\geqslant$95% sequence similarity (**b**). Error bars represent 95% confidence intervals. For clarity, every tenth sample was plotted.

between the three marshes at all OTU resolutions (analysis of similarity test; 99% cutoff: $R=0.65$, $P=0.001$; 97% cutoff: $R=0.59$, $P=0.001$; 95% cutoff: $R=0.51$, $P=0.001$; 90% cutoff: $R=0.26$, $P=0.034$). Pairwise comparisons revealed that this result was driven by differences between all marshes (for instance, at 99% cutoff, freshwater and brackish: analysis of similarity $R=0.72$, $P=0.01$; freshwater and salt: $R=0.74$, $P=0.01$; brackish and salt: $R=0.46$, $P=0.02$). In contrast, the presence or absence of plants had only a marginal influence on OTU composition at the finest level of taxonomic resolution (99% cutoff: $R=0.19$, $P=0.08$) and did not influence fungal composition at broader taxonomic levels (97% cutoff: $R=0.16$, $P=0.14$; 95% cutoff: $R=0.01$, $P=0.5$; and 90% cutoff: $R=0.09$, $P=0.27$).

Phylogenetic composition

Similar to OTU composition, phylogenetic composition of the fungal communities was significantly different among the three marshes (UniFrac metric, $P<0.001$). In contrast to the OTU analyses, however, plant presence significantly altered fungal phylogenetic composition (UniFrac metric, $P<0.001$). The first two principal coordinates explained 50% of the variability in phylogenetic composition among the samples (Figure 5). The first principal coordinate axis (explaining 25.73% of the variation) was associated with both marsh type and the presence/absence of plants (marsh: $F=19.0$, $P<0.01$; plant: $F=12.4$, $P<0.01$). In particular, fungal composition was similar in both the brackish and freshwater marsh, and the salt marsh was significantly different from both marshes (Tukey's honest significant difference: $Q=2.67$, $P=0.05$). Along the second principal coordinate (explaining 24.28% of the variation), the samples were separated by the presence or absence of plants in the brackish and salt marshes. Overall, however, this axis was not significantly related to marsh type or the presence/absence of plants ($F_{5,17}=0.08$, $P=0.99$).

Discussion

Our study provides evidence that fungal diversity—that is, taxonomic richness and evenness—is greatly influenced by the presence of plants and much less so by salinity or other covarying variables. In the three marshes in this study, fungal OTU diversity (defined at 95%, 97% or 99% cutoff) was significantly higher in association with plants than in bare sediments. This result suggests that sediment fungi, like soil fungi, specialize on unique niches provided by plants. In soils, plants supply organic substrates through root exudates (Zak *et al.*, 2003) and increase microclimate variability and habitat complexity (Waldrop *et al.*, 2006). In sediments, plants greatly alter the physical environment by reducing surface temperatures and surface salinity and oxygenating the sediment, as well as altering the supply of organic and inorganic substrates.

We detected a trend of higher fungal richness at the intermediate salinity (the brackish marsh), similar to that seen in estuarine bacterioplankton (Crump *et al.*, 2004). However, these differences were only statistically significant for observed OTU richness at the finest genetic resolution (99%). This result suggests that the traits that allow for higher

Table 1 Summary statistics for each marsh and plant treatment at the 99% sequence similarity cutoff

Marsh	*Plant treatment*	*No. of sequences*	*No. of OTUs*	*No. of singletons*	*Shannon's index (*H*')*	*Simpson's index (*D*)*	*Coleman richness*
Salt	Plants	186	65	43	2.58 ± 0.19	15.37 ± 5.04	18.55 ± 2.01
Salt	Plant removal	136	27	12	1.96 ± 0.25	7.04 ± 2.90	12.38 ± 1.13
Brackish	Plants	204	82	45	2.90 ± 0.18	30.44 ± 13.57	22.90 ± 2.63
Brackish	Plant removal	201	61	35	2.19 ± 0.54	14.29 ± 9.75	16.93 ± 4.18
Fresh	Plants	167	60	37	2.52 ± 0.09	10.82 ± 2.08	18.94 ± 0.89
Fresh	Plant removal	203	41	23	1.98 ± 0.33	6.22 ± 2.55	13.62 ± 3.18

Abbreviation: OTU, operational taxonomic unit.
The number of sequences, OTUs and singletons are summed across the three replicates for each marsh and plant treatment category. The mean (and s.d.) of the Shannon's index, Simpson's index and Coleman richness observed for the plots in each category are reported. These numbers are rarefied to 38 sequences, the lowest number of sequences available from one of the 18 plots. This procedure standardizes the measures for sampling effort and allows one to compare the relative values among the marsh-plant treatment categories.

