

MOLECULAR DESCRIPTION OF ASCOMYCETE FUNGAL COMMUNITIES ON

SPARTINA SPP. IN THE U.S.

by

JUSTINE ISABELLE LYONS

(Under the Direction of Merryl Alber)

ABSTRACT

Ascomycetous fungi play a crucial role in the decomposition of salt marsh vegetation. The first part of this research used molecular methods to examine whether physical associations exist between individual bacterial and common ascomycetous fungal species that co-occur on decaying smooth cordgrass, *Spartina alterniflora*, in a southeastern U.S. salt marsh. We found that bacterial communities were unaffected by the identity of initial fungal decomposers, suggesting that few species-specific associations exist between those members of the *Spartina* decomposer community. The next part of the study involved a characterization of ascomycetes involved in the decomposition of different species of *Spartina* in different environments. In California, the fungi associated with *S. alterniflora*, *S. foliosa*, the hybrid between them, and *S. densiflora* were all characterized using terminal restriction fragment length polymorphism (T-RFLP) analysis of their internal transcribed spacer (ITS) region of rRNA genes as well as clone libraries. Although we found no effect from the hybridization of two host species, we did see significant differences in the fungal decomposer communities both within and among species. *S. densiflora* hosted several unique ascomycetes. Two previously described ascomycetes, *Phaeosphaeria spartinicola* and *Mycosphaerella*, were ubiquitous on all samples analyzed. On the east coast, ascomycete communities on samples of *S. alterniflora* and *S. patens* collected in four states (Georgia, North Carolina, New York, and Massachusetts) were compared, again using T-RFLP analysis of the interspacer region. Results show that diversity of the ascomycete taxa on *S. patens* hosts a higher number of unique ascomycete species than *S. alterniflora*, and that it has significantly higher diversity, but there were no consistent differences among states. *P. spartinicola* and *Mycosphaerella* again dominated most T-RFLP profiles. The data suggest that two fragments (147 and 149 bp), although not confirmed to represent separate taxa, were specific to host plant species. Preliminary data from samples of the same species of grass collected in states along the Gulf coast (FL, LA, MS, AL) confirm all conclusions drawn for samples collected along the east coast, including the higher diversity on *S. patens*, omnipresence of *P. spartinicola* and *P. halima*, and species-specificity of fragments at 147 and 149 bp.

INDEX WORDS: Ascomycete, *Spartina*, fungal diversity, prokaryotic decomposition

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DEDICATION

I dedicate the ambition and commitment behind this work to my daughter Sophia, who is the light and love of my life. I learn from her as much every day as she learns from me. I also dedicate it to my father, Lionel Lyons, and to my grandfather, George Oliver, whose love and pride I continue to feel and whose souls will always be in my heart.

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CHAPTER 1

INTRODUCTION

Background

Salt marshes are intertidal habitats that are globally important for their economic and ecological value. Marshes improve water quality by retaining pollutants and excess nutrients. They provide habitat for numerous species of organisms, including many that are commercially important. Salt marsh plants help stabilize the soil, thereby helping to prevent erosion, and they also provide a buffer for coastal floods. They are also highly productive. Primary producers in marshes include benthic and epiphytic microalgae, phytoplankton, benthic macroalgae and vascular plants.

The dominant vascular plant in eastern Atlantic coast marshes of the U.S.A. is smooth cordgrass (*Spartina alterniflora* Loisel), which contributes approximately 81% of the total primary production in east coast intertidal marshes (17). Estimates of primary production for *Spartina* in Georgia marshes range from 1,100 to 7,600 g dry mass·m⁻²·yr⁻¹ (18). In Massachusetts' Great Sippewissett Marsh, Teal and Howes (19) measured average annual biomass production of various *S. alterniflora* forms. Aboveground, short form *S. alterniflora* produced from 370-501 g·m⁻² annually while creek bank forms produced from 1,023-1,623 g·m⁻²; belowground *S. alterniflora* produced 872-1,021 g C·m⁻² annually.

Although *S. alterniflora* dominates eastern U.S. coastlines, several other species of *Spartina* flourish in the U.S. *Spartina patens*, also known as Saltmeadow cordgrass or Marsh Hay, is native to the Atlantic coast but found along the east, Gulf, and west coasts of the U.S. *Spartina foliosa*, the California cordgrass, is native to salt marshes on the west coast. *S. densiflora*, or Denseflower cordgrass, is native to Chile but has spread north along the Pacific coast of the U.S and is considered invasive in California. As these different species have spread, several viable hybrids (ie *S. alterniflora* x *S. foliosa*) have formed.

As *Spartina* plants decay, their detritus becomes a large and renewable pool of organic material. When the leaves of *Spartina* cease photosynthesizing, they die in succession from the base of the plant toward the tip of the shoot (6). Dead leaf blades are not abscised from the plant; rather, they remain upright and attached to the shoot as they undergo early stages of decay. These attached, dead leaf blades are referred to as standing dead leaves. When compared with live *S. alterniflora*, standing dead leaves comprise 65.5 % of the standing grass biomass (18). During later stages of decay, these leaves bend toward the sediment where they will further decompose.

Most *Spartina* detritus is comprised of lignocellulose (7), which is degraded primarily by microbes since it is highly refractory, not easily leached, and inaccessible to most other organisms. By fueling microbial metabolism, decomposition of plant tissues becomes a major route for carbon remineralization in aquatic and terrestrial ecosystems (11, 12). Although both bacteria and fungi are integral components of the *Spartina* decay system (1), the majority of *S. alterniflora* above-ground biomass decomposes aerielly rather than in a submerged state (15), and the early stages of aerial decomposition are

dominated by ascomycetous fungi (14). Fungal mycelium can be found on the surface, in the hollow center of the culm, and intra- and extracellularly within the tissues of *S. alterniflora* (5). During decomposition, fungi colonize senescing blades of *Spartina*, where they break down the lignin polymer into smaller, low molecular weight fragments that are then available either for conversion into fungal biomass, or for further decomposition by bacteria (13, 14). Despite their critical roles, little is known about the type and extent of interactions among fungi and bacteria involved in decomposition.

Several methods have been used to identify plant-associated fungi. The most traditional methods are those of direct identification. Ascospore taxonomy and direct microscopy of leaf surfaces are commonly employed to identify fungi based upon morphology. Other traditional methods combine culturing with direct identification. Ascospore-drop procedures, conidia micromanipulation, and ascospore nichrome needle micromanipulation are all culture-dependent identification techniques. Recently, however, molecular tools have been developed to identify fungi. Buchan et al. (4) used terminal restriction fragment length polymorphism (TRFLP) analysis of fungal internal transcribed spacer (ITS) regions of rRNA genes to describe the ascomycete population living within senescent *Spartina alterniflora* leaves. This technique complements results found by more traditional methods; however, once methods are optimized molecular techniques can provide for rapid sample processing.

Both molecular and traditional culture methods indicate the presence of ascomycetes on senescent *S. alterniflora* leaves in east coast (primarily Georgia) marshes. The two most common species, *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. 2, are often found in association with each other (2, 4, 5, 9, 10, 13,

16). Other common fungal species in this system include *Phaeosphaeria halima*, *Buergenerula spartinae*, and an unidentified ascomycete called '4clt' (4). While these studies (3, 4, 8) described the ascomycete communities present on *S. alterniflora* from the east coast, little work has been done looking at the diversity of the decomposer communities on different *Spartina* species or on *Spartina* from other areas.

Overview

This study used molecular techniques (PCR, T-RFLP) to examine the interactions between the fungi and bacteria involved in the decomposition of *S. alterniflora* and to describe the ascomycete communities associated with various *Spartina* species. In Chapter 2, we address the question of whether physical associations exist between individual bacterial and fungal species that co-occur on decaying smooth cordgrass, *Spartina alterniflora*, in a southeastern U.S. salt marsh. In Chapters 3 and 4 we used PCR and T-RFLP to survey the ascomycete populations on samples of early decay leaf blades collected from several species of *Spartina* in several geographic locations. In Chapter 3 we compared the decomposer ascomycete communities on *S. alterniflora*, *S. foliosa*, *S. densiflora*, and a *S. alterniflora* x *S. foliosa* hybrid in California. Our intent was to answer the following questions: 1) are the ascomycetous fungal communities on *Spartina alterniflora* transplanted to the west coast similar to those already described on the east coast?, 2) do variations in the ascomycete communities exist within and among *Spartina* species? and 3) how did hybridization between invasive *S. alterniflora* and native *S. foliosa* change or affect the associated resident fungi? In Chapter 4, we collected samples of *Spartina alterniflora* and *S. patens* from sites in Georgia, North

Carolina, New York, and Massachusetts to investigate whether the ascomycete community is species-specific among *Spartina* species, and whether the same dominant species of fungi are found on plants growing in different geographic regions. As part of this final chapter we also did a preliminary analysis of samples from the Gulf coast, collecting samples of *S. alterniflora* and *S. patens* from sites in Florida, Alabama, Mississippi, and Louisiana to investigate whether the same fungal species occurred on marsh grasses in this region.

Taken together, this thesis provides a comprehensive investigation of the ascomycete decomposer communities on standing dead *Spartina*.

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CHAPTER 2

SCREENING FOR BACTERIAL-FUNGAL ASSOCIATIONS IN A SOUTHEASTERN U.S. SALT MARSH USING PRE-ESTABLISHED FUNGAL MONOCULTURES¹

¹ Lyons, J.I., S.Y. Newell, R.P. Brown, M.A. Moran. 2005. Screening for bacterial-fungal associations in a southeastern U.S. saltmarsh using pre-established fungal monocultures. *FEMS Microbiol. Ecol.* 54:179-187.

Abstract

Both bacteria and fungi play critical roles in decomposition processes in many natural environments, yet only rarely have they been studied as an integrated community. We examined whether physical associations exist between individual bacterial and fungal species that co-occur on decaying smooth cordgrass, *Spartina alterniflora*, in a southeastern U.S. salt marsh. Fungal-infiltrated decaying *Spartina* was used as “bait” for potential bacterial associates. The bundles (infiltrated with one of four dominant fungal members of the decomposer assemblage, or an autoclaved control) were placed in a salt marsh and collected biweekly for eight weeks during the first experiment (late summer 2002), and weekly for four weeks during the second experiment (early summer 2003). Terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes was used to track establishment of bacterial taxa in association with the established fungal species. T-RFLP analysis of 18S-to-28S internal transcribed spacer (ITS) regions was used to monitor changes in fungal communities once bundles had been placed in the field. Results from both years were nearly identical, and showed that invasion by fungi other than the bait species was slow, resulting in a virtual fungal monoculture throughout the time period of the experiments. Surprisingly, bacterial communities were unaffected by the identity of the fungal bait. Regardless of the fungal species, and even in the absence of prior fungal colonization, bacterial 16S rRNA profiles were remarkably similar. These results suggest that few species-specific associations, either positive or negative, exist between bacterial and fungal members of the *Spartina* decomposer community during initial colonization.

Introduction

The intertidal marshes of the southeastern U.S. support a diverse community of heterotrophic microorganisms that play critical roles in marsh decomposition activities. Prokaryotic and eukaryotic decomposers are major links in the mineralization and transformation of organic matter (25) and interact with marsh plants and animals through nutrient regeneration and food web interactions. Despite these important roles, little is known of the type and extent of interactions among prokaryotic and eukaryotic members, or how such interactions may influence carbon and nitrogen cycling in the marsh ecosystem.

Salt marsh fungi colonize senescing blades of the dominant primary producer, *Spartina alterniflora*, leading to fungal productivity that can average $\sim 550 \text{ g m}^{-2} \text{ y}^{-1}$ (22). As the aerobic filamentous fungi decompose plant matter, they efficiently break down the lignocellulose polymer into smaller, low molecular weight fragments that are then available either for conversion into fungal biomass or for further decomposition by bacteria (22, 25).

Previous research using culture- and molecular-based methods has shown that ascomycetes, including *Phaeosphaeria halima*, *P. spartinicola*, *Buergenerula spartinae*, *Mycosphaerella* sp. 2, and taxonomically undescribed species '4clt' (5, 23) dominate the fungal component of the *Spartina* decomposer community on Sapelo Island. Fungal mycelia can be found on the surface of decaying *S. alterniflora* blades, as well as in the hollow center of the culm, and intra- and extracellularly within the tissues (10). Most evidence suggests that fungi use nonspecific, extracellular enzymes to modify the lignocellulose complex in the decaying plant material (10).

The bacterial assemblage of decomposing *S. alterniflora* is dominated by α -*Proteobacteria*, with secondary contributions from γ -*Proteobacteria*, *Bacteroidetes*, and Gram positive taxa (4, 5). The bacterial assemblage associated with decomposing *S. alterniflora* makes small contributions to total particulate organic mass (21) yet large contributions to the degradation of lignocellulose carbon (1, 24).

Several recent studies have taken steps towards describing the microbial communities associated with decaying saltmarsh grasses (4, 5, 23). Characterization of decomposer communities has been improved by the growing availability of DNA-based methods that do not require cultivation of microorganisms, including taxonomic gene markers to characterize species diversity (4, 5) and functional gene markers to characterize genomic diversity (3, 17). Here we use molecular methods to begin to explore the types and extent of interactions between members of the decomposer community. We ask whether physical associations, which may reflect ecological interactions, occur between prokaryotic and eukaryotic *S. alterniflora* decomposers. Two “baiting” experiments were conducted in which bundles of leaf blades of *S. alterniflora* that had first been infiltrated with one of four dominant fungal members of the decomposer assemblage were placed in a southeastern U.S. salt marsh and sampled periodically to identify bacterial colonizers. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes was used to follow colonization and establishment of bacterial communities on the *S. alterniflora* bundles, and T-RFLP analysis of 18S-to-28S internal transcribed spacer (ITS) regions was used to monitor changes in the ascomycete fungal community during the experiment.

Materials and Methods

Bundle preparation and deployment. The study was performed twice, once in September 2002 (mean temperature 27.0°C) and once in June 2003 (mean temperature 26.7°C) (temperature data available at: http://climate.engr.uga.edu/sapelo_island/daily_2000s.html). All methods except for collection times were identical.

Ascomycetes *Phaeosphaeria spartinicola* (Psp), *Phaeosphaeria halima* (Ph), and *Mycosphaerella* sp. 2 (My) had previously been isolated from the study site and were maintained on dilute V8-agar (DV8) plates (5). Cultures were propagated by transferring agar plugs from plates of isolates onto new DV8 plates. Four plates were cultivated per species and allowed to grow for four weeks until they covered the entire plate (60 x 15 mm).

Dead “early-decay” blades of *S. alterniflora* (brown to black in color and remaining attached to the stem) were collected from Dean Creek Marsh, Sapelo Island, GA, in spring each year. Dean Creek Marsh is a Georgia Coastal Ecosystems Long Term Ecological Research sampling area and is typical of southeastern U.S. salt marshes (6) (maps available at <http://gce-lter.marsci.uga.edu/lter/asp/studysites.htm>). Blades were cut into 5 cm long pieces and autoclaved at 121°C for 30 minutes in 50 ml centrifuge tubes. To keep the blades moist, 5 ml of artificial seawater (salinity=15) were also added to each tube before autoclaving. Six blade sections (one bundle) were autoclaved in each tube.

After autoclaving, each bundle was inoculated with one of the three fungal isolates (*Phaeosphaeria halima* [Pha], *Phaeosphaeria spartinicola* [Psp], *Mycosphaerella* sp. 2 [My]), taken as an agar plug from DV8 plates. All inoculations were performed using aseptic technique in a laminar flow hood. Bundles were incubated at room temperature under ambient daylight

conditions until the fungus had visibly infiltrated the entire length of the *S. alterniflora* bundle. Fifteen bundles were inoculated for each fungal species (treatments Pha, Psp, My). Fifteen additional bundles were left uninoculated (treatment Un).

In both years (2002 and 2003), two “initial” bundle samples of each fungal species were kept for immediate analysis to determine whether the pre-established fungal biomass was a pure culture. These samples were never placed in the salt marsh. Two uninoculated bundles were also analyzed to verify that no unintentionally included fungal species had contaminated the bundles. These bundles had been sterilized and incubated in centrifuge tubes for the four week period, but had never been inoculated and were never placed in the field.

Of the remaining bundles, twelve of each species were placed in a *S. alterniflora* marsh at Dean Creek. One bundle at a time was removed from its centrifuge tube, attached with a cable tie to a 0.6 m long segment of 1.2 cm diameter PVC pipe and then immediately placed in the marsh. The bundle was oriented so that the bottom of the bundle was ~1 cm above the sediment when the pipe was inserted into the ground. Twelve uninoculated bundles were also attached to PVC pipes and placed in the marsh.

Three bundles for each species, plus three uninoculated bundles, were collected biweekly for six weeks in 2002 (T2, T4, T6) and weekly for three weeks in 2003 (T1, T2, T3). Three T0 samples for both years were also collected immediately after all samples had been placed in the marsh (approximately one hour after initial deployment). In 2003, a visible fungal contaminant was found to have invaded three of the bundles inoculated with *P. halima* prior to placement in the marsh. These bundles were discarded and the T0 time point was left out for the *P. halima* treatment in 2003.

Bundles were collected by cutting the cable tie and placing the bundle into a sterile 50 ml centrifuge tube. Latex gloves were worn and changed between collection of each fungal treatment. Bundles were transported on ice and stored at -20°C.

DNA extraction and T-RFLP analysis. Genomic DNA was extracted from the bundles of decaying *S. alterniflora* using Soil DNA Extraction Kits (MoBio, Solana Beach, CA). Fungal internally transcribed spacer (ITS) regions (2, 9) were amplified with the ascomycete-specific primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4A (5' CGCCGTTACTGGGGCAATCCCTG 3') (15). These primers amplify a product of 630 bp, including the ITS1, 5.8S, and ITS2 regions of the rRNA operon. A previous study found no evidence for variation due to multiple ITS sequences within a single individual for the salt marsh fungi used in this study (5). Bacterial 16S rRNA genes were amplified with general bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3', where M is A or C) and 1522R (5'-AAGGAGGTGATCCANCCRCA-3', where N is A, T, C, or G and R is A or G) (12). The ITS1F and 27F primers were fluorescently labeled on the 5' end with FAM (carboxyfluorescein).

