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Analysis of Internal Transcribed Spacer (ITS) Regions of rRNA Genes in Fungal Communities in a Southeastern U.S. Salt Marsh

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ABSTRACT

The ascomycete community colonizing decaying *Spartina alterniflora* blades in a southeastern U.S. salt marsh was characterized by analysis of internal transcribed spacer (ITS) regions of fungal rRNA genes. ITS sequences were amplified with ascomycete-specific primers from DNA extracted from *S. alterniflora* blades at two stages of decay (early and late) and were identified based on sequence analysis of a companion ascomycete culture collection. The *S. alterniflora* ITS libraries were dominated by clones from three species of ascomycetes: *Mycosphaerella* sp. 2, *Phaeosphaeria spartinicola,* and *Phaeosphaeria halima.* ITS sequences from five other less abundant ascomycete species were also found in the clone libraries, only two of which could be identified based on the culture collection, *Hydropisphaera erubescens* and a new species nicknamed '4clt'. Ascospore expulsion assays indicated dominance by the same three species as the ITS analysis, although this non-molecular approach differed from the molecular method in relative ranking of the dominant species and in characterization of minor species. Analysis of ITS amplicons from three replicate plots by terminal restriction fragment length polymorphism (T-RFLP) analysis showed significant spatial homogeneity in ascomycete community composition for both early- and late-stage decay. ITS sequence analysis identified morphologically cryptic subgroups for two of the three dominant salt marsh ascomycetes.

Introduction

The importance of fungi in the biogeochemical cycling of carbon and nutrients in coastal salt marshes is well established [27 and references therein]. In southeastern U.S. marshes, for example, fungal productivity can reach 500 g m^{-2} y^{-1} [27], and hyphal biomass can equal almost one-third of the living organic matter of marsh grass in winter [32]. Saltmarsh fungi colonize senescing blades of the dominant primary producer, *Spartina alterniflora,* while the blades are still attached to the stem. As they carry out decomposition, fungi efficiently convert plant organic matter into fungal biomass in the form of mycelia and reproductive structures [32]. Further links from fungi to bacteria [27, 32] and animal shredders [11] establish an

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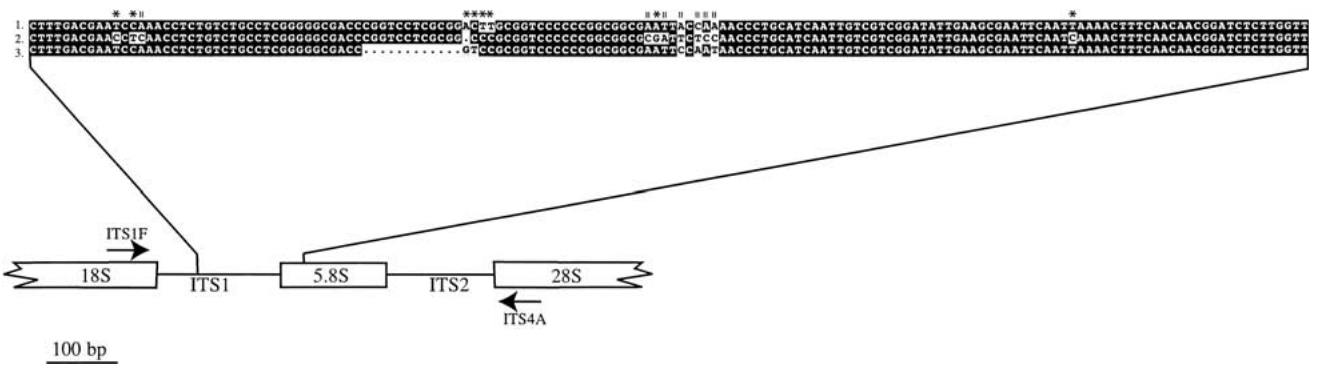

Fig. 1. Primer location for amplification of ascomycete ITS regions, and comparison of sequences of a variable region in ITS1 from (1) a clone from the early decay library (SIF32) that clustered with *Mycospharella* sp. 2 sequences, (2) *Mycospharella* sp. 2 Group A strains (SAP152 and SAP153), and (3) *Mycospharella* sp. 2 Group B strains (SAP133, SAP136 and SAP154). Base pair deletions (●), transitions (⋆) and transversions (||) are indicated.

important detritus-based food web in one of the most productive of marine ecosystems.