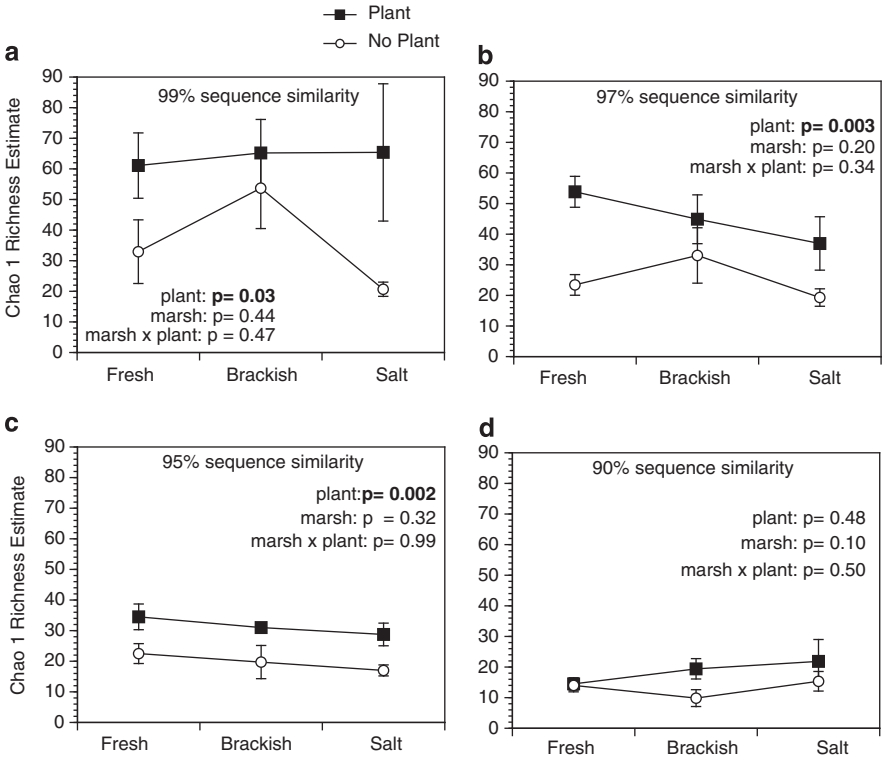

Figure 4 Chao 1 richness estimates of fungal diversity at 99% sequence similarity (**a**), 97% (**b**), 95% (**c**) and 90% (**d**) for both plant (filled squares) and plant removal (open circles) plots. Error bars represent ± 1 s.e. around the mean of the three plots. *P*-values from two-way ANOVA are inset.

numbers of fungi to coexist (theoretically because of greater resource partitioning) at intermediate salinities vary at a relatively fine genetic scale. Thus, we hypothesize that had we targeted a less conserved genetic marker than the 18S rDNA gene, we would have observed a stronger correlation between fungal diversity and salinity. Indeed, many studies that detect significant treatment effects on fungal diversity target the more variable ITS region (O'Brien *et al.*, 2005; Allison *et al.*, 2007; Artz *et al.*, 2007). Because diversity estimators such as Chao1 depend on sample size, the absolute number of OTUs estimated would likely increase with further sampling (Colwell and Coddington, 1994; Hughes *et al.*, 2001). However, the conclusions above focus on the relative diversity between samples, and relative rankings using diversity statistics can be stable even with substantial undersampling of microbial communities (Shaw *et al.*, 2008).

In contrast to diversity, sediment fungal composition appears to be primarily influenced by the salinity gradient, and secondarily by the presence of plants. Fungal composition varied significantly among the marshes, regardless of the way in which fungal composition was measured. For instance,

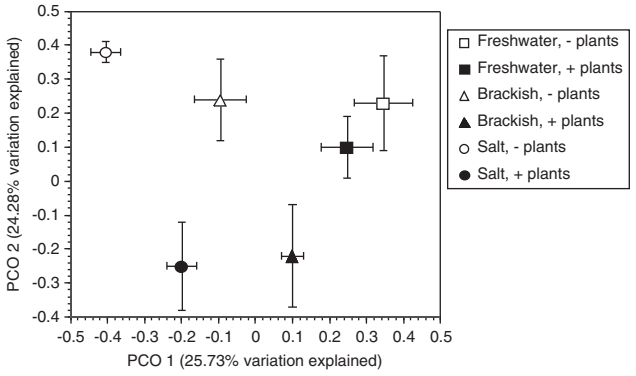

Figure 5 Scatterplot of principal coordinate 1 (PCO 1) vs principal coordinate 2 (PCO 1). The percent of variation explained by each principal component is indicated on the axis labels.