In 2002, all PCR amplifications were carried out using a MasterAmp™ PCR Optimization Kit (Epicentre Technology, Madison, WI). In 2003, all PCR amplifications were carried out with Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, N.J.), except those for *Mycosphaerella* sp.2, for which the MasterAmp™ PCR Optimization Kit was used due to difficulty with amplification. All PCR reactions contained 0.2 µM concentration of each primer and 50-100 ng of DNA. Thermal cycling reactions for ITS amplifications consisted of an initial 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 s at 52°C, and 1 min at 72°C. Conditions for 16S rRNA gene amplification began with an initial 3 min at 95°C, followed by 25

cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. For both cycling reactions, a final step of 10 min at 72°C was included to complete any partial polymerizations. Products of the appropriate size were recovered from a 1% agarose gel with a QiaSpin gel extraction kit (Qiagen, Valencia, Calif.).

For terminal restriction fragment length polymorphism (T-RFLP) analysis (16), restriction enzyme digestion of the PCR product was carried out in a 20- μ l total volume containing 100 ng of purified PCR product and either 10 U of *Cfo*I (16S ribosomal DNA [rDNA] sequences) or 10 U of *Hae*III (ITS sequences) at 37°C for 3 h. Digested DNA was precipitated in ethanol and suspended in 12.5 μ l of deionized formamide with 1 μ l of DNA fragment length standard Gene-Scan-2500 TAMRA (tetramethylrhodamine; Applied Biosystems). The terminal restriction fragment (T-RF) lengths were determined on an ABI PRISM 310 in GeneScan mode.

T-RFLP output data were analyzed using a Visual Basic program that reconciles minor shifts in fragment sizes between successive chromatograms (28). Peaks comprising <1% of total chromatogram area were excluded from the analysis. Dendograms were created using the Primer 5 software (Plymouth Marine Laboratory, Plymouth, UK).

Although triplicate samples were collected, initial T-RFLP results were so consistent within treatments that molecular analysis of only two of the three replicates was carried out for each data point.

Results

Fungal community. T-RFLP analysis of ITS region amplicons was used to confirm that the baited bundles supported a fungal monoculture at the time of deployment in the marsh for both the 2002 and 2003 experiments. The characteristic terminal restriction fragment (T-RF) of the single species inoculated onto the bundles (72 bp for *P. spartinicola*, 410 bp for *Mycosphaerella* sp. 2, and 630 bp for *P. halima*) was the only fragment evident in the T-RFLP chromatograms for all initial samples (Fig. 1A) as well as at the T0 time point in both years.

Patterns of invasion by other salt marsh fungi were followed by analysis of bundles retrieved at one or two week intervals after deployment. In 2002, when bundles were retrieved biweekly over a six week period, T-RFs representing the fungal inoculum still accounted for >92% of the total chromatogram area at the T2 collection. By T4, additional peaks were evident in some chromatograms, although they still composed a relatively minor fraction (<5%) of the total area. Based on two previous studies that identified T-RFs of the dominant fungal groups associated with decaying *Spartina alterniflora* in the Sapelo Island marshes (4, 5), the colonizing fungi were identified as ascomycete species '4clt' (5) and *Buergenerula spartinae*. By T6, all baited bundles contained '4clt' and *B. spartinae* peaks in addition to peaks indicative of the original fungal inoculum (Fig. 1B-D).

For the initially uninoculated bundles in 2002, T-RFs representing *P. spartinicola* (72 bp) and three additional fungi (131 bp, 144 bp, and 392 bp) appeared by T2. The T-RF at 144 bp has been found previously on decaying *S. alterniflora* and represents one of three morphologically cryptic subgroups of *Mycosphaerella* sp. 2 (along with T-RFs at 410 and 428 bp) (5). The 131 bp and 392 bp T-RFs have not been identified. By T6, uninoculated bundles also yielded T-RFs

indicative of '4clt' and *P. halima* (Fig. 1E). These profiles were nearly identical to those of natural standing dead *Spartina* detritus collected from the marsh at the same time (Fig. 1G) except that the latter contained an additional *Mycosphaerella* peak at 410 bp. Thus colonization of *S. alterniflora* detritus was nearly complete within 6 weeks of deployment in the marsh for uninoculated material, but was still heavily biased toward the inoculated species for the baited bundles (Table 1).

In 2003, samples were collected weekly over a three week period to obtain finer resolution of the earliest stages of fungal invasion and bacterial colonization. T-RFs representing the fungal inoculum still accounted for >85% of the total chromatogram area at all three time points (T1, T2, and T3) (Figure 1F, Table 1). For the initially uninoculated bundles, T-RFs representing *P. halima* were evident at T1 and T2, and by time point T3, T-RFs representing *P. spartinicola*, '4clt', *B. spartinae*, and *P. halima* had appeared. As was found for the 2002 experiment, the uninoculated bundles supported a more diverse community of fungi than the inoculated, and the inoculated bundles remained dominated by a single fungus over a several week period in the marsh.

Bacterial community. In both years, bacterial communities became established on the bundles early in the experiment. 16S rRNA genes could be amplified from bundles after deployments as short as one hour (at T0). All of the dominant bacterial T-RFs that colonized the baited bundles were found in a previous study identifying bacterial taxa associated with decaying *S. alterniflora* in the Sapelo Island marshes (4). These include a predominance of α -*Proteobacteria* represented by T-RFs 56, 79, 231, 346, 369, and 517, as well as one γ -*Proteobacteria* (203 bp),

and one *Bacteroidetes* (92 bp). A novel, unidentified T-RF at 557 bp was present in almost all samples and particularly prominent in 2003 (Fig. 2).

After the initial colonization of the *Spartina* detritus, the bacterial community changed relatively little over time (Fig. 2). By T2, the dominant taxa were typically established in proportions similar to those of the final time point (Fig. 2). α -*Proteobacteria* taxa accounted for 24 to 63% of the T2 samples, and 36 to 76% of the final time point samples (T6 in 2002 and T3 in 2003). Over all time points in 2002 and 2003, the *Bacteroidetes* T-RF accounted for an average of 7 and 12% and the γ -*Proteobacteria* T-RF accounted for an average of 14 and 17% of the total chromatogram area, respectively. Over 80% of the chromatogram area represented typical detritus-associated bacterial taxa (as identified by Buchan et al. (4)) at the final time point in 2002 (T6), and over 65% at the final time point in 2003 (T3). Only at T0, when samples had been in the marsh for 1 hour, were atypical T-RFs present in chromatograms (20-83% of chromatogram area; not shown).

We had hypothesized that distinct bacterial communities would become established on bundles pre-colonized by different fungal monocultures, but this was not the case. Except for the T0 samples, bacterial community profiles were extremely similar across bundles regardless of the dominant fungal species that also occupied the detritus. Cluster analysis using the T-RFLP data matrix (peak area) as the input data set confirmed that bacterial communities established on all three fungal treatments were similar for both 2002 (Fig. 3) and 2003 (Fig. 4) experiments. For inoculated bundles, bacterial communities were intermingled on the dendrogram regardless of the fungal treatment. Uninoculated bundle communities were also intermingled in the dendrogram clusters with the inoculated bundle communities. Only in the case of the initial

samples (which were never placed in the marsh) were the T-RFLP chromatograms distinct, and in this case all were devoid of amplifiable 16S rDNA (Figs. 3 and 4).

Discussion

Bacteria and ascomycetous fungi co-occur on decomposing *S. alterniflora* blades in southeastern U.S. salt marshes, where both groups are known to play major roles in decomposition (1, 11, 22). The physical proximity of bacteria and fungi during decomposition suggests the possibility of ecological interactions between the two groups. For example, the invasive nature of hyphae allows fungi to initiate physical disruption of intact plant material that may benefit bacteria (23, 25), while bacteria can provide growth factors and soluble nitrogen to adjacent fungi (7) or produce antimicrobial compounds that inhibit fungal growth (14, 26). Thus interactions might include competitive relationships, predator/prey associations, or metabolic synergisms that accelerate decomposition (25, 29).

As a step toward identifying potential ecological interactions, we used an established fungal monoculture on *S. alterniflora* detritus to encourage recruitment of bacterial taxa that interact directly or indirectly with the fungus. Results suggest, however, that the composition of the bacterial community was unaffected by the established fungus, an outcome that was consistent across two independent experiments in 2002 and 2003. Further, detrital bundles with no pre-established fungus supported communities that were indistinguishable from those with fungi, likewise suggesting that establishment of the bacterial community was independent of the fungal community. The similarity of bacterial communities regardless of the fungal inoculum was not attributable to invasion of the other major fungi into the detritus bundle once deployed in

the field, since the inoculated species remained the dominant fungus on the bundles throughout both experiments. Indeed, it appears that the bacterial community is *Spartina*-adapted, as indicated by the fact that it replaced a significant fraction of the bacterial assemblage that appeared in the T0 samples.

Other studies have observed antagonistic or competitive interactions between fungi and bacteria on decomposing leaf litter, but few signs of synergistic relationships. For example, Gulis and Suberkropp (13) found that aquatic hyphomycetes isolated from leaves decaying in a freshwater stream inhibited bacterial growth of co-occurring Gram-negative bacteria. Similarly, Mille-Lindblom and Tranvik (19) found increased biomass of fungi and bacteria isolated from *Phragmites* when each was grown alone compared to when the two microbes were grown together. We previously found that the presence of natural bacterial communities decreased rates of fungal degradation of *S. alterniflora* lignocellulose, although they apparently increased the efficiency of processing of dissolved decomposition intermediates (18). Results from the present study suggest that the observed effects were more likely due to non-specific interactions between the groups, rather than direct interactions involving particular species of bacteria and fungi. For example, it has been suggested that bacteria and fungi attack different components of plant material (20, 27) and therefore may interact in more general ways to influence decomposition rates.

In this study, the only major effect of pre-colonization with a single fungal species was on the diversity of the fungal members of the decomposer community. Fungal invasion into pre-colonized detritus was slow, and most samples remained dominated by the original fungal inoculum throughout the experiments. Bundles not inoculated with a fungus (Fig. 1E) had final T-RFLP profiles more similar to natural *S. alterniflora* detritus, with the exception that another

of the *Mycosphaerella* sp. 2 subgroups (410 bp) was present in the natural samples (Fig. 1G). At this same time point in 2002, fungal T-RFLP profiles on baited bundles typically consisted of the fungal bait species, with minor contributions from '4clt' and *B. spartinae* (Fig. 1B-D). These two species have previously been shown to be temporally and spatially transient, with the former being particularly abundant on leaves in later stages of decay (4, 23). After three weeks incubation in 2003, fungal profiles consisted almost entirely of the fungal bait species (Fig. 1F). Slow invasion of baited bundles may reflect both the extent of colonization prior to deployment and the naturally slow growth rates of ascomycetous fungi. The course of invasion by other species in 2002 and 2003 argues that replacement of the original monoculture was occurring on the time scale of weeks to a few months.

Overall, whether baited, uninoculated, or natural, T-RFLP profiles showed slow fungal invasion with only five previously identified species appearing in slightly varying combinations. This is comparable to a study by Buchan et al. (4), which found that *P. spartinicola*, *P. halima*, *Mycosphaerella* sp. 2 and "4clt" together accounted for ~88% of the total T-RFLP chromatogram area in >70% of samples collected from Dean Creek. It appears that the native ascomycete assemblage is strongly enough adapted to the standing-decay environment that it outcompetes occasional colonizers that can appear early in the fungal-invasion process. This is evidenced, for example, by the appearance of two unknown T-RFs (131 and 392 bp) that appeared at T2 in 2002, but not thereafter.

Likewise, the bacterial communities that established on the pre-colonized *Spartina* detritus (Fig. 2) consisted largely of taxa that were identified in a previous seasonal study of natural marsh detritus (4). Thus detritus-associated bacterial communities appear to be quite stable in this ecosystem, at least over the 3-year period (2000-2003) during which these two

studies were conducted. How these communities compare to those established on detritus of other marsh species is not known, but a study by Dang and Lovell (8) found that inert surfaces incubated in a salt marsh tidal creek were also dominated (43%) by α -*Proteobacteria* related to those found here.

T-RFLP analysis of ITS regions and 16S rRNA genes proved to be an effective tool to survey the composition of fungal and bacterial communities across treatments and over time. While T-RFLP analysis may be susceptible to PCR biases that could affect the representation of microbial taxa, it is appropriate for comparative studies. Individual bacterial T-RFs do not necessarily map to a single species, as in the case of several genera within the α -*Proteobacteria* (i.e. *Roseovivax*, *Rhodovulum*, and *Stappia*) that share a *CfoI* restriction site at 517 bp although their 16S rRNA gene sequences have similarities as low as 76% (4, 8). However, the fungal ITS-based T-RFs have been shown to agree well with both microscopy- and culture-based methods, and have proven informative at the species, and sometimes even subspecies level (5).

In summary, this study used colonization of baited detritus to investigate the extent to which bacterial and fungal decomposers form an integrated and interacting community during *Spartina* decomposition. Bacterial colonization patterns provided little evidence that bacteria and fungi establish species-specific ecological associations on the *Spartina* blades, despite the fact that they co-occupy and co-degrade the material. Interactions between bacterial and fungal decomposers that are not manifested as consistent physical associations between species were not addressed here.

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TABLE 2.1. Relative contributions of fungal species to the 2002 and 2003 baited *S. alterniflora* detritus bundles at the final time point (six weeks in 2002; three weeks in 2003) based on percent of total T-RFLP chromatogram area. Dashes indicate that peaks comprised <1% of total chromatogram area. Percentages are averages of two samples.

Inoculum	<i>P. halima</i> (630 bp)	<i>P. spartinicola</i> (72 bp)	<i>Mycosphaerella</i> sp. 2 (144 bp)	<i>Mycosphaerella</i> sp. 2 (410 bp)	Species '4clt' (123 bp)	<i>B. spartinae</i> (150 bp)
2002						
<i>P. halima</i>	42%	--	--	--	38%	12%
<i>P. spartinicola</i>	7%	51%	--	--	28%	5%
<i>Mycosphaerella</i>	--	--	34%	--	34%	26%
Uninoculated	14%	30%	42%	--	8%	--
Natural field	2%	39%	28%	26%	2%	--
2003						
<i>P. halima</i>	100%	--	--	--	--	--
<i>P. spartinicola</i>	--	100%	--	--	--	--
<i>Mycosphaerella</i>	--	--	--	98%	--	--
Uninoculated	--	21%	49%	--	12%	11%

Figure 2.1. Chromatograms of fungal T-RFs. (A) Overlay of chromatograms of 'initial' bait bundles, showing single peaks for each inoculum. (B-E) Bundles inoculated with *P. halima*, *P. spartinicola*, *Mycosphaerella*, and uninoculated bundles collected after six weeks in 2002. (F) Final time point (3 weeks) of bundle baited with *P. spartinicola* in 2003. (G) Natural sample of *S. alterniflora* standing dead leaves in 2002. Coverage percentages and T-RF sizes are listed in Table 1.

Terminal restriction fragment (T-RF) size (bp)

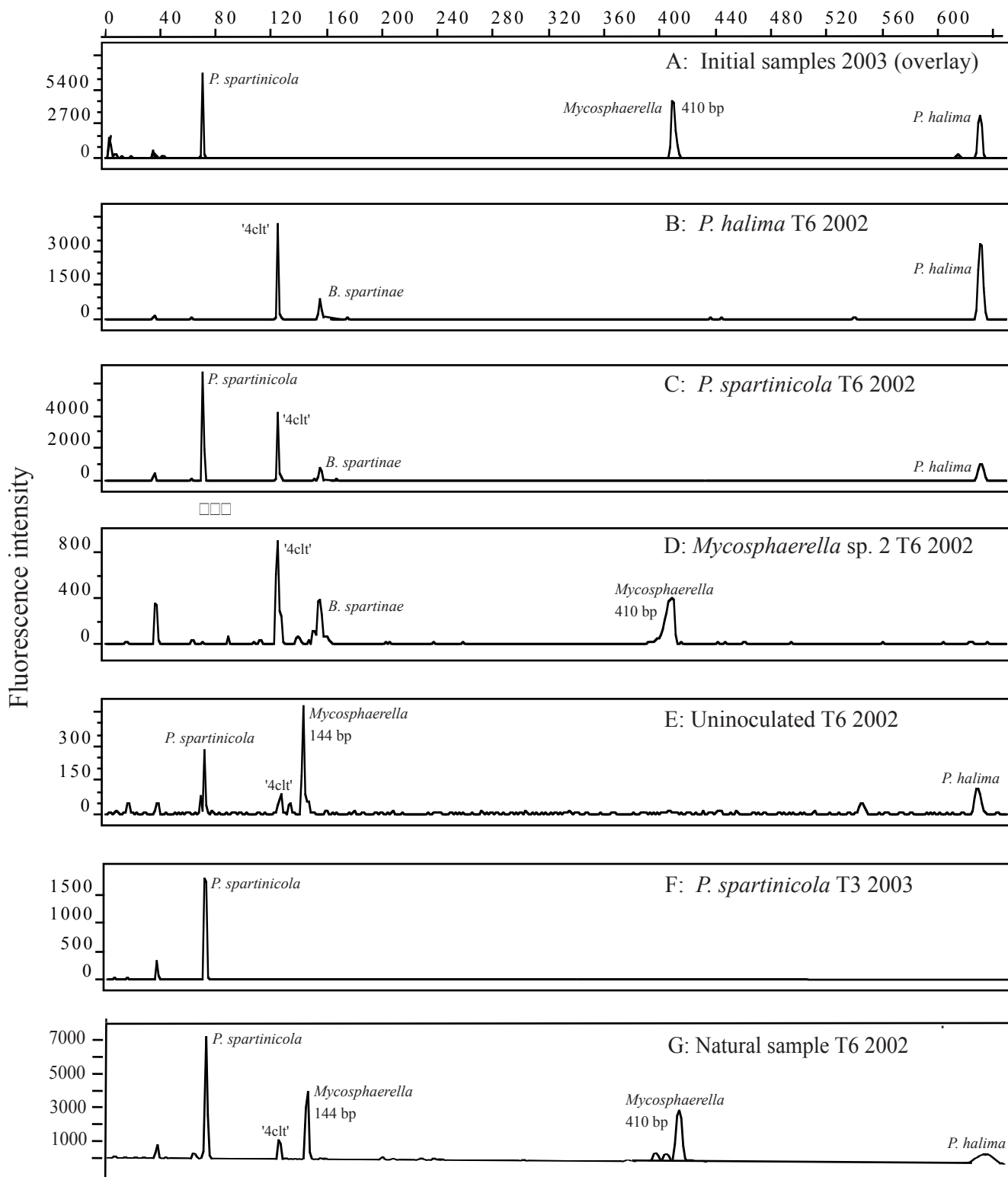


Figure 2.2. Relative contributions of T-RFs of eight previously identified bacterial groups (a-*Proteobacteria* 56, 79, 231, 346, 369, and 517 bp; g-*Proteobacteria* 203 bp; *Bacteroidetes* 92 bp) and one unidentified bacterial taxon (557 bp) in profiles of microbial communities associated with baited bundles of *S. alterniflora*. Samples were collected at 2, 4, and 6 weeks in 2002 (top) and 1, 2, and 3 weeks in 2003 (bottom). The bar heights represent averages from two duplicate bundles.