The *S. alterniflora* decay system in southeastern U.S. salt marshes is one of the few natural ecosystems for which fungal community composition is relatively well known. Using direct microscopic observation, several species of ascomycetes have been identified as major decomposers of *S. alterniflora* blades [27, 28]. The two most regularly recorded species (78% of all ascospores expelled from decaying blades over a 3-year period, and occurrence of ascomata in 66–77% of blades examined) are *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. 2 [16], both of which are involved in lysis of lignocellulosic components of the blades [3, 32, 33]. Other ascomycetes, such as *Phaeosphaeria halima* and *Buergenerula spartinae,* occur in about 2–40% of blades examined [28]. Species of mitosporic fungi (i.e., species that are probably asexual forms of ascomycetes [1]) have also been observed (e.g., *Stagonospora* sp. 2; [16]). In the leaf sheaths and true stems of decaying *S. alterniflora,* distinctly different ascomycetes from those of blades are detectable (e.g., *Lachnum spartinae* in sheaths) [32].

Based on immunoassay of *P. spartinicola* biomass, it has been hypothesized that the visually detectable ascomycetes of the *S. alterniflora* decay system are the only important biomass producers [32]. However, immunoassays can be inaccurate because of a variety of interferences [26], and it is possible that other ascomycete species participate cryptically but substantially in the decay of S. *alterniflora* blades. Testing this possibility requires use of methods other than direct observation or culturing.

Molecular methods that rely on DNA analysis are now widely available for microbial community analysis. Although used most frequently for bacterial community analysis, molecular methods are equally suited for investigations of fungal ecology [4, 18]. For the saltmarsh community, these methods have the potential to provide a novel perspective on the composition of the fungal community mediating *S. alterniflora* decomposition and may identify species that have not yet yielded to traditional mycological techniques. Moreover, rapid molecular fingerprinting methods, such as terminal restriction fragment length polymorphism (T-RFLP) analysis, can more readily and fully track fungal community dynamics over time and space than can more time intensive culture- and microscopy-based approaches. When using direct microscopy, for example, species of ascomycetes can be identified only when they exhibit visible reproductive structures.

Previous studies have identified the internal transcribed spacer (ITS) regions of fungal rRNA genes as suitable targets for molecular analysis of fungal communities [4, 8]. The ITS regions are stretches of DNA between the 18S, 5.8S, and 28S rRNA genes (Fig. 1). Their high sequence variability relative to the flanking rRNA genes makes them valuable for genus- and species-level identification. ITS analysis has been used with success to describe soil fungal community composition [12] and species diversity within endophytic fungi of common reed (*Phragmites australis*) [42]. At least in some cases, ITS analysis has produced a very different view of fungal community structure than culture-based methods [40].

Here we use sequencing and T-RFLP analysis of ITS regions to describe the fungal community associated

Table 1. Species, strains, and sources of ascomycetes and mitosporic fungi from smooth cordgrass (*Spartina alterniflora*)