Chytridiomycota sequences were more abundant in the salt marsh samples, whereas BFL1 sequences were more abundant in the freshwater marsh samples. Similarly, OTU composition (at all genetic resolutions) and overall phylogenetic composition (as determined by the UniFrac analysis) varied significantly between the marshes along the salinity gradient. These results are consistent with patterns of bacterial composition; across a variety of habitats, the primary determinant of bacterial composition appears to be salinity, rather than other factors such as temperature, pH or geography (Lozupone and Knight, 2007). Several studies have described the mixing of freshwater and marine bacterioplankton along estuarine gradients (Bouvier and del Giorgio, 2002), Crump *et al.* (2004) report evidence of a unique bacterioplankton community that formed at intermediate salinities. To our knowledge, this pattern has not been detected before with sediment-associated microbes. The fact that fungal composition varied at all levels of taxonomic resolution along the salinity gradient suggests that fungal adaption to salinity (or covarying environmental parameters) occurs at many genetic scales (Martiny *et al.*, 2009). We hypothesize that the salt marsh environment selects for fungi with physiologies that have adaptive value in this habitat, such as perhaps anaerobic respiration. However, until we know more about the basic biology of these novel taxa, it is unclear whether salinity (or some other covarying factor) is causing the observed compositional shifts.

Although the presence of plants selected for a different phylogenetic structure of fungal communities than occurred in bare sediments (as per the UniFrac analysis), these differences appeared to occur primarily at the finest genetic resolution (99% cutoff). Further, the difference between fungal composition in the plant and plant removal plots increases with increasing salinity (Figure 5). Salt marsh sediments are characterized by high salinity, high sulfide concentrations and waterlogging. Perhaps plants have a differentially high impact on fungal communities in saline environments because they alleviate some of these stressors.

Fungal diversity in sediments and saline environments is generally assumed to be low because of low plant diversity and the physiological constraints of submersion in water (Shearer *et al.*, 2007). In fact, the sediment fungal communities along this salinity gradient appear to be highly diverse and quite distinctive in composition compared with other environments. For instance, in temperate forest soil, O'Brien *et al.* (2005) primarily found a mixture of Ascomycota and Basidiomycota (35% and 34%, respectively) and few Glomeromycota or Chytridiomycota (4% and 1%, respectively). In comparison, our study recovered more Ascomycota (78%), fewer Basidiomycota (6%) and a greater number of basal fungal lineages (16%), including Glomeromycota, Chytridiomycota and BFL1-3. The Chytridiomycota include saprobes, pathogens and parasites and are common inhabitants in marine and freshwater environments; thus, it is not surprising to detect them in relatively high abundances in marshes. However, most of the 'lower' fungal sequences observed are in BFL1. These are highly divergent from cultured isolates and likely represent novel fungi. It is tempting to speculate that sequences in this group—and the better-supported subgroups within the group—are fungi that specialize in aquatic sediments. These sequences also suggest that estuarine sediments are a good location for further fungal sampling. More knowledge of these groups may aid in understanding early fungal evolution.

In conclusion, estuarine sediments appear to harbor an unusual community of fungi that may include taxa that specialize on saline or submerged habitats. Like plants and other microorganisms, the composition of these communities appears to be greatly altered by changes in the abiotic conditions along a salinity gradient (Crain *et al.*, 2004; Crump *et al.*, 2004; Bernhard *et al.*, 2005; Walsh *et al.*, 2005; Sahan and Muyzer, 2008). Sediment fungal diversity (richness and evenness) varies little across the salinity gradient, whereas the presence of plants greatly increases fungal diversity regardless of salinity. Taken together, the particular estuarine conditions appear to select for distinct fungal communities, and plants provide additional niches upon which taxa within these communities can specialize and coexist. Given how little is known about the taxa detected in this study, however, the functional consequences of these different fungal communities are unknown.

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