Bacterial Community

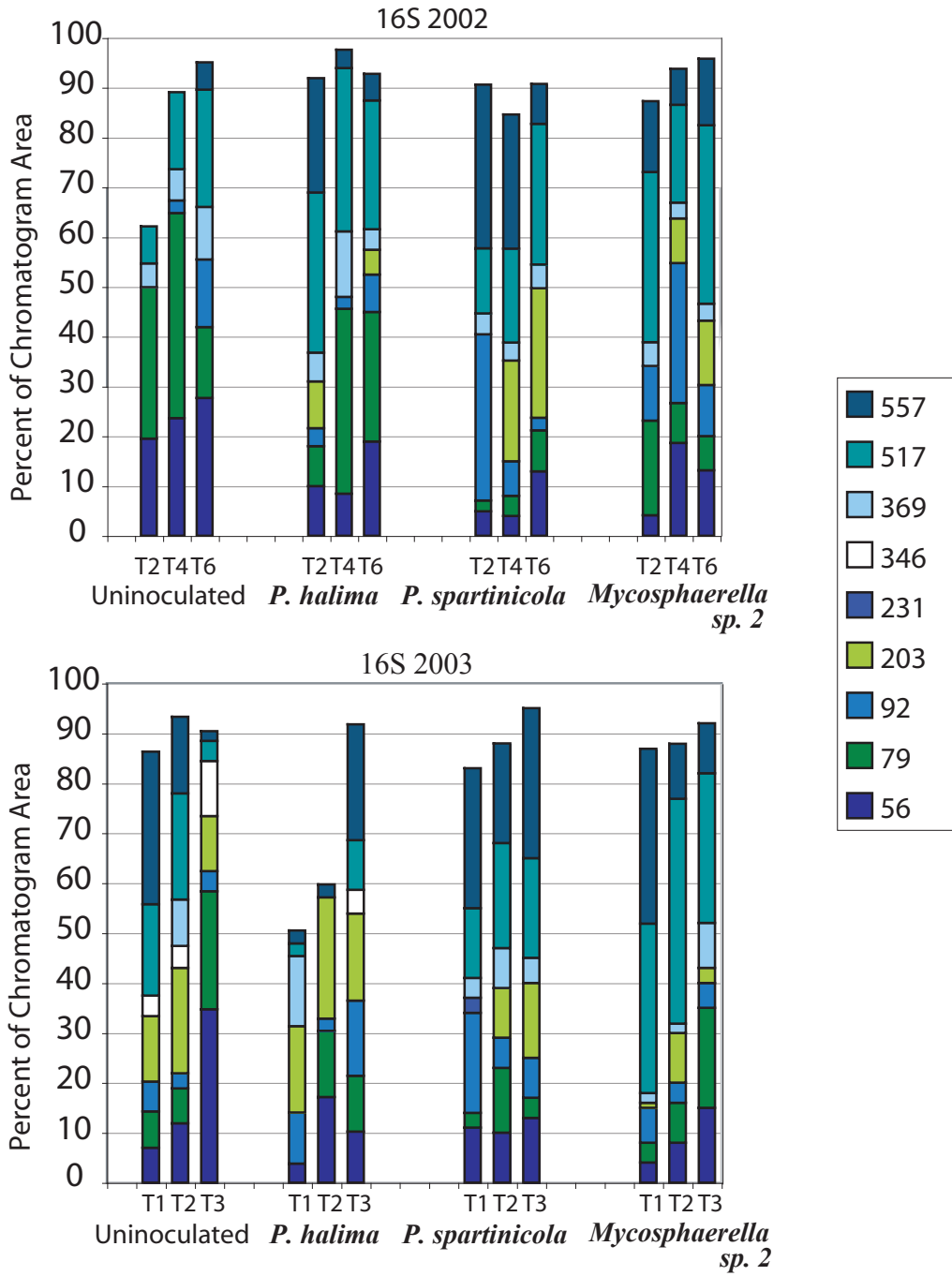


Figure 2.3. Cluster analysis of bacterial 16S rRNA terminal restriction fragment (T-RF) peak area data from baited and uninoculated bundles of decaying *Spartina alterniflora* (2002 experiment). Coverage data of nine dominant T-RFs were input into the data matrix. Samples are color-coded according to the inoculated fungus and labeled with the collection time point (T0, T2, T4, T6 or Initial). Gray shading indicates a cluster of samples with no amplifiable 16S rRNA genes.

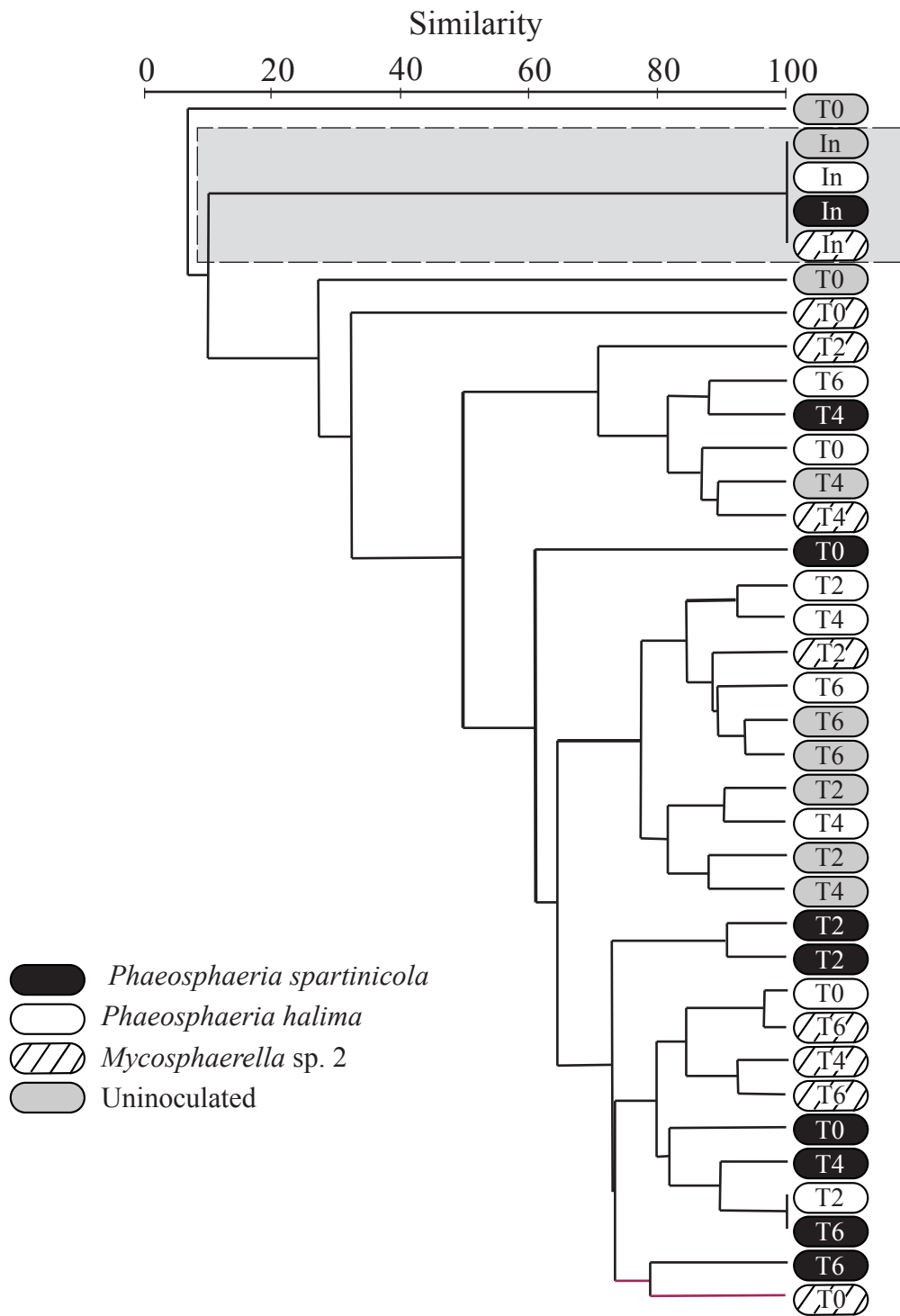
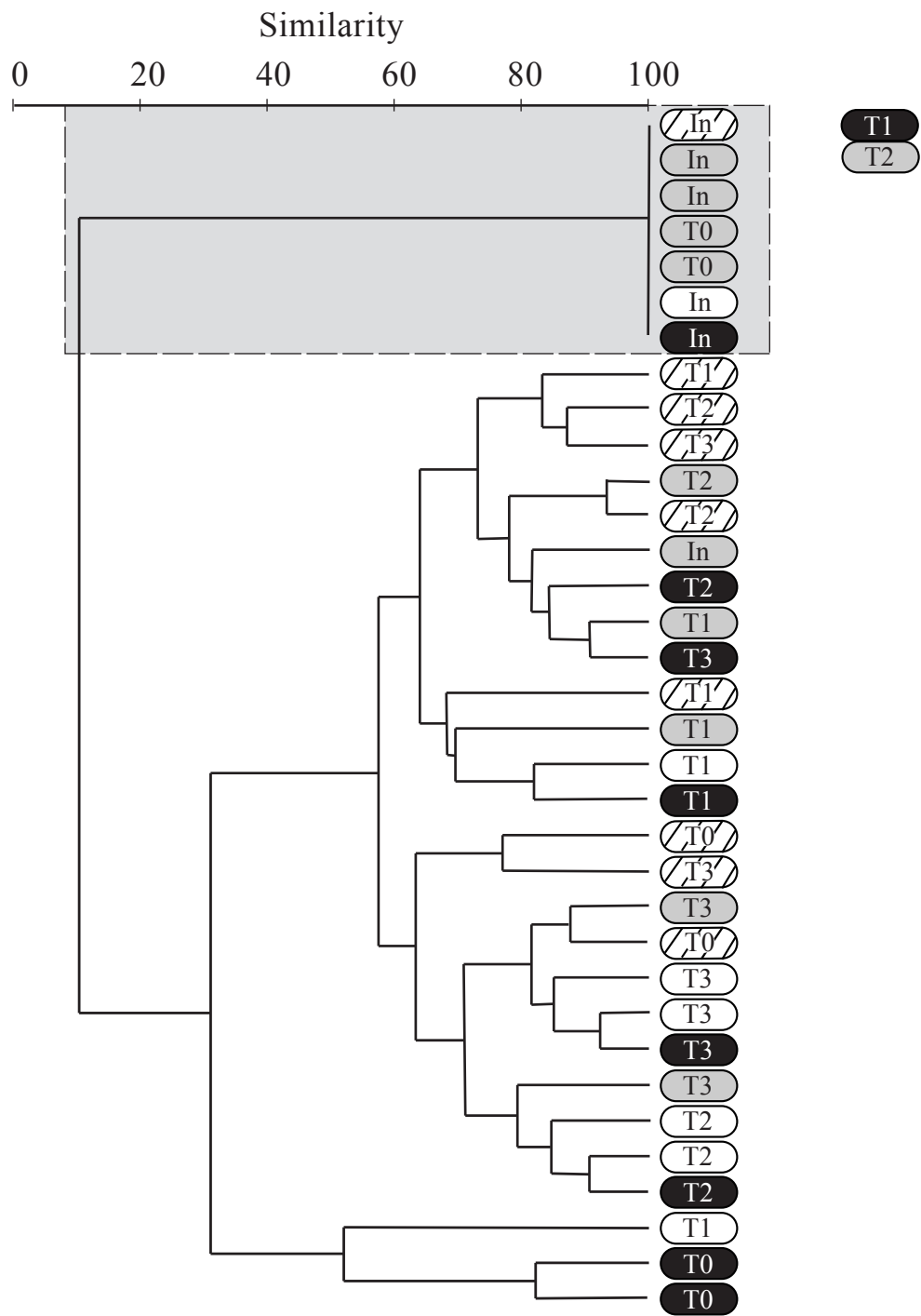


Figure 2.4. Cluster analysis of bacterial 16S terminal restriction fragment (T-RF) peak area data from baited and control bundles of decaying *Spartina alterniflora* (2003 experiment). Coverage data of nine dominant T-RFs were input into the data matrix. Samples are color-coded according to the inoculated fungus according to Fig. 3 and labeled with the collection time point (T0, T1, T2, T3 or Initial). Gray shading indicates a cluster of samples with noamplifiable 16S rRNA genes.



CHAPTER 3

ASCOMYCETE FUNGAL COMMUNITIES ASSOCIATED WITH EARLY DECAY LEAF BLADES OF THREE *SPARTINA* SPECIES AND A *SPARTINA* HYBRID IN THE SAN FRANCISCO BAY¹

¹ Lyons, J.I., M. Alber, and J.T. Hollibaugh. Ascomycete fungal communities associated with early decay leaf blades of three *Spartina* species and a *Spartina* hybrid in the San Francisco Bay estuary. To be submitted.

Abstract

Ascomycetous fungi play an important role in the early stages of decomposition of *S. alterniflora*, but their role in the decomposition of other *Spartina* species has not been investigated. Here we use a molecular approach to compare the composition of the ascomycete fungal communities on early decay blades of *Spartina* species [(the native *S. foliosa*, two introduced species, *S. alterniflora* and *S. densiflora*, and an *S. alterniflora* x *S. foliosa* hybrid] collected from three salt marshes (Blackie's Pasture, Cogswell Marsh, Hayward Landing) in the San Francisco Bay and one in Tomales Bay, California, U.S.A. Terminal restriction length polymorphism (T-RFLP) analysis of the ITS rDNA region was used to analyze the ascomycete communities associated with each sample by comparing T-RFLP profiles, and building a clone library to identify individual members of the community. Neither *S. alterniflora* nor the hybrid supported fungal flora different from that of *S. foliosa*, but another exotic species (*S. densiflora*) did harbor a unique fungal community. A previously described fungal species, *Phaeosphaeria spartinicola*, was often dominant and was found on all samples collected. Two other ascomycetes, *Phaeosphaeria halima* and *Mycosphaerella* sp. II, were also common, although the latter formed two novel subgroups as shown by previously undetected restriction sites in the ITS region. Significant differences in the fungal decomposer communities were detected both within (i.e. two clones of *S. foliosa*) and among species (i.e. *S. densiflora* was different than all other species, and hosted at least two unique ascomycetes), but the differences were minor and can be tentatively attributed to morphological differences among the plants.

Introduction

The cordgrass *Spartina foliosa* is native to salt marshes on the west coast of the U.S., but a number of other *Spartina* species found there (e.g. *S. alterniflora*, *S. densiflora*) are exotic, invasive plants (1). *S. alterniflora* was introduced by the US Army Corps of Engineers as part of a tidal marsh restoration project near the town of Fremont, CA in 1973 (11). A few years later several thousand more plants were transplanted to the shoreline of Alameda Island and to San Bruno (1). Over the next decade, tidal distribution of seeds spread *S. alterniflora* to other parts of the San Francisco Bay and by 1997 it was present in at least seven other areas in the south Bay (8). The wind-carried pollen of *S. alterniflora* soon resulted in the formation of a fertile, vigorous hybrid with the native *S. foliosa* (hereafter referred to as “hybrid *Spartina*”) (10). Hybrid *Spartina* is of concern in the San Francisco Bay because its population has expanded to cover previously unvegetated mudflats and it also threatens to invade habitat normally occupied by *S. foliosa*. *S. densiflora*, which is native to Chile, was introduced to San Francisco Bay in 1976 as part of a marsh restoration project adjacent to Corte Madera Creek in Marin County (14). The species has now spread beyond the original marsh plantings to the entire two-mile length of Corte Madera Creek and continues to move into new areas. *Spartina* decomposition is an important process in salt marsh ecosystems because *Spartina* biomass is a large reservoir of organic matter. Together with bacteria, fungi represent the major mechanism for the breakdown of vascular plant lignins (2, 22). Fungi begin the degradation process by using their hyphae to penetrate the substrate. The

hyphae then excrete degradation enzymes into the substrate, making macromolecules such as lignin more available to other decomposers (23, 28).

Previous studies of the fungal community of U.S. salt marshes have focused on the east coast (4, 6, 18, 25). Several species of ascomycetous fungi have been identified as major decomposers of *S. alterniflora* blades, based on traditional culture- and microscopy-based methods (16, 20-22) as well as molecular approaches (6, 7, 18). Although at least 28 marine ascomycetes have been identified on *Spartina* (16), the two most common species are *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II, both of which are involved in lysis of lignocellulosic components of the blades (3, 22, 27). Additional species that are typical but less prevalent members of the community include *Phaeosphaeria halima*, environmental isolate “4clt” (an ascomycetous species that does not yet have a formal taxonomic description), and *Buergenerula spartinae* (6, 21). At least ten species of mitosporic fungi have also been detected on decaying blades (16).

Studies have shown that fungal community composition can vary according to host plant species (4, 15, 16). Blum et al. (2004) used general fungal primers and T-RFLP profiles to compare the fungal communities associated with five species of standing dead marsh plants [(three *Spartina* species (*S. alterniflora*, *S. bakeri*, and *S. patens*), *Phragmites australis*, and *Juncus roemerianus*)] collected at ten locations along the eastern coast of the U.S. from Maine to Florida. These sites were characterized by gradients in temperature, precipitation frequency and amount, humidity, salinity, and light intensity. Fungal communities were analyzed by principal components analysis of T-RFLP data converted to binary format. Latitudinal differences of fungal communities within a plant species were not detectable, and the authors suggested that plant type,

rather than geographic location, is a primary determinant of fungal community composition. Although differences in fungal community composition were strongest when different plant genera were compared, slightly different fungal communities were recorded on the different *Spartina* species.

The fungal decomposer communities associated with west coast *Spartina* species have not been described, and information on the fungi associated with *Spartina* species other than *S. alterniflora* is limited. The purpose of this study was threefold: 1) to assess the similarity of ascomycetous fungal communities on *Spartina alterniflora* living on the west coast with those on the east coast, 2) to investigate variations in the ascomycete communities within and among *Spartina* species and 3) to explore whether the hybridization between invasive *S. alterniflora* and native *S. foliosa* changed or affected the associated resident fungi.

Methods

Site description and sample collections. In June 2005, grass in the “early-decay” stage (brown to black in color and remaining attached to the stem) was collected from *S. foliosa*, *S. alterniflora*, hybrid *Spartina*, and *S. densiflora* from three marshes (Blackie’s Pasture, Cogswell Marsh, and Hayward Landing) in the San Francisco Bay (Appendix A). In each marsh, two samples of each grass species (designated samples ‘1’ and ‘2’) were collected from separate, individual clones as identified by previously conducted genotyping (1). Each sample consisted of a group of 18 blades, which was subdivided into three sub-samples of six blades each. The lowest portion of each blade (6 cm,

beginning where the blade attached to the stem) was cut, air-dried overnight, and stored at -20°C until analysis.

Although some dense monocultures of *S. foliosa* were noted along Hayward Shoreline, the two clones sampled from this area (previously designated as ‘clone 4’ and ‘clone 23’ by D. Ayres, via personal communication) were growing in discrete circular patches separated by open mud. Clone 23 was growing surrounded by many other clones at the end of a small peninsula protruding from the Hayward shoreline, whereas clone 4 was completely isolated and was found away from the shoreline in a mudflat approximately ¼ mile north of clone 23. The two clones were morphologically different (Appendix A). Those of clone 23 (as well as all other samples of *S. foliosa* collected) were short (<6 cm), thin, and very dark brown in color. Conversely, leaf blades from clone 4 were longer (>6 cm), broader, and lighter brown in color. Because the standard length of leaf processed for DNA extraction was 6 cm, the entire leaf collected from clone 23 was used for analysis, whereas only the bottom 6 cm of leaf from clone 4 was processed.

Additional samples of *S. foliosa* were collected in July 2005 from ten marshes around Tomales Bay, an estuary located on the central California coast approximately 40 miles northwest of San Francisco. Tomales Bay has not been invaded by *S. alterniflora*, thereby making it an ideal site from which to collect non-hybridized samples of the native grass.

DNA extractions and PCR amplifications. Genomic DNA was extracted from the bundles of decaying *S. alterniflora* using DNA Plant Mini Kits (Qiagen, Valencia, CA).

The ITS rDNA (5, 12) was amplified with the ascomycete-specific primers ITS1F (5'CTTGGTCATTTAGAGGAAGTAA 3') and ITS4A (5'CGCCGTTACTGGGGCAATCCCTG 3') (17). These primers amplify a product ranging in size from 632- 713 bp, including the ITS1, 5.8S, and ITS2 regions of the rRNA operon (37). A previous study (6) found no evidence for variation due to multiple ITS sequences within single individuals for the salt marsh fungi we encountered. All PCRs were carried out with Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, N.J.), using 0.2 μ M of each primer and 50-100 ng of DNA. Thermal cycling reactions for ITS amplifications consisted of an initial 3 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 s at 52°C, and 1 min at 72°C. A final step of 10 min at 72°C was included to complete any partial polymerizations. Although triplicate sub-samples were available, initial TRFLP results were so consistent that molecular analysis of only two of the three sub-samples was carried out for each data point.

Fungal community characterization using TRFLP analysis. The ascomycete fungal communities were depicted using terminal restriction fragment length polymorphism (TRFLP) analysis of the ITS rDNA (6, 7). PCR amplifications were carried out as described above with the exception that the ITS1F primer was fluorescently labeled on the 5' end with FAM (carboxyfluorescein). Products were recovered from a 1% agarose gel using the QiaSpin gel extraction kit (Qiagen). Restriction enzyme digestion of the PCR product was carried out in a 20 μ l total volume containing 17 μ l of purified PCR product and 10 U of *Hae*III at 37° C for 3 h. Digested DNA was precipitated in ethanol

and suspended in 12 μ l of deionized formamide with 1 μ l of DNA fragment length standard GeneScan-2500 (TAMRA; Applied Biosystems).

Terminal restriction fragment (T-RF) lengths were determined on an ABI PRISM 310 (Applied Biosystems, Foster City, CA) in GeneScan mode. The automated sequencer detects all fluorescent DNA fragments. If there is a partial digestion by the restriction enzyme, a signal that does not correspond to a true T-RF can be detected. The fact that two sub-samples were analyzed independently (i.e. independent PCRs and independent digestions) helped to guard against such artifacts. In addition, all T-RFLP runs were duplicated. Besides allowing us to eliminate erratic peaks, these additional observations allowed us to confidently identify small T-RF peaks that were present consistently.

T-RFLP peaks were analyzed using a Visual Basic program that reconciles minor shifts in fragment sizes and quantifies them in terms of the percent of total area under the chromatogram represented by each peak (35). Peaks comprising <1% of total chromatogram area were excluded from the analysis. The standardized T-RFLP data were quantitatively compared by PCA, ANOSIM, and SIMPER based on a fourth-root-transformed data in a Bray Curtis similarity matrix using Primer 5 software (Primer-E LTD, Plymouth, UK).

Clone library construction and analysis. Clone libraries were constructed from selected samples of *Spartina* (*S. alterniflora* and *S. densiflora* from Blackie's Pasture, and *S. foliosa* clones 4 and 23 from Hayward Landing). PCR was carried out as described above using ascomycete-specific ITS primers (ITS1F and ITS4a). The PCR product was recovered from a 1% agarose gel with a QiaSpin gel extraction kit (Qiagen, Valencia,

Calif.), and the amplified ITS gene was cloned into an *E. coli* vector using a TA cloning kit (Invitrogen Corp., Carlsbad, CA) and plated onto LB plates. ITS rDNA was sequenced in a 96-well format by SeqWright DNA Technology Services (Houston, TX) using the ITS1F primer.

Approximately 500 bp of sequence was obtained from each clone. 325 bp of each sequence were aligned using ClustalW and used to a) predict theoretical *Hae*III restriction sites (to be matched to T-RFLP profiles), b) determine the identities of the ascomycetes represented in the T-RFLP profiles by comparison with sequences in the GenBank database using BLASTn and c) construct a phylogenetic tree. Differences in the composition of the clone libraries were tested for statistical significance using a web-based version of the LIBSHUFF analysis (33).

Sequences were analyzed using Accelrys GCG (Genetics Computer Group, Madison, Wisc.). A phylogenetic tree was created with the PHYLIP package and BioEdit (13), using evolutionary distances (Kimura algorithm) and the neighbor-joining method. The tree contained 329 sequences, eleven of which were taken from the NCBI sequence database to serve as references.

Results

T-RFLP profiles. The number of T-RFs per profile ranged from 1 to 6 over all the samples (Table 1). This number varied little among collection sites (Blackie's Pasture, Cogswell Marsh, Hayward Landing, Tomales Bay) and plant species (*S. alterniflora*, *S. foliosa*, hybrid *Spartina*, *S. densiflora*). The number of peaks observed in T-RFLP

profiles among the sites and species sampled was relatively low compared to previously described samples of *S. alterniflora* (which average 10-12 peaks) (6, 16, 21).

All T-RFLP profiles contained a prominent fragment at 72 bp. The percentage of total chromatograph area covered by this fragment ranged from 13-100% over all profiles (Table 1). Several previous studies (6, 18) have shown that a 72 bp fragment represents *Phaeosphaeria spartinicola*, one of the most dominant ascomycetes in southeastern U.S. saltmarshes (6, 7, 19, 23, 24). Only three samples contained fragments that accounted for a larger percentage of total chromatograph area than did the 72 bp fragment: samples of *S. densiflora* collected from two sites at Blackie's Pasture were dominated by a T-RF at 91 bp (Table 1) and one duplicate of *S. foliosa* clone 4 collected at Hayward Landing contained a higher percentage of a fragment at 528 bp (Table 1). However, these three samples also yielded a dominant 72 bp fragment (13-40% of the chromatograph area).

Table 1 shows that while some T-RF peaks were found on a relatively consistent basis, others were distinct in terms of either species or location. The peak at 63 bp was represented in ascomycete communities associated with all species, at all sites, and was observed in 24 of the 36 samples. The next most common peak, at 528 bp, was observed on all species and at all sites except Tomales Bay. All *S. densiflora* samples had peaks at both 81 and 91 bp. The peak at 81 bp was unique to this species, whereas that at 91 bp was only observed in one other sample.

Five of the six samples of *S. foliosa* collected from Tomales Bay contained a 502 bp fragment. Particularly in the two duplicates from Tomales Bay site 1, this 502 bp fragment covered a relatively large percentage of the chromatograph area (35 and 42%), and in all cases it replaced the 528 bp fragment observed at other sites. This peak was

observed in only one other sample (*S. foliosa* at Cogswell marsh), and may represent an ascomycete that is unique to this grass. Also of note is that the two clones of *S. foliosa* sampled at Hayward Landing had distinct T-RF profiles; clone 4 had at least three peaks not observed in the profiles of *S. foliosa* clone 23 (Figure 1). One of these fragments (123 bp) was not observed in any other sample.

A principal components analysis was performed to further evaluate differences among T-RF profiles (Figure 2). The analysis showed overlap amongst all species and sites, with two exceptions: *S. foliosa* 'clone 4' from Hayward Landing, and *S. densiflora* from Blackie's Pasture. SIMPER analysis confirmed that clone 4 and *S. densiflora* are responsible for the highest percentages of dissimilarities among samples, and that these dissimilarities are due to the presence of four minor fragments (85, 123, 129, 463 bp) in the former sample and of two minor fragments (81 and 91 bp) in the latter.

Hybrid *Spartina*. T-RFLP profiles of *S. alterniflora*, *S. foliosa*, and the *Spartina* hybrid collected from Blackie's Pasture were aligned to compare the fungal communities of the hybrid with that of its two parents (Figure 4). The chromatograms for these samples are all similar, which suggests that the ascomycete communities are also similar. All of the profiles are dominated by the 72 bp fragment. A small number of other minor peaks are present in the *S. alterniflora* and the hybrid chromatograms (Table 1), but the principle components analysis showed that these samples are not resolved along the PC1 axis, indicating that there are few differences between them (Figure 2). Analysis of similarity (ANOSIM) conducted on the T-RF data also confirms that there is not a significant difference among these three taxa at Blackie's Pasture ($R= 0.3$, $p < 0.01$).

Samples of *Spartina foliosa* and the hybrid collected from Hayward Landing and Cogswell Marsh followed a similar trend. All of the profiles were dominated by a T-RF at 72 bp, and showed relatively low diversity (Table 1). Seven of the eight samples collected from Hayward Landing contain a fragment at 528 bp. Interestingly, profiles for all samples of the hybrid *Spartina* collected from Hayward Landing contained a significant peak (10-20% of total area under the chromatogram) at 63 bp, but the samples of *S. foliosa* collected there did not contain this fragment. At Cogswell Marsh, profiles for *S. alterniflora*, *S. foliosa*, and the hybrid contained fragments at 63 and 72 bp, but only the hybrid contained a 528 bp fragment.

Clone libraries and phylogenetic tree. Partial ITS sequences were obtained for 83 clones from *Spartina alterniflora* '1', 71 clones from *S. densiflora* '1', 80 clones from *S. foliosa* 'clone 23', and 85 clones from *S. foliosa* 'clone 4' collected at Blackie's Pasture. These samples were chosen because their T-RF profiles contained a high diversity of fragment sizes.

Variation in ITS sequences readily distinguished ascomycete strains at the species level (Figure 3). The vast majority of clones from *S. alterniflora* (81%) and *S. foliosa* 23 (83%), a high number of clones from *S. densiflora* (44%), and a smaller number from *S. foliosa* clone 4 (9%) showed >99% sequence similarity to a previously cultured clone ('SIF10'; (6)) of *Phaeosphaeria spartinicola* (Figure 3). All of the sequences obtained in this study that were >99% similar to SIF10 contained a *Hae*III restriction site (GG/CC) at 72 bp. Additionally, all the ITS sequences were grouped on a clade with a known

sequence of *P. spartinicola*, so we conclude that the dominant 72 bp fragment represents *P. spartinicola*.

Another taxon represented in the clone libraries and tentatively identified on the chromatograms according to previously determined *Hae*III fragment lengths was *Mycosphaerella*. A previous study (6) identified two *Hae*III restriction sites (at 144 and 410 bp) among clones of *Mycosphaerella* sp. II. The taxon was thereby subdivided into two “subgroups” based on these restriction sites (subgroup A= 144 bp, subgroup B= 410 bp). In the current study, ITS sequence analysis showed that 8% of clones from *S. alterniflora*, 13% from *S. foliosa* clone 23, and 24% from *S. foliosa* clone 4 were >98% similar to SIF03, which was previously identified as *Mycosphaerella* sp. II Group A (6). Interestingly, these sequences did not have the cut site at 144 bp previously associated with this group. Rather, cut sites were identified at both 85 and 528-533 bp. The T-RF profile for one sample (*S. foliosa* clone 4) had a cut site at 85, whereas several had cut sites at 528. Also, one clone from *S. foliosa* clone 23 produced a sequence with a restriction site at 528 bp, but the sequence did not group with those from *Mycosphaerella*. This is not unusual, as individual T-RFs do not necessarily map to a single species. Moreover, the genus *Mycosphaerella* includes several thousand plant pathogenic species that tend to be host-specific (9). DNA-based techniques have clearly shown that in most cases these morphologically similar taxa are phylogenetically distinct (32), and it is not uncommon for *Mycosphaerella* to exhibit genetic polymorphism within a species. This is likely the explanation for the multitude of restriction sites observed here and in previous studies (6, 18).

The third most dominant ascomycete according to the clone libraries, *Phaeosphaeria halima*, is another that is common on *Spartina* on the east coast (6, 7). Although *P. halima* does not have a *Hae*III restriction site within the ITS region, 55 of the 322 clones produced sequences that were highly (>98%) similar to this ascomycete (Figure 3). *S. foliosa* clone 4 produced the highest percentage (58%), followed by *S. densiflora* (6%) and *S. alterniflora* (2%). If present on the T-RFLP profiles, *P. halima* would produce a peak corresponding to the size of the amplified ITS gene, which could range from 632-713 bp (37). Detection of *P. halima* on T-RFLP profiles is therefore difficult, as other fungi may exist that also lack a restriction site and as a result would also produce a peak within this range.

When the clone libraries were evaluated by *Spartina* species, we observed that *S. densiflora* ascomycete sequences generally grouped separately from those of *S. alterniflora* and *S. foliosa*. Figure 3 shows that the clone library from samples of *S. densiflora* produced 18 sequences containing a restriction site at 91 bp, and 15 sequences containing a restriction site at 515 bp. Neither of these fragments was found in the clone libraries of samples from the other grass species. A peak at 91 bp was observed in all T-RFLPs of *S. densiflora* (Table 1), but a peak at 515 bp was not. It is not clear why this is the case.

The two *S. foliosa* clones were placed in separate groups on the tree (Figure 3). Sixty-eight percent of clones from *S. foliosa* 4 show >98% sequence similarity to the previously identified ascomycete *Phaeosphaeria halima*, whereas none of the clones from the *S. foliosa* 23 library corresponded to this organism. Conversely, the smallest percentage of clones (9%) to identify with *P. spartinicola* came from the library created

from *S. foliosa* 4, whereas the largest (83%) came from that of *S. foliosa* 23 (Table 1). The clone library of *S. foliosa* '4' also produced sequences containing a unique fragment at 531 bp; however, the sequence is very similar overall to others in a clade on the phylogenetic tree that is tentatively identified as representing the ascomycete *Mycosphaerella* sp. II. Significant differences between the two clone libraries are confirmed by both LIBSHUFF analysis ($p < .001$) and ANOSIM ($R = 0.424$, $p < .02$).

Several terminal fragments were observed in the data but were not represented in the clone library. These include 63, 81, 123, 129, 463, and 632 bp (Table 1). The peak at 81 bp represents a substantial portion of the total chromatograph area (7-27%), and therefore does not appear to represent a rare species. Still, any of the inconsistencies between the clone library data and the chromatograph representations may be a product of cloning biases and subsequent amplicon coverage by the clone library. Biases in ITS-based methods may arise from interspecies variability in DNA extraction efficiency, primer binding, PCR amplification kinetics, and cloning efficiency (34, 36). It is unlikely that complex mixtures of amplified rRNA genes are cloned with uniform efficiency, and it is therefore often assumed that cloning systems generally influence the abundance of single sequences in gene libraries (29). Finally, as not every clone colony on the plate was picked and sequenced, it is possible that these fragments represent rare species that were present in clones and were simply missed by the random selection of clones chosen for sequencing.

It is also interesting to note that duplicate T-RFLP profiles of *S. densiflora* '1' lacked a 528 bp fragment (and no clones of *S. densiflora* '1' produced sequences with a restriction site at 528 bp), but profiles for both duplicates of *S. densiflora* '2' possessed

this fragment. This result suggests the possibility of patchiness in ascomycete colonization for at least this species. This may not be too surprising considering that the species likely represented by this fragment (*Mycosphaerella*) is a plant pathogen capable of “host-jumping” (9) in an attempt to reach and infect its ideal host.

Discussion

Description of the fungal community. This paper used analysis of T-RFLPs and clone libraries to evaluate the ascomycete fungal communities associated with several *Spartina* species found on the west coast of the United States. Almost all of the dominant *Spartina*-associated fungi found were similar to those previously identified on the east coast of the U.S (6, 16, 21, 25). All T-RFLP profiles contained a prominent peak at 72 bp (Table 1), which previous studies have shown to represent an ascomycete (*Phaeosphaeria spartinicola*) that is dominant on east coast *S. alterniflora* (6, 25). ITS sequences retrieved from California *Spartina* samples showed that over half (175; 54%) shared >99% sequence similarity to an isolate of *P. spartinicola* (SIF10) from Sapelo Island, GA. These sequences also contained a *Hae*III restriction site at 72 bp. Many of the remaining clones (94; 29%) shared high sequence similarities (>98%) to two other ascomycetes, *Phaeosphaeria halima* and *Mycosphaerella* sp. 2 (Figure 3), which were also previously described as dominants on east coast *Spartina*. This is consistent with reports by Newell (2000) of the omnipresence of *P. spartinicola* and a *Mycosphaerella* species on all samples of standing decaying smooth cordgrass blades collected from eight marshes between Maine and Florida.

Variations within *Spartina* species. The ascomycete communities from the two clones of *S. foliosa* sampled from Hayward Landing ('clone 4' and 'clone 23') were different, as observed on T-RFLP profiles (Figure 1) and confirmed using LIBSHUFF analysis of clone libraries ($p < .001$). It is interesting that two clones of the same *Spartina* species growing in the same area would host different fungal communities, but this may be due to differences in tidal elevation and exposure. Clone 23 is further inland at this site, and thus may be subjected to a shorter submersion time than clone 4 (Appendix A). This would affect the drying/wetting cycle, temperature exposure, and possibly redox potential of the sediment [which has been shown to influence nitrogen uptake by *Spartina* and distribution throughout leaf tissues (30)]. The lighter color and different morphology of the leaves from clone 4 may also indicate either a different stage of decomposition or different chemical composition, two more factors that could influence the decomposer community (26).

Variations among *Spartina* species. *S. densiflora* hosted the most divergent ascomycete communities among the *Spartina* species sampled. Principle components analysis of all samples (Figure 2) showed that *S. densiflora* groups separately along the PC2 axis, indicating that it harbors a distinct ascomycete community. Also, most (36 of 53) of the unidentified sequences from the clone library were from *S. densiflora*; these sequences appear as distinct groups on the phylogenetic tree (Figure 3).

Spartina densiflora has a unique morphology that may contribute to determining the structure of the associated ascomycete community. It is distinguished from other

cordgrasses by its grayish foliage, narrower leaf blades, and dense, compact growth form, giving the plant the appearance of growing in distinct clumps. In decaying *S. alterniflora*, which was the source of samples for the database of ITS sequences used in this study, distinctly different ascomycetes are detectable in the leaf sheaths and true stems as compared to those from the blades (21, 28). Newell (2001) suggests several reasons why this might be the case, one of which is that stems are only partly exposed to air, being enwrapped by leaf sheaths over much of their lengths. The leaves of *S. alterniflora* are broad and flat, and protrude from the stem at a 15-18° angle, whereas those of *S. densiflora* are narrow, wound tightly around the stem, and do not protrude from the stem at all (31). Indeed, it is difficult to distinguish the leaves of *S. densiflora* from the stem, although for this study attention was given to discerning between the two. Leaves that are wrapped around the stem, as are those of *S. densiflora*, are less exposed to air and moisture - two conditions that are known to affect fungal productivity (23, 26) - than leaves of other *Spartina* species. The differences in morphology of *S. densiflora* in conjunction with the differences among plant leaves as described above might therefore account for the presence of novel ascomycetes on *S. densiflora*.

Effect of hybridization on the fungal community. Our analysis indicates that there are no significant differences among the ascomycete communities of *S. alterniflora*, *S. foliosa* and hybrids of these two species at any of the sites where all three were growing. Although samples of *S. foliosa* collected from Tomales Bay contained some unique T-RFs (samples collected from site 1 contained a fragment at 104 bp, and five of the six samples collected over sites 1, 5, and 10 contained a fragment at 502 bp), the rest were

similar to those found on *S. alterniflora*. Thus, the invasive grass does not appear from this data to have introduced any new competitors, and it is unlikely that the unique fragments associated with *S. foliosa* represent ascomycetes that have been outcompeted by those introduced with *S. alterniflora*. However, since our analysis is based only on 500 bp of the ITS sequence, strain variants associated with *S. alterniflora* may in fact be more competitive than the strain variants from *S. foliosa* due to differences in genes encoded outside of the ITS regions.

As it stands, the striking similarities between the ascomycete communities described for *S. alterniflora* on the east coast (see Introduction) and those described here suggest that the fungal communities on *S. alterniflora* are the same regardless of geographic location. It also appears that the leaves of *S. alterniflora* and *S. foliosa* naturally harbor similar ascomycete communities, and therefore hybridization between the two species did not significantly impact these communities. However, we must acknowledge the possibility that species of other fungal classes not considered here (i.e. basidiomycetes) could have been more affected by the hybridization event.

Summary and future studies. Analysis of clone libraries constructed from samples of early-decay leaves of *Spartina alterniflora*, *S. foliosa*, and *S. densiflora* showed significant differences in the fungal decomposer communities both within and among species. The ascomycete *Phaeosphaeria spartinicola*, a dominant on east-coast *S. alterniflora*, was found on all samples collected. Two other ascomycetes, *P. halima* and *Mycosphaerella* sp. II (including two novel restriction sites), were also identified on *Spartina* samples processed in this study. Hybridization between *S. alterniflora* and *S.*

foliosa did not significantly affect the composition of the ascomycete communities associated with the plants.

The global distribution of *P. spartinicola* is intriguing. Investigating its adaptive and competitive strategies, as well as its physiological requirements and tolerances, may help to elucidate the environmental and biological factors that influence it and other fungal decomposers on *Spartina*. Chemical and nutritional analyses of the various *Spartina* species, including comparisons between plant clones growing under different environmental conditions, may also shed light on factors that control the composition of the associated ascomycete communities.

Also of interest to future studies may be further investigation of the fungal communities associated with *S. densiflora* across a broader sample range. Although we only collected *S. densiflora* from one site, it appears to support a unique ascomycete community. However, this claim should be substantiated by examining samples from other sites, including its native Chile.

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Appendix A

In June 2005, grass in the “early-decay” stage was collected from *S. foliosa*, *S. alterniflora*, hybrid *Spartina*, and *S. densiflora* from four marshes (Tomales Bay, Blackie’s Pasture, Cogswell Marsh, and Hayward Landing) in the San Francisco Bay, CA.



The two *S. foliosa* clones sampled from Hayward Landing (designated as ‘clone 4’ and ‘clone 23’) were growing in discrete circular patches separated by open mud. Clone 23 was growing surrounded by many other clones at the end of a small peninsula protruding from the Hayward shoreline, whereas clone 4 was completely isolated and was found away from the shoreline in a mudflat approximately 1/4 mile north of clone 23.



The two clones were morphologically different.

Leaf blades of clone 23 were short (<6 cm), thin, and very dark brown in color.



Those of clone 4 were longer (>6 cm), broader, and lighter brown in color.



	63	72	81	85	91	104	123	129	162	411	463	490	502	528	543	632
Blackie's Pasture											--					
<i>S. alterniflora</i> 1	22	74	--	2	--	--	--	--	2	--	--	--	--	--	--	--
<i>S. alterniflora</i> 1d	12	53	--	2	--	--	--	--	--	--	--	--	--	18	--	15
<i>S. alterniflora</i> 2	10	67	--	--	--	--	--	--	--	--	--	--	--	23	--	--
<i>S. alterniflora</i> 2d	17	69	--	--	--	--	--	--	--	--	--	4	--	10	--	--
<i>S. foliosa</i> 1	23	77	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. foliosa</i> 1d	--	81	--	--	--	--	--	--	--	--	--	--	--	7	--	12
<i>S. foliosa</i> 2	--	100	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. foliosa</i> 2d	--	90	--	--	--	--	--	--	--	--	10	--	--	--	--	--
Hybrid 1	5	89	--	--	--	--	--	--	--	--	--	--	--	6	--	--
Hybrid 1d	2	98	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Hybrid 2	25	75	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Hybrid 2d	13	87	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. densiflora</i> 1	--	34	20	--	46	--	--	--	--	--	--	--	--	--	--	--
<i>S. densiflora</i> 1d	--	40	14	--	46	--	--	--	--	--	--	--	--	--	--	--
<i>S. densiflora</i> 2	16	13	27	--	17	--	--	--	--	--	--	--	--	23	--	--
<i>S. densiflora</i> 2d	--	33	7	--	40	--	--	--	--	4	--	--	--	20	--	--
Cogswell Marsh																
<i>S. alterniflora</i>	43	57	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. alterniflora</i> d	3	97	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. foliosa</i> 1	6	87	--	--	--	--	--	--	--	--	--	--	7	--	--	--
<i>S. foliosa</i> 1d	7	93	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Hybrid	3	68	--	--	--	--	--	--	--	--	--	--	--	24	5	--
Hybrid 1d	4	75	--	--	--	--	--	--	--	--	9	--	--	12	--	--
Hayward Landing																
<i>S. foliosa</i> clone 23	--	55	--	--	--	--	--	--	--	--	--	--	--	45	--	--
<i>S. foliosa</i> clone 23d	--	59	--	--	--	--	--	--	--	--	--	--	--	41	--	--
<i>S. foliosa</i> clone 4	--	20	--	4	--	--	6	2	--	--	4	--	--	64	--	--
<i>S. foliosa</i> clone 4d	--	40	--	--	--	--	10	3	--	--	5	--	--	42	--	--
Hybrid clone 5	20	70	--	--	--	--	--	--	--	--	--	--	--	10	--	--
Hybrid clone 5d	16	74	--	--	--	--	--	--	--	--	--	--	--	10	--	--
Hybrid clone 25	15	51	--	--	--	--	--	--	--	--	--	--	--	34	--	--
Hybrid clone 25d	10	69	--	--	--	--	--	--	--	--	--	--	--	21	--	--
Tomales Bay																
<i>S. foliosa</i> 1	--	57	--	--	--	8	--	--	--	--	--	--	35	--	--	--
<i>S. foliosa</i> 1d	--	56	--	--	--	2	--	--	--	--	--	--	42	--	--	--
<i>S. foliosa</i> 5	2	95	--	--	--	--	--	--	--	--	--	--	3	--	--	--
<i>S. foliosa</i> 5d	9	83	--	--	--	--	--	--	--	--	--	--	8	--	--	--
<i>S. foliosa</i> 10	20	80	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>S. foliosa</i> 10d	22	54	--	--	6	--	--	2	--	--	--	3	13	--	--	--

Table 3.1. Percent area coverage of total chromatogram area covered by terminal restriction fragments from *Spartina alterniflora*, *S. foliosa*, a *S. alterniflora* x *S. foliosa* hybrid, and *S. densiflora* collected from four marshes (Blackie's Pasture, Cogswell Marsh, Hayward Landing, Tomales Bay), San Francisco Bay, CA. d=duplicate. Shading indicates samples used to make clone libraries.

Figure 3.1. T-RFLP chromatograms of amplified ITS regions from the ascomycete community associated with early decay leaves of two clones ('4' and '23') of *Spartina foliosa* at Hayward Landing.

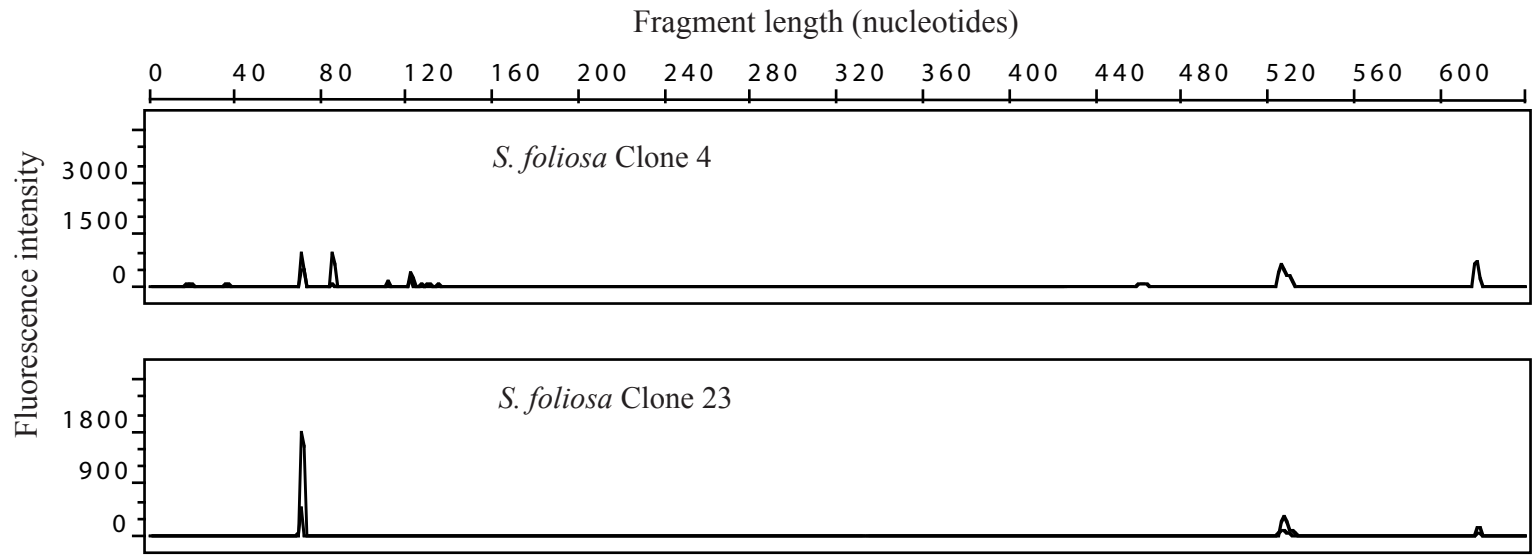


Figure 3.2. Principal component plot (PC1 x PC2) of scores of individual samples generated from T-RFLP profiles of ascomycete communities associated with four species (*S. alterniflora*, *S. densiflora*, *S. foliosa*, and *S. alterniflora* x *S. foliosa* hybrids) of early-decay *Spartina* collected from four sites around San Francisco Bay (CA). Input variables were expressed as percentage of total peak area.

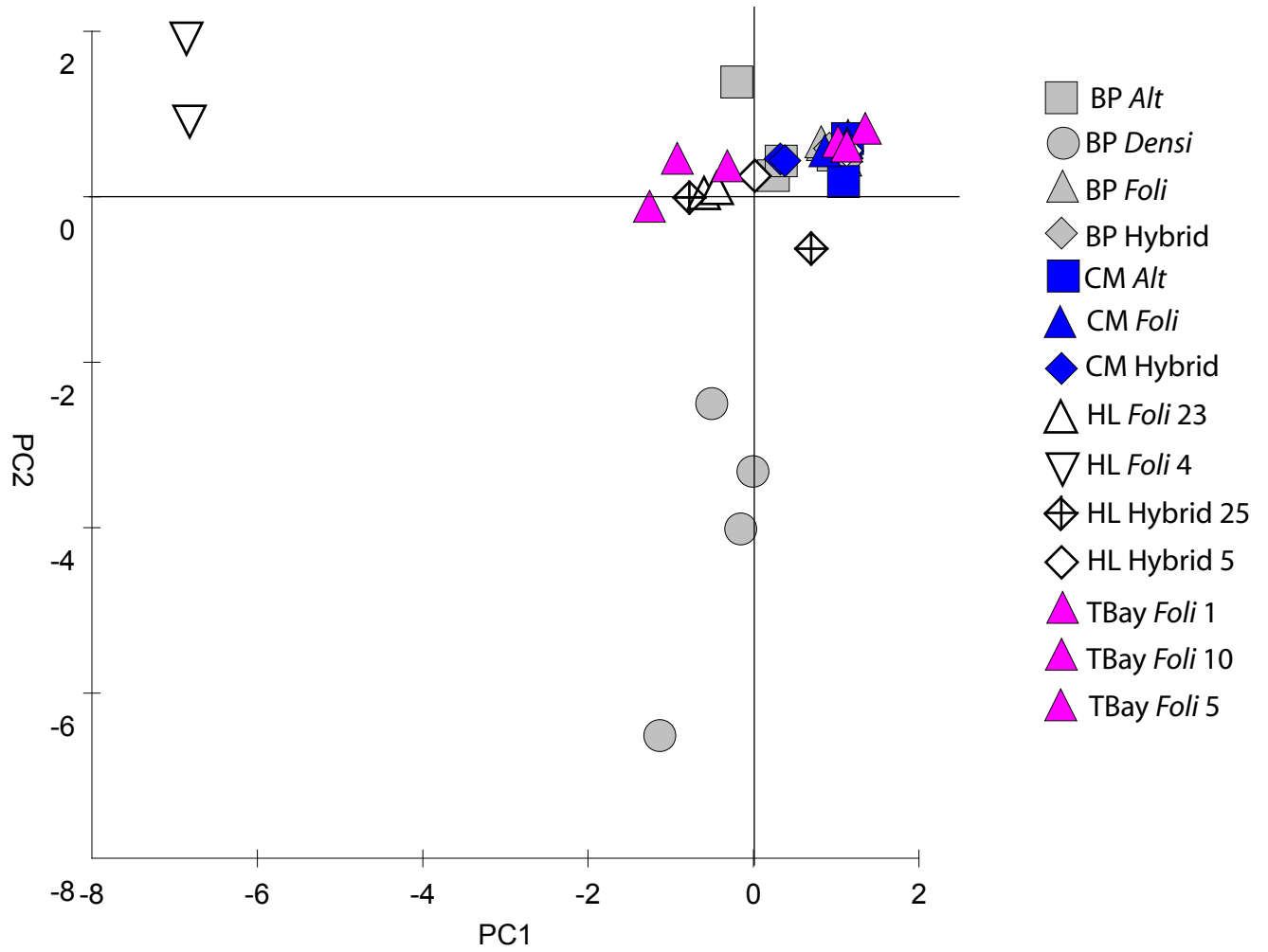


Figure 3.3. Phylogenetic tree of ascomycete ITS sequences obtained from PCR amplicons from decaying *Spartina* blades. Species of grass analyzed include *S. alterniflora* (“Alt.”), *S. densiflora* (“Dens.”), *S. foliosa* (two clones: “Foli4” and “Foli23”). SAP= previously identified sequences from Sapelo Island (GA). The tree was constructed from 400 positions (ITS1, 5.8S rRNA gene, and ~80 bp of ITS2) using the Phylip program with Kimura distances and *Scutellospora castanea*, a zygomycete, as the outgroup. Numbers in parenthesis indicate clones, the size of the terminal restriction fragment follows in italics. Clones with 100% sequence similarity are listed on the same line. Groups of sequences with identical terminal fragment restriction sizes following digestion with *Hae*III are indicated by shading. In the *Mycosphaerella* SIF03 cluster (indicated by a *), the group of sequences were >98% similar overall but exhibited varying restriction sites. Clusters of sequences that identified with >98% similarity to a previously identified ascomycete are indicated by a border; the species of ascomycete is indicated along the right-hand side of the box. Clones of *S. densiflora* and *S. foliosa* with unique T-RFs are listed in bold.

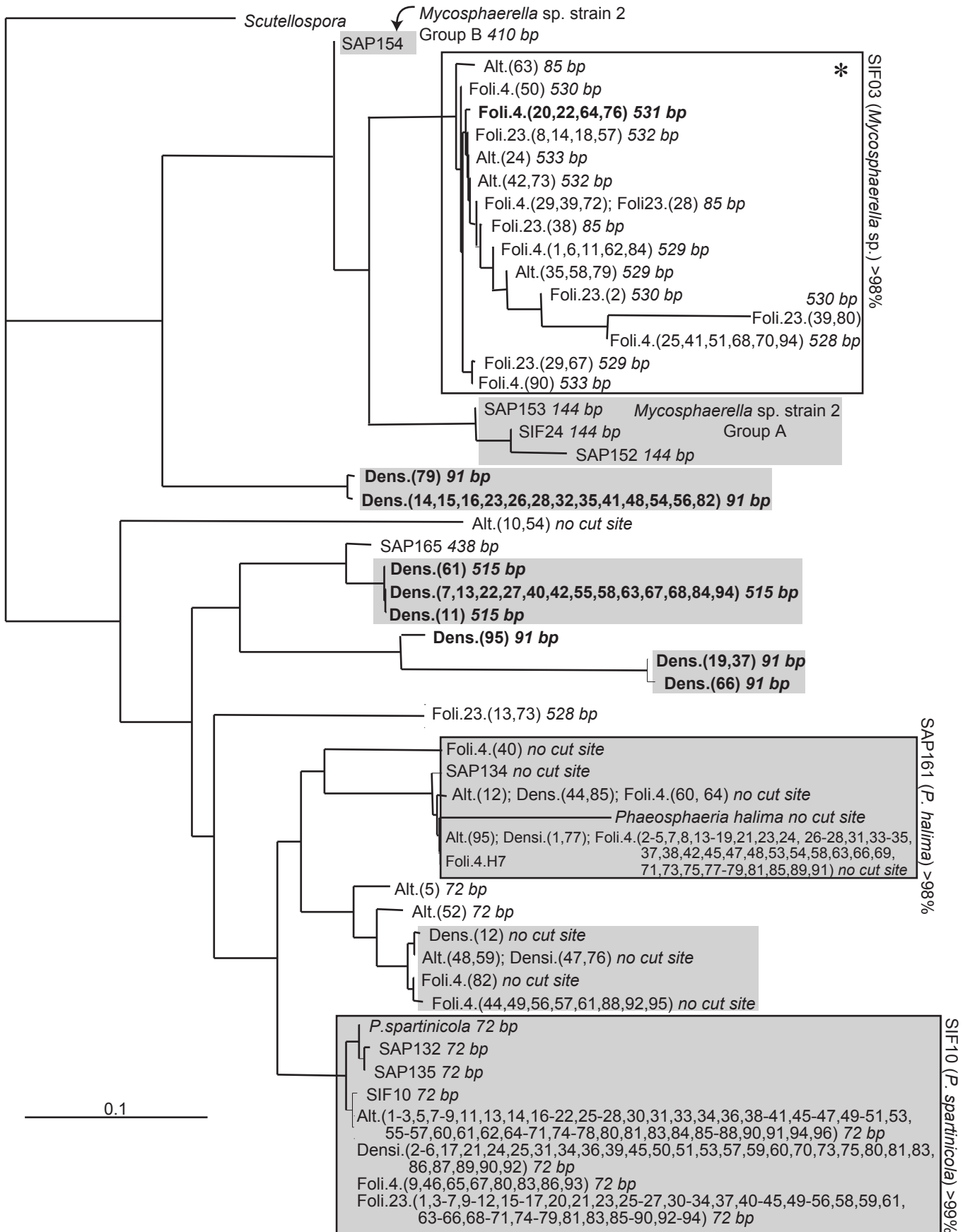
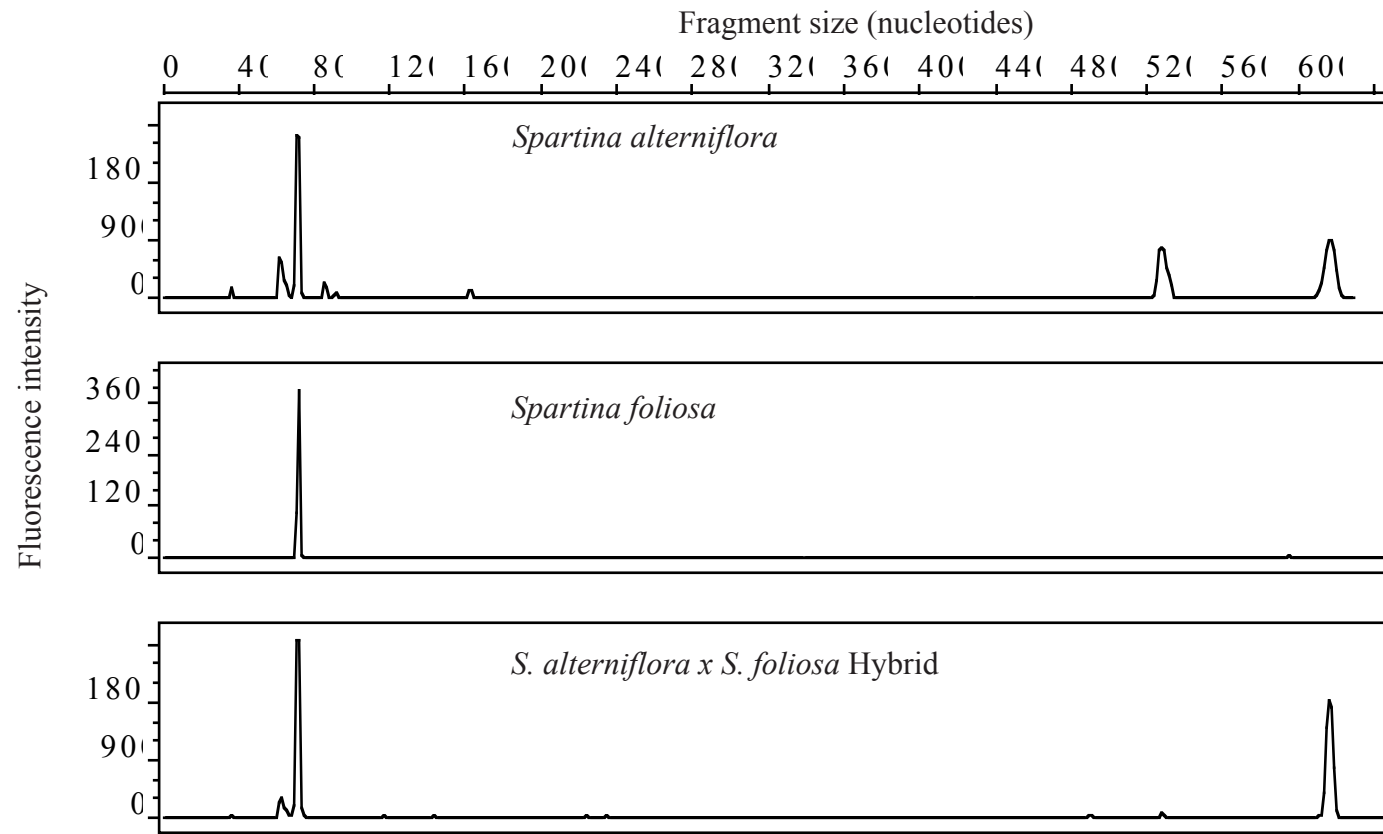


Figure 3.4. T-RFLP chromatograms of amplified ITS regions from the ascomycete community associated with early decay leaves of three species of *Spartina* at Blackie's Pasture, CA.



CHAPTER 4

ASCOMYCETE FUNGAL COMMUNITIES ASSOCIATED WITH EARLY DECAY LEAF BLADES OF TWO *SPARTINA* SPECIES IN FOUR STATES ALONG THE EASTERN U.S. SEABOARD¹

¹ Lyons, J.I., J.T. Hollibaugh, and M. Alber. Ascomycete fungal communities associated with early decay leaf blades of two *Spartina* species in four states along the eastern U.S. seaboard. To be submitted.

ABSTRACT

Microbial decomposers are key components in the degradation of salt marsh plants. Fungi, especially ascomycetes, are responsible for breaking down lignin macromolecules into smaller pieces that are then available for bacterial metabolism. Here we use a molecular approach to compare the ascomycete communities on two *Spartina* species (*S. alterniflora* and *S. patens*) that co-occur in salt marshes on the east coast of the U.S. Early-decay blades of both species were collected from marshes in four locations along the east coast (Georgia, North Carolina, New York, and Massachusetts). Terminal restriction fragment length polymorphism (T-RFLP) analysis of the 18S-to-28S internal transcribed spacer (ITS) region was used to produce fingerprint profiles of the ascomycete communities. Results showed no differences across latitude. Two species of previously identified ascomycetes (*Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II) were dominant on both species of decaying *Spartina* examined here. However, the number of ascomycete T-RFs observed on *S. patens* was significantly higher than that on *S. alterniflora*, suggesting a higher fungal diversity. *S. patens* also hosted a higher number of unique T-RFs. Two unidentified fragments (147 and 149 bp) appear to be species-specific to *S. alterniflora* and *S. patens*, respectively. We conclude that host plant type is a primary determinant of fungal community composition.

Introduction

Spartina alterniflora (smooth cordgrass) and *Spartina patens* (saltmeadow cordgrass) are two grasses found in salt marshes along the east coast of North America, from Canada to Florida. Both plants grow in open coastal marshes, the former between high and low tides and the latter from mean high tide to an elevation of about 0.5 m above mean sea level. *S. alterniflora* can be found growing in oxic or anoxic soils, whereas *S. patens* is not able to sufficiently oxygenate its rhizosphere in anoxic soils (4). Both will tolerate regular (*S. alterniflora*) and occasional (*S. patens*) inundation with water of salinities ranging from fresh to full-strength seawater (24).

Microscopic fungi, particularly ascomycetes, are instrumental in breaking down the lignocellulosic component of salt marsh cordgrass (13, 14, 17). The smaller molecules formed are then available for further decomposition by bacteria (15, 17). The fungal community associated with decomposing *Spartina alterniflora* has been examined in detail on Sapelo Island, GA (4, 5, 14, 16, 18, 20), and elsewhere (4, 9, 20). Although at least 28 marine ascomycetes have been identified on *S. alterniflora* (9), the two most ubiquitous species are *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II (2, 17, 21). Additional species that are typical but less common members of the community include *Phaeosphaeria halima*, environmental isolate “4clt” (an ascomycetous species that does not yet have a formal taxonomic description), and *Buergenerula spartinae* (5, 16). At least ten species of mitosporic fungi (i.e., species that are probably asexual forms of ascomycetes) have also been detected on decaying blades (9).

Several studies have investigated whether fungal community composition and activity vary with host plant species (4, 8, 9) and latitude (1, 4, 26). Kohlmeyer and Volkmann-Kohlmeyer (9) described more than 39 species of fungi on several different marsh plants. They

reported that, while the ascomycete communities on *Juncus roemerianus* are distinct from those of *S. alterniflora* and *S. densiflora*, some ascomycetes were common to the two *Spartina* species. Blum et al. (2004) compared the fungal communities associated with five species of standing dead marsh plants [three *Spartina* species (*S. alterniflora*, *S. bakeri*, and *S. patens*), *Phragmites australis*, and *Juncus roemerianus*] collected at sites ranging from Maine to Florida. They found differences in fungal community composition between genera, with smaller differences among *Spartina* species. Although the sites sampled in this study covered a range of temperature, precipitation frequency and amount, humidity, salinity, and light intensity, fungal community composition (analyzed by principle components analysis of T-RFLP data converted to binary format) did not vary with latitude for any of the host plants examined. The authors suggested that plant type, rather than geographic location, is a primary determinant for fungal community composition.

The present study expands on previous work by focusing on the ascomycete communities on *Spartina alterniflora* and *S. patens* collected from a broad range of east-coast environments. The purpose was to determine whether the ascomycete community is species-specific among *Spartina* species, and whether the same dominant species of fungi are found on plants growing in different geographic regions.

Methods

Site description and sample collections. Dead “early-decay” leaf blades (brown to black in color and remaining attached to the stem) of *S. alterniflora* and *S. patens* were collected from two marshes where they co-occur (designated site 1 and site 2) in each of four states (Georgia,

North Carolina, New York, and Massachusetts) along the east coast of the United States. In each marsh, duplicate samples of both species of grass were collected from two subsites (A & B), which were chosen randomly. The lowest portion of each blade (6 cm, beginning where the blade attached to the stem) was cut, air-dried overnight, and stored at -20°C.

In Georgia, samples of each species of grass were collected from two sites on Sapelo Island (University of Georgia Marine Institute (site 1) and Dean Creek (site 2)) in April 2006. The latter site is a long-term monitoring station of the Georgia Coastal Ecosystems Long Term Ecological Research project (GCE-LTER; <http://www.gce-lter.marsci.uga.edu/lter>). In North Carolina, samples were collected from two sites near the Duke University Marine Lab (Pivers Island (site 1) and Radio Island (site 2)) in May 2006. In New York, samples were collected at the Jamaica Bay Wildlife Refuge (site 1) and the Oceanside Marine Nature Study area (site 2) in November 2005. In Massachusetts, plants were sampled from 2 creeksheds (Sweeney and West) that are part of the Plum Island Estuary Long Term Ecological Research site (PIE-LTER; <http://ecosystems.mbl.edu/PIE>) in August 2006. These creeksheds are approximately 5 km apart, but site 2 (Sweeney Creek) is manipulated. From 2004 to the present day, a nitrate and phosphate fertilizer solution has been pumped into the incoming tidal water on each tide from mid-May to early October. The addition is designed to raise NO₃ levels to 70 umol/L and PO₄ levels to 5 umol/L, which are approximately 15X and 5X that of ambient NO₃ and PO₄ concentrations, respectively (C. Picard, personal communication). West Creek (site 1) serves as the reference creek for Sweeney. For this study, *Spartina* was collected from transects '1' and '3', located on the left branches of each creekshed.

DNA extractions and PCR amplifications. A total of 32 (2 species x 4 states x 2 marshes x 2 subsites) samples were collected for this study. All samples were processed as described previously (10). Briefly, genomic DNA was extracted using DNA Plant Mini Kits (Qiagen, Valencia, CA). The ITS region was amplified using Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, N.J.) and ITS1F and ITS4A primers. The ITS1F primer was fluorescently labeled on the 5' end with FAM (carboxyfluorescein). PCR products were digested with *HaeIII*, and the terminal restriction fragment (T-RF) lengths were determined on an ABI PRISM 310 (Applied Biosystems) in GeneScan mode.

The sizes of individual T-RFLP peaks were quantified in terms of percent of total chromatogram area and analyzed using a Visual Basic program that reconciles minor shifts in fragment sizes (22). Duplicate T-RF profiles were run for each sample, and the percentage of chromatogram area covered by each peak were then averaged for all subsequent analyses. The standardized TRFLP data were analyzed by PCA, SIMPER, ANOSIM, and MDS based on a fourth-root-transformed, Bray-Curtis similarity matrix using Primer 5 software (Primer-E LTD, Plymouth, UK).

The percent area under the peaks was used as a surrogate for the percent community composition represented by each fragment. Assuming that each peak represented a single fungal taxon, relative peak heights were taken as a measure of relative abundance. This may, however, be an under-representation of taxonomic diversity, as individual T-RFs do not necessarily map to a single species, and some species may not possess a *HaeIII* restriction site (ie *P. halima*). Other sources of bias in T-RFLP analysis include DNA extraction efficiency of different fungal species, as well as primer-binding efficiency during PCR.

RESULTS

T-RFLP profiles. As described in the Methods, individual T-RFs generally correspond to specific fungal taxa, and their abundance on the profiles were taken as indicators of their relative importance in the community. The most commonly observed peak, at 72 bp, was present in 27 of the 32 T-RFLP profiles. This fragment has been shown in several previous studies (5, 10, 11) to represent the ascomycete *Phaeosphaeria spartnicola*, one of the most dominant ascomycetes in southeastern U.S. saltmarshes (5, 6, 18, 21). The percentage of total chromatogram area covered by this fragment ranged from 2-87% over all profiles (average= $33.6\% \pm 27.9\%$), and covered more total chromatogram area than any other peak in 17 of the 32 samples.

Of the fifteen profiles that were not dominated by fragments representing *P. spartnicola*, six were dominated by one of several peaks (144, 410, 424, 528-533 bp) that have previously been shown to represent morphologically cryptic subgroups of *Mycosphaerella* sp. II (5, 10, 11). The second most common peak to appear on all of the profiles was at 410 bp, which was present on 21 of the samples. A peak at 424 bp appeared on 16 of the profiles, and that at 533 bp on 15 of them. The 528 bp peak appeared on only 9 profiles, but in four of those it was either the most or second-most dominant fragment. Overall, fragments representing *Mycosphaerella* sp. II accounted for an average of $25.2\% \pm 17.6\%$ of the total chromatogram area across all of the samples.

Of the other individual samples that were not dominated by *P. spartnicola* or peaks associated with *Mycosphaerella* sp. II, four (chromatograms from fungi associated with *S. alterniflora* collected from sites 1A and 2B and *S. patens* collected from site 2B in NC; and those

associated with *S. patens* collected from site 2B in Georgia) were dominated by a 91 bp fragment. Three samples (*S. patens* 1A and 1B from Georgia, *S. patens* 2B from Massachusetts) were dominated by a 149 bp fragment. The T-RFLP of fungi associated with *S. patens* collected from site 2A in Massachusetts was dominated by a peak at 478 bp; this was the only sample where this fragment was observed. These fragments have not been identified.

Fungal communities by plant species. The 72 bp peak, which, as described above, likely represents *P. spartinicola*, covered an average of $46.7\% \pm 28.9\%$ of the chromatogram area across all samples of *S. alterniflora*, and an average of $20.5\% \pm 20.4\%$ on samples of *S. patens*. Statistical analyses revealed that the mean coverages of *P. spartinicola* on the two species of grass are significantly different (*t* test, $P=0.006$). T-RFs likely representing *Mycosphaerella* were also noted on most profiles but were not significantly different between *S. alterniflora* ($26.8 \pm 25.2\%$ of the total chromatogram area) and *S. patens* ($25.4\% \pm 11.4\%$) (*t* test, $P=0.85$). The predominance of *P. spartinicola* and *Mycosphaerella* on both species of *Spartina* suggests that these two fungi play important roles in the decomposition of both marsh grasses.

Although samples from *S. alterniflora* and *S. patens* both produced substantial peaks representing *P. spartinicola* and *Mycosphaerella*, there were significant differences between the fungal communities on the two grasses. In particular, we noted two strongly species-specific ascomycetes represented by peaks at 147 bp (*S. alterniflora*) and 149 bp (*S. patens*). The 147 bp fragment was found on 69% of the *S. alterniflora* samples and was 100% specific to *S. alterniflora*, whereas the 149 bp was found in 88% of samples from *S. patens* samples and appeared in only one *Spartina alterniflora* sample (MA, site 2B) at 1% coverage. Most other peaks were found at various sites and on both *Spartina* species, but samples of *S. patens* had

more (three) unique T-RFs (404, 419, and 478 bp) than did *S. alterniflora* (147 bp) (Table 1).

One of the *S. patens* peaks, at 478 bp, was seen only on plants collected from the fertilized creek site (2A) in Massachusetts.

The number of fungal T-RFs per profile, which ranged from 3 to 12 over all the samples, can be used as a minimum estimate of taxonomic richness, assuming each peak represents a separate taxonomic unit. *S. patens* hosted an overall average of 7.9 ± 1.7 T-RFs, which was significantly different (*t* test, $P < 0.05$) from the 6.5 ± 2.0 on *S. alterniflora*. To further investigate these differences, we performed an MDS analysis of the profiles (Figure 1). Separation of the fungal assemblages associated with the two grass species is apparent in these results. An analysis of similarity (ANOSIM) conducted on the data confirmed that there is a significant difference between the fungal profiles of *S. alterniflora* and *S. patens* ($R = 0.389$, $p = 0.01$). Hence, the ascomycete communities associated with *S. alterniflora* and *S. patens* collected in this study can be generally characterized as exhibiting variability in diversity according to host plant species. Differences among states are discussed below.

Fungal communities by state. As noted above, *P. spartinicola* was the most commonly observed fragment on all fungal profiles. However, its relative contribution to T-RFLP area varied by state. In profiles of fungi from *S. alterniflora*, this fragment covered more than half of the total chromatogram area in New York and Massachusetts (averaging $70\% \pm 8.8\%$ and $57.3\% \pm 30.1\%$, respectively), and was lower in chromatograms from samples collected in GA and NC ($30.5\% \pm 30.5\%$ and $29.0\% \pm 25.2\%$, respectively). On samples from *S. patens*, the 72 bp fragment again contributed the highest percent coverages in New York ($41.8\% \pm 10.2\%$),

followed by North Carolina (23.0% ± 24.9%), then Massachusetts (10.75% ± 15.8%), and then Georgia (6.5% ± 10.4%).

Amplicons representing *Mycosphaerella* (144, 410, 424, 528, 534 bp) were also commonly noted, but like *P. spartinicola* their coverage varied by state. Average coverage was highest Georgia (32.8% ± 13.4%), followed by North Carolina (18.9% ± 19.2%), Massachusetts (18.9% ± 19.2%), and New York (6.8% ± 6.8%). Percent coverage varied widely in the two northern-most states, as evidenced by the standard deviations.

Some states exhibited a high degree of variation among subsites. In Georgia, for example, profiles from *S. patens* samples collected at site 2 had several T-RFs (63, 78, 91, 130, 149, 362, 404, 528 bp) that were not found at both subsites (A & B). Similar observations were made on profiles of fungi associated with *S. alterniflora*, with 63, 78, 130, 144, 147, 362, 410, 424, and 528 bp fragments being unique to one subsite or the other. We also observed a high degree of variation among subsites in the North Carolina marshes. The samples of both plant species collected at site 2B hosted ascomycete communities that were distinct ($R > 0.896$, $p = 0.03$) from those found in samples collected from the other three sites within that state (1A, 1B, 2A).

Although there was variability observed among subsites in all four states, perhaps the most striking are those subsites that lacked the otherwise ubiquitous 72 bp fragment. In North Carolina, profiles from both species of *Spartina* collected at site 2B lacked this fragment (which was predominant on all other samples collected there). In Massachusetts, profiles from *S. patens* collected at site 2 also lacked the 72 bp T-RF, which was in stark contrast to the 33 and 10% recorded on samples collected at sites 1A and 1B.

The average numbers of T-RFs produced by samples collected in each state were significantly different ($p < 0.02$). The highest number of fragments (8.4% ± 1.8%), suggesting the

greatest richness of ascomycetes, was observed in Georgia, followed by $7.9\% \pm 1.9\%$ in North Carolina, $6.7\% \pm 1.4\%$ in New York, and $5.6\% \pm 1.9\%$ in Massachusetts.

To provide an integrated overview of the fungal community structure by state, T-RF data for all states were pooled and subjected to a principle components analysis (Figure 2). The analysis shows clustering of ascomycete communities from duplicate samples and overlap among all plant species collected from all sites except one: duplicate samples of *S. patens* collected from site 1A in Georgia fell apart from all other samples along PC1. SIMPER analysis confirms that this sample is responsible for the highest percentage of diversity, and that the dissimilarity is due to a number of minor inconsistencies between this sample and all the others. Among these inconsistencies are low coverage (2%) by *P. spartinicola* and relatively high (12%) coverage by a 144 bp peak. The MDS analysis resulted in no clear separation among the four states, suggesting low geographic variability (Figure 1).

Discussion

Species identification. Several of the fungal T-RFs observed in this study are the same size as those previously identified on early decay *S. alterniflora* on Sapelo Island, GA (5) and on early decay *S. alterniflora*, *S. foliosa*, and *S. densiflora* in the San Francisco Bay in CA (10). As described above, the peak at 72 bp has been shown to represent *Phaeosphaeria spartinicola*, whereas those at 144, 410, 424, 528, 534 bp have been linked to *Mycosphaerella*. Both of those fungi were dominant on almost all samples in this study.

Several other T-RFs recorded here have also been observed in other studies. For example, a 91 bp fragment was common (but unidentified) on samples of *S. densiflora* collected in San

Francisco Bay (10). Other peaks, such as those representing 80, 123, 130, 148, and 150 bp fragments, have been previously observed in ascomycetes isolated from decaying *S. alterniflora* collected in a Georgia salt marsh (5).

Conversely, some fungal species commonly noted in other studies were not recorded here. *P. halima* is a dominant ascomycete in *Spartina* decomposer communities in Georgia (5, 6, 20) that was abundant in clone libraries created from samples of *S. alterniflora* and *S. foliosa* in California (10), but was not represented in the chromatograms. This is due to the fact that the ITS region of *P. halima* does not possess a *HaeIII* restriction site, and therefore peaks representing the entire ITS subunit (at ~634 bp) may represent either *P. halima*, any other ascomycete lacking a restriction site, or an incomplete digestion of the amplicon. As a result, we cannot identify this species in our chromatograms.

Buergenerula spartinae is another ascomycete that has been cultured from samples of *Spartina* decomposer communities (7, 16) that was not detected in this study. Cultures of this species produced T-RFLP profiles with an *HaeIII* restriction site at 150 bp, and it is possible that the 149 bp fragment observed here represents this species. However, *B. spartinae* is often missing from molecular assessments of fungal community composition (5, 23), which has been attributed to either poor DNA extraction from highly melanized areas or to competition with other fungal species resulting in early decline of the species.

Differences in ascomycetes associated with plant species. Overall, the greatest differences in the ascomycete communities were observed between the two *Spartina* species. Amplicons representing *P. spartinicola* were found in 94% of all *S. alterniflora* but only 75% of *S. patens* samples. The relative abundance of *P. spartinicola* was also significantly different ($p=0.006$) on

the two plants (an average of 47 and 21% for samples from *S. alterniflora* and *S. patens*, respectively). Samples of *S. patens* yielded a higher number of total (eight) and of unique (three) T-RFs than did *S. alterniflora* (six total, one unique) (Table 1), and MDS analysis indicated clear separation between the ascomycete community colonizing the two species (Figure 1). Taken together, these results show a clear separation between the two species.

The significant differences in the fungal decomposer communities on these two marsh plants are likely due to the different habitats in which the grasses are found. Although *S. patens* and *S. alterniflora* often occupy the same tidal marshes, the former is normally limited to high salt marshes because it lacks aerenchyma tissue that would allow it to oxygenate the anoxic rhizosphere at lower tidal elevations (3). In addition, differences in inundation result in slower weight loss during the leaching phase of decomposition in *S. patens* as compared to *S. alterniflora* (25). A lower rate of leaching, the somewhat dryer, less favorable habitat for microbes, and chemical makeup of *S. patens* could all combine to maintain the differences observed between the ascomycete communities on these two plant species. This concurs with the study by Blum et al. (4), who found that *S. alterniflora*, *S. bakeri*, and *S. patens* hosted somewhat different fungal communities.

Differences in ascomycetes between states. We found no evidence for a latitudinal gradient affecting the ascomycete communities on decaying *Spartina*. Figures 1 and 2 indicate that there were no differences in number of T-RFs associated with the fungal communities on the two species of *Spartina* sampled across four states. To further investigate any possible latitudinal effects, environmental variables for each collection area (latitude and longitude coordinates, average temperature, tidal range, precipitation, and salinity) are compared in Table 2.

Regression analyses (not shown) resulted in no significant ($p < 0.05$) correlations between either the number of T-RFs or the percent coverage by any of the dominant terminal restriction fragments (37, 72, 91, 144, 148, 410, 528, and 534 bp) and any of the environmental conditions for either species of grass. These results are again consistent with those of Blum et al. (4), who concluded that plant type, rather than geographic location, was a primary determinant for fungal community composition.

Although we found no evidence for a latitudinal effect on ascomycete community composition, seasonal time of collection and corresponding temperatures could be important factors affecting the fungal community. Table 1 shows that the lowest coverage by the 72 bp fragment, which we assume is *P. spartinicola*, occurred on samples collected from Georgia and North Carolina. These samples were collected in the spring (April and May, respectively), when *Spartina alterniflora* is experiencing a period of growth; any decaying grass collected at these times was still in the very early stages of decomposition (and was, in fact, difficult to find). It thus is logical that decomposer communities were not yet well-established. In contrast, samples collected later in the season (early November in New York) had the highest percent coverage by the 72 bp fragment. According to Gessner (7), frequency of *P. spartinicola* remains high until the standing crop of *S. alterniflora* is killed by frost (which had not occurred by the time of collection in 2005). Note, however, that Newell (2001) found that it exhibit a higher rate of ascospore expulsion in winter months (Newell 2001), suggesting that the portion of standing crop that remains on *S. alterniflora* in the winter is still active. The relative percent coverages by the 72 bp fragment among samples analyzed in this study supports the notion of some seasonal variability in the relative abundance of *P. spartinicola* (7).

The variations in coverage by *Mycosphaerella* may also be attributed to seasonality. Buchan et al (2003) found that amplicons from *Mycosphaerella* sp. II had highest relative abundance in July samples (65 and 61% of the total peak area) and played a lesser role in the nonsummer samples. In accordance with this, *Mycosphaerella* sp. II was previously found to exhibit a summer peak in rates of ascospore expulsion (approximately threefold higher than in winter) (Newell 2001).

Although ascomycete diversity and the dominant species observed in the profiles were relatively consistent overall, we did find that samples collected from some states and sites were anomalous. Samples of *S. patens* collected from the manipulated creekshed (sites 2A & 2B) in Massachusetts and samples of both grass species collected from site 2B in North Carolina were the only ones that lack T-RFs representing *P. spartanicola*. *S. patens* collected at the Massachusetts site also yielded the only 478 bp fragment observed in the study. Although not enough is known about the physiology of ascomycetous fungi to enable us to know for sure what specific factors contribute to and control their growth, the nitrogen content of decaying vascular plant substrates is known to be a key determinant of microbial activity (27). Additions of nitrogen to stands of decaying *S. alterniflora* resulted in increases in ascomycete standing crop (12, 19), and in significant increases in decomposition (12), suggesting that salt marsh fungal production is nitrogen-limited. This could possibly contribute to the differences in T-RFs observed in the Massachusetts site 2 samples, which came from a nitrogen-enriched creekshed.

Figure 2 shows that fungi associated with *S. patens* collected from site 1A in Georgia were distinct from all other samples on the PC1 axis. This sample also produced the most T-RFs of all the samples (Table 1). This collection site was located along a levee bordering a small

creek, and was relatively dry compared to the other subsites of site 1. However, it is not clear how these conditions would influence ascomycete richness.

Comparison with the Gulf coast. In August 2005, we collected samples from four states along the Gulf coast (Louisiana, Mississippi, Alabama, and Florida) in conjunction with this study (Table 3). All materials and methods were as described previously (10), but due to inconsistencies in the number and availability of samples (for example, *S. patens* was not available in Florida in the area sampled), the sampling program was not complete. However, we can use these preliminary results to broaden our comparison of fungal communities associated with *S. alterniflora* and *S. patens*.

As was observed along the east coast and in California, T-RFs representing *P. spartanicola* (72 bp) and *Mycosphaerella* (144 and 424 bp) were prominent in most fungal profiles from plants collected from the Gulf coast, averaging 23%, 23% and 14%, respectively. These numbers are slightly lower than those recorded on the east coast. No significant differences ($p > 0.96$) in the relative importance of the *Mycosphaerella* amplicons (pooled) was observed between *S. alterniflora* and *S. patens* in the Gulf coast: coverage of these fragments averaged $18.5\% \pm 15.6\%$ and $18.0\% \pm 14.8\%$, respectively. However, a substantial difference was again obvious in the predominance of the *P. spartanicola* fragment on samples from *S. alterniflora* (an average of 38% of chromatogram area on samples collected in LA, AL, and MI) as opposed to those from *S. patens* (an average of 4%, including 0% in LA).

Another result consistent with those reported here for the east coast is the species-specificity of the 147 and 149 bp T-RFs. 100% of the *S. alterniflora* samples collected along the Gulf coast produced a 147 bp fragment, whereas no samples of *S. patens* did. Conversely, 66%

of the *S. patens* samples produced a 149 bp T-RF, whereas no samples of *S. alterniflora* did. Although they are too similar to be confidently distinguished as separate fragments, T-RFs 147 and 149 bp were consistently specific to *Spartina alterniflora* and *S. patens*, respectively, on samples collected along both coasts, and the persistence with which we observed this species-specificity substantiates the hypothesis that these peaks represent two different ascomycete taxa. If this is true, then these fragments could serve as potential indicators and should be investigated further. It would also be interesting to sample *S. patens* on the west coast to determine whether this T-RF-specificity holds in a different environment.

T-RFLP profiles produced for *Spartina* samples collected along the Gulf coast produced several unique T-RFs (135, 150, 153, 159, 204, 448, 499, and 511 bp) not reported either along the east coast or in California (Chapter 3). Conversely, the 81 bp fragment was not observed in the fungal profiles from the Gulf coast. This T-RF was observed on samples of *S. densiflora* (but not *S. alterniflora*) collected in California and on both *S. alterniflora* and *S. patens* collected along the east coast. Again, however, without sequence information for samples collected along the east coast, we cannot say for sure that this fragment represents the same ascomycete in all three studies.

Finally, ascomycete diversity (as represented by the number of T-RFs on each sample) was higher on average on samples of *S. patens* (mean=12) collected along the Gulf coast than on *S. alterniflora* (mean= 9.75). This is again consistent with the results of profiles from samples collected along the east coast.

Conclusions

From the studies described here and previously (10) it appears that two species of ascomycetes (*Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II) dominate decaying *S.alterniflora*, *S. foliosa*, *S. patens*, and *S. densiflora*. However, this study suggests that the diversity of the ascomycete taxa on *S. patens* is significantly higher across a geographic range than that on *S. alterniflora*, and that *S. patens* hosts a higher number of unique ascomycete species. These findings correlate well with the results of previous studies which have concluded that 1) variations in latitude do not explain differences in fungal decomposer communities (4, 20), and 2) plant type is a more likely primary determinant of fungal community composition (4, 10).

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	37	44	63	72	78	81	91	123	130	144	147	149	362	404	410	419	424	478	528	533	# of T- RFs
Georgia																					
<i>S. alterniflora</i> 1A	3	--	1	30	--	--	--	--	--	1	4	--	--	--	47	--	11	--	--	--	7
<i>S. alterniflora</i> 1B	4	--	--	17	--	--	--	--	10	--	13	--	--	--	24	--	4	--	--	--	7
<i>S. alterniflora</i> 2A	2	--	1	73	--	--	--	--	--	--	7	--	--	--	11	--	1	--	--	1	7
<i>S. alterniflora</i> 2B	1	--	--	2	4	--	--	--	1	1	--	--	18	--	--	--	--	--	62	2	8
<i>S. patens</i> 1A	5	--	--	2	1	2	10	--	--	12	--	20	--	6	2	4	5	--	--	3	12
<i>S. patens</i> 1B	--	2	--	--	--	3	15	--	29	--	--	34	--	3	3	1	--	--	--	--	8
<i>S. patens</i> 2A	11	--	16	22	--	--	--	--	2	--	--	10	--	--	10	--	6	--	--	5	8
<i>S. patens</i> 2B	3	--	--	2	3	--	16	--	--	--	--	--	1	13	12	--	3	--	14	2	10
North Carolina																					
<i>S. alterniflora</i> 1A	9	2	--	21	--	1	24	--	19	6	1	--	--	--	13	--	--	--	--	1	11
<i>S. alterniflora</i> 1B	2	1	1	35	--	--	1	--	--	--	2	--	--	--	2	--	--	--	--	55	8
<i>S. alterniflora</i> 2A	6	1	4	60	--	--	--	--	--	--	10	--	--	--	6	--	3	--	1	7	9
<i>S. alterniflora</i> 2B	--	2	--	--	--	13	22	--	--	10	--	--	--	--	--	--	2	--	1	21	7
<i>S. patens</i> 1A	23	--	--	3	--	--	1	--	--	--	--	3	13	3	34	--	16	--	--	2	9
<i>S. patens</i> 1B	16	--	--	44	--	--	6	--	--	1	--	7	--	--	8	--	6	--	--	5	8
<i>S. patens</i> 2A	7	--	--	45	12	--	9	--	--	25	--	--	--	--	--	--	--	--	--	--	5
<i>S. patens</i> 2B	--	1	--	--	--	1	36	--	--	--	--	1	--	--	2	--	--	--	--	28	6
New York																					
<i>S. alterniflora</i> 1A	8	--	16	58	--	--	--	--	--	--	3	--	--	--	17	--	4	--	--	--	6
<i>S. alterniflora</i> 1B	9	--	4	75	--	--	--	--	2	--	1	--	--	--	--	--	--	--	--	3	6
<i>S. alterniflora</i> 2A	10	1	2	78	--	--	--	--	3	--	5	--	--	--	--	--	--	--	--	--	6
<i>S. alterniflora</i> 2B	3	--	2	69	--	--	--	--	--	--	--	--	--	--	15	--	3	--	--	--	5
<i>S. patens</i> 1A	--	--	5	29	--	--	--	21	--	3	--	16	--	--	12	--	7	--	--	--	7
<i>S. patens</i> 1B	3	--	--	50	4	--	--	--	15	2	--	16	--	--	4	--	4	--	--	--	8
<i>S. patens</i> 2A	--	--	24	38	1	--	2	--	1	--	--	6	--	--	16	--	7	--	1	--	9
<i>S. patens</i> 2B	7	2	--	50	--	--	--	--	1	--	--	7	--	--	10	--	18	--	--	1	8
Massachusetts																					
<i>S. alterniflora</i> 1A	--	--	4	69	--	3	4	--	--	--	1	--	--	--	--	--	--	--	1	--	6
<i>S. alterniflora</i> 1B	--	--	--	57	--	--	--	--	--	34	1	--	--	--	--	--	--	--	--	--	3
<i>S. alterniflora</i> 2A	--	--	--	87	4	--	--	--	--	1	--	--	--	--	--	--	--	--	--	--	3
<i>S. alterniflora</i> 2B	--	--	2	16	--	--	--	1	--	78	--	1	--	--	--	--	--	--	--	--	5
<i>S. patens</i> 1A	1	19	8	33	7	--	--	--	--	31	--	--	--	--	--	--	--	--	--	--	6
<i>S. patens</i> 1B	--	--	--	10	15	--	--	--	--	2	--	22	--	2	1	3	--	--	40	--	8
<i>S. patens</i> 2A	--	--	--	--	8	--	--	--	--	1	--	12	--	--	5	7	--	25	14	4	8
<i>S. patens</i> 2B	--	1	--	--	23	--	--	--	--	--	--	24	--	--	--	18	--	--	17	3	6

Table 4.1. Percent area coverage of total chromatogram area covered by terminal restriction fragments from *Spartina alterniflora* and *S. patens* collected from marshes in four states (Georgia, North Carolina, New York, Massachusetts) in the U.S. All numbers are averages of duplicate samples.

<i>Site</i>	<i>Coordinates</i>		<i>Average Temperature (°C)</i>		<i>Tide range</i>	<i>Salinity</i>	<i>Total Precipitation (in)</i>	
	<i>Lat</i>	<i>Long</i>	<i>Jan</i>	<i>July</i>	<i>(m)</i>	<i>(PSU)</i>	<i>Jan</i>	<i>July</i>
GA	31.4	81.3	18.4	31.8	2.1	29.4	3.7	1.6
NC	34.7	76.7	10.6	26.4	1.0	33.4-36.6	1.9	5.3
NY	40.6	73.9	0	22	1.5	23-27	5.9	4.8
MA	42.8	70.9	-8.08	25.6	1.5	29	0.79	3.3

Table 4.2. Environmental characteristics of each of the participant sites.

		37	44	63	72	78	91	130	135	144	147	149	150	153	159	204	424	448	478	490	499	511	# of T- RFs
Louisiana	Location																						
<i>S. alterniflora</i>	LUMCON	1	--	--	16	--	5	1	1	29	1	--	--	--	1	1	39	--	1	--	1	3	13
<i>S. patens</i>		10	--	--	--	2	37	--	1	18	--	--	--	--	--	--	8	1	21	--	1	--	9
Mississippi																							
<i>S. alterniflora</i>	GCRL	2	--	2	19	1	11	4	3	21	14	--	9	1	--	--	13	--	--	--	1	--	13
<i>S. patens</i>		6	--	--	9	1	1	7	3	43	--	13	--	2	1	--	5	2	--	2	3	2	15
Alabama																							
<i>S. alterniflora</i>	DISL	2	--	4	70	--	--	--	--	4	18	--	2	--	--	--	--	--	--	--	--	--	6
<i>S. patens</i>		8	1	--	3	3	3	1	20	7	--	24	--	1	--	--	27	--	--	--	--	1	12
Florida																							
<i>S. alterniflora</i>	Pensacola Beach	4	--	--	46	--	5	--	--	38	2	--	--	--	1	--	4	--	--	--	--	--	7

Table 4.3. Percent area coverage of total chromatogram area covered by terminal restriction fragments from *Spartina alterniflora*, and *S. patens* collected from marshes in four states (Louisiana, Mississippi, Alabama, Florida) in the U.S. All samples were collected in August of 2005. All numbers are averages of duplicate samples. Only *S. alterniflora* was collected in Florida because it was an *S. alterniflora* restoration area. LUMCON= Louisiana University Marine Consortium (Cocodrie). GCRL= Gulf Coast Research Labs (Ocean Springs), DISL= Dauphin Island Sea Lab (Dauphin Island).

Figure 4.1. Multidimensional scaling analysis (MDS) of terminal restriction fragment coverage of ascomycetes on *Spartina alterniflora* (blue) and *Spartina patens* (green) collected in four states (GA, NC, MA, NY). Data points are averages of duplicates collected from two subsites (A & B) in two marshes (1& 2).

Stress: 0.19

S. patens

S. alterniflora

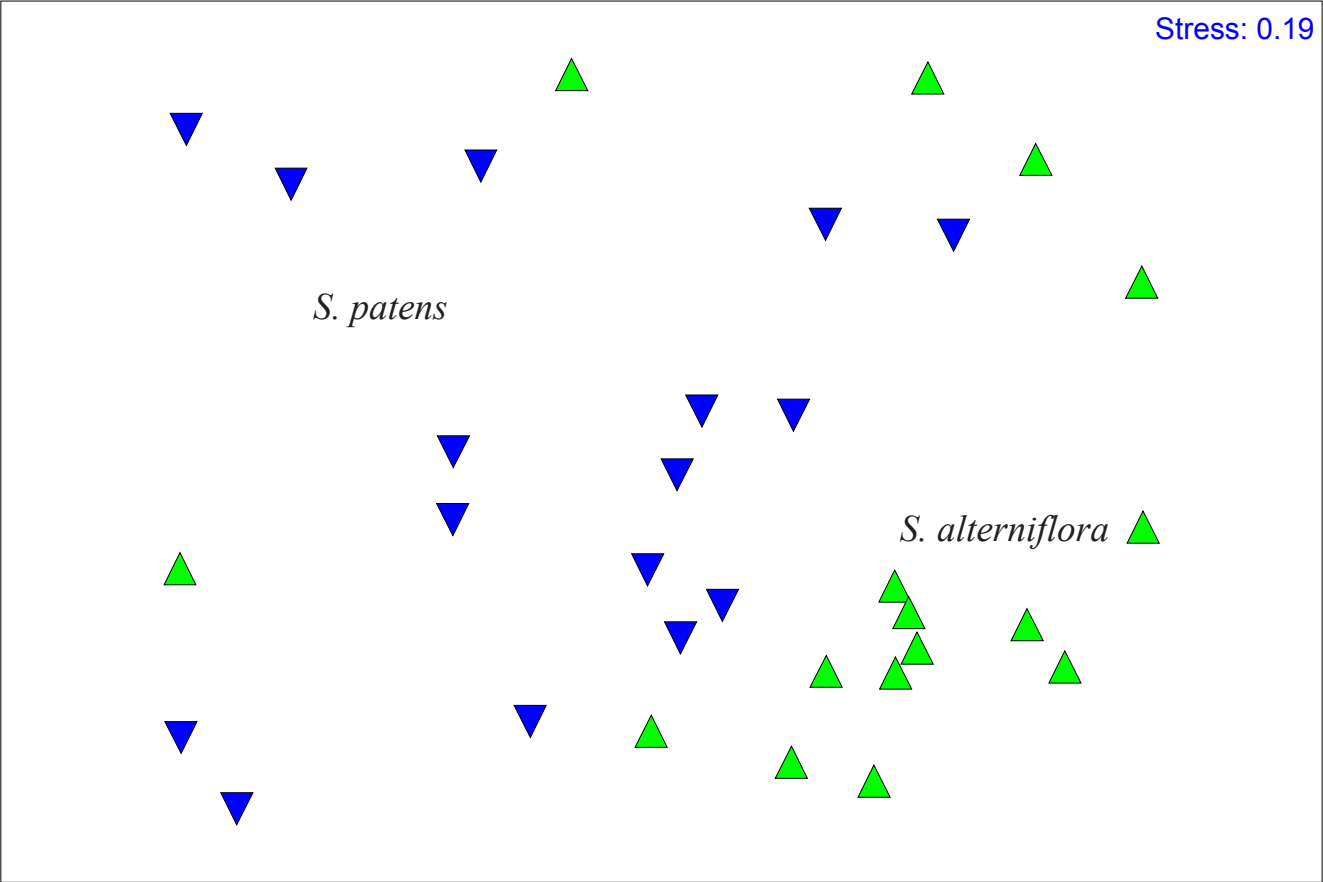
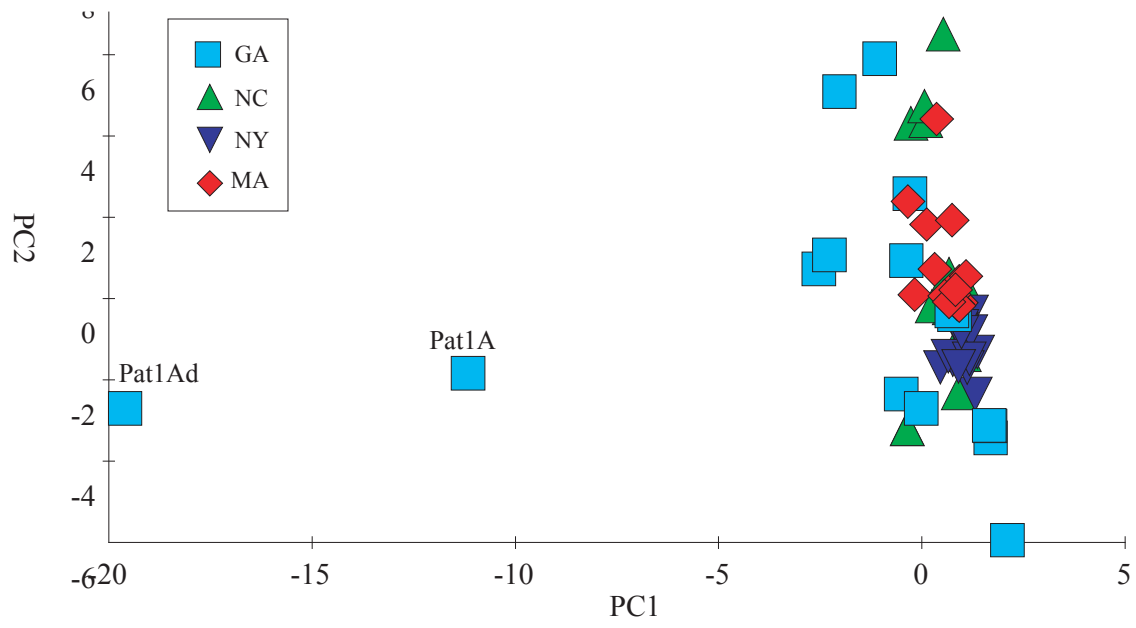


Figure 4.2. Principle component plots (PC1 x PC2) of scores of individual samples generated from 64 T-RFLP profiles of fungal communities associated with early-decay *S. alterniflora* and *S. patens* leaf blades. Input variables expressed as percentage of total peak area. Pat, *S. patens*; d, duplicate.



CHAPTER 5

CONCLUSIONS

The intent of the research described here was to investigate the degradation of salt marsh plants (*Spartina* species) by fungi and bacteria in salt marsh ecosystems, with a focus on a description of the ascomycetous fungi. To this end, we looked at both general associations between the fungal and bacterial consortia involved in decomposition, and at specific components of the fungal population.

Ascomycetous fungi are predominant secondary producers of the *Spartina alterniflora* decay system (8), and it has been shown that the overall fungal community can be diverse on marsh plants (2, 3, 10). Because of the abundance of fungi in the salt marsh decay system, their ecological role in the marsh is significant. Solubilization of organic matter by bacteria is another important feature of carbon cycling in marine ecosystem, and it has been established that bacteria contribute significantly to the transformation of this plant-derived material (5, 6). However, the question of associations between bacteria and fungi is still in the early stages of being addressed in the salt marsh ecosystem (1, 4).

In the first part of the research presented here (Chapter 2), we addressed the question of whether physical associations exist between individual bacterial and fungal species that co-occur on decaying smooth cordgrass, *Spartina alterniflora*, in a southeastern U.S. salt marsh. We

showed that bacteria and fungi do not establish species-specific ecological associations on the *Spartina* blades, despite the fact that they co-occupy and co-degrade the material. Interactions between bacterial and fungal decomposers that are not manifested as consistent physical associations between species were not addressed.

In Chapter 3, we addressed the questions 1) are the ascomycetous fungal communities on *Spartina alterniflora* transplanted to the west coast similar to those already described on the east coast? 2) do variations in the ascomycete communities exist within and among *Spartina* species? and 3) how did hybridization between invasive *S. alterniflora* and native *S. foliosa* change or affect the associated resident fungi? The clone libraries constructed from samples of *Spartina alterniflora* showed that samples of the grass transplanted to the west coast do host many of the same ascomycete decomposers as *S. alterniflora* in its native east coast environment. A previously described fungal species, *Phaeosphaeria spartinicola*, was often dominant and was found on all samples collected. Two other ascomycetes, *Phaeosphaeria halima* and *Mycosphaerella* sp. II, were also common, although the latter formed two novel subgroups as shown by previously undetected restriction sites in the ITS region. Significant differences in the fungal decomposer communities were detected both within (i.e. two clones of *S. foliosa*) and among species (i.e. *S. densiflora* was different than all other species, and hosted at least two unique ascomycetes), but the differences were minor and were attributed to morphological differences among the plants. Finally, we used T-RFLP analysis of the ITS region of ascomycetous fungi to show that neither *S. alterniflora* nor the hybrid supported fungal flora different from that of *S. foliosa*, but another exotic species (*S. densiflora*) did harbor a unique fungal community.

In Chapter 4 we asked whether the ascomycete community associated with *S. alterniflora* is the same as that found on *S. patens*, and whether the same dominant species of fungi are found on plants growing in different geographic regions. Two species of previously identified ascomycetes (*Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II) were dominant on both species of decaying *Spartina*, but two unidentified fragments (147 and 149 bp) appeared to be species-specific to *S. alterniflora* and *S. patens*, respectively. We also found that the diversity of the ascomycete taxa (as evidenced by T-RF fragments) was significantly higher on *S. patens* than on *S. alterniflora*, and that *S. patens* hosts a higher number of unique ascomycete species, and therefore concluded that host plant type is a primary determinant of fungal community composition.

Future Research

Several interesting questions arise from the results of this work. The global distribution of *P. spartinicola* and *Mycosphaerella* is intriguing, and it would be informative to investigate their adaptive and competitive strategies as well as their physiological requirements and tolerances.

It would also be interesting to see further analysis of the ascomycete community. For example, examining fungi associated with *S. patens* growing on the west coast would be useful in order to determine whether the observations made on the east and Gulf coasts hold true across all regions. Analysis of samples of *S. densiflora* from additional sites in California (or other regions, such as its native Chile) would help us to determine whether it truly does host a unique ascomycete community.

More thorough investigation into the species-specificity of T-RFs 147 and 149 is also warranted. Although these two fragments consistently appeared on different *Spartina* species, their closeness in size prevents us from determine conclusively whether they actually represent unique ascomycetes. Future studies could use DGGE and sequencing to investigate this question. If the two fragments did prove to represent unique ascomycetes, we would have even further evidence that *S. patens* and *S. alterniflora* host distinct ascomycete communities.

The data collected in this study will prove valuable to future efforts to evaluate the dynamics of microbial degradation in salt marshes. It has been established that microfungi are affected by environmental conditions (7, 9). Therefore, a reliable record of the environmental conditions (water salinity, air and water temperature, dissolved oxygen levels, pH, nutrients, etc.) at each sample site would provide more clues into the factors that contribute to determining microbial community composition. The information presented here would then serve as a basis for investigations into how the ascomycete community responds to seasonal change or environmental perturbations, ultimately providing insight into the ecological relevance of specific taxa to community structure and function.

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