Species	Strains Analyzed	Source
'1ch'[a]	SAP142	Sap
'4clt'[a]	SAP162, 163, 164	DC
Buergenerula spartinae Kohl & Gessner	SAP12, 124, 126	Sap
Debaryomyces hansenii[b] (Zopf) Lodder & Kreger-van Rij	NRRL Y-7426	LA
Halosarpheia viscosa (I. Schmidt) Shearer & Crane *ex* Kohlm. & Volkmann-Kohlm	SAP147	Sap
Helminthosporium sp.[c]	SAP156	Sap
Hydropisphaera erubescens (Desm.) Rossman & Samuels	SAP145	Sap
Kananascus sp.[c]	SAP 141	DC
Kluyveromyces lactis var. drosophilarum[b] (Shehata et al.) Sidenberg & Lachance	NRRL Y-8378	LA
Koorchaloma spartinicola Sarma, et al.	SAP130	Sap
Lachnum spartinae Cantrell	SAP138	DC
Mycosphaerella sp.2[d]	SAP133, 136, 152, 153, 154	DC, Sap
Phaeosphaeria halima (Johnson) Shoemaker & Babcock	SAP134, 137, 159, 160, 161	DC, Sap
Phaeosphaeria spartinicola Leuchtmann	SAP132, 135, 149, 150, 151	DC, Sap
Pichia spartinae[b] Ahearn, et al.	NRRL Y-7665-1, -7665-3	LA
Pleospora pelagica Johnson	SAP165	DC
Pleospora spartinae (Webster & Lucas) Apinis & Chesters	SAP146	DC
Stachybotrys sp.	SAP155	DC
Stagonospora sp. (4-celled)[d]	SAP144	Sap
Stagonospora sp. (8-celled)[d]	SAP143	Sap
Stagonospora sp. (8-celled, appendaged)[d]	SAP158	Sap
Tubercularia sp.[c]	SAP148	DC

[a] Nicknames for undescribed ascomycetes ('1ch' is the "one-celled haustorial" of Newell [28]; '4clt' is briefly described herein).
[b] Ascomycetous yeasts obtained from the National Center for Agricultural Utilization Research (courtesy of C.P. Kurtzman).
[c] Identity not confirmed.
[d] See [16, 17].
Strains were isolated from Dean Creek Marsh plots (DC), other Sapelo Island salt marshes (Sap), or Louisiana marshes (LA). The *Lachnum* species was isolated from leaf sheaths [5]; all others were from leaf blades.

with decaying *S. alterniflora* blades in a southeastern U.S. salt marsh. We compared community composition at two successive stages of decomposition in the decay process and examined spatial variability in fungal community structure within the marsh. Traditional mycological methods carried out in parallel (i.e., direct microscopy, ascospore identification, and culturing) provided a valuable comparison between the approaches.

Methods

Site Description and Sample Collection

Decaying blades of tall-form *Spartina alterniflora* were collected from Dean Creek Marsh, Sapelo Island, GA, in July 2000. Dean Creek Marsh is located within Site 6 of the 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). Senescent blades that represented two distinct temporal stages of decomposition were collected: "early decay" blades were yellow or brown in color, remained attached to the stem, and were not yet collapsed onto the sediment; "late decay" blades were brown to black in color and also remained attached to the stem, but were collapsed onto the sediment surface.

Three 5-m diameter replicate plots, designated plots 1–3, were established in Dean Creek Marsh, and 10 blades from each category were collected from each plot. Each 6-cm blade sample was cut in half, with one portion used for microscopy and isolation of fungi and the other for DNA extraction.

Fungal Observations and Isolations

Occurrence of ascomycetes in decaying leaf blades was recorded by observation of spore-capture coverslips and direct microscopy of leaf surfaces [28]. Briefly, the 3-cm lengths of blades were rinsed in tapwater, with gentle rubbing to remove clay films, and then soaked for 10 min in tapwater. Each wet blade piece was positioned within a 60 × 15 mm glass dish, 7 mm above a clean coverslip (6.25 cm^2), with the abaxial surface facing the coverslip. The dish contained deionized water in the bottom to maintain 100% relative humidity, and all dishes were enclosed within a 4-L sealed plastic bag, along with an open dish of deionized water, to prevent any drying of incubation dishes. Dishes were incubated at 20°C for 72 h under 30 μE m^{-2} sec^{-1} photosynthetically available radiation in a 12 h on/off cycle. Three 3-cm pieces were incubated for each of the blade types from each of the three marsh plots.

Each coverslip was subsequently examined under the dissecting microscope (Wild M8) at 100× along its entire width below where the center of the blade piece had been positioned [28]. Rather than enumerate all ascospores, we visually estimated relative order of abundance of each spore type, from most frequent to least frequent. Species of ascospores were identified according to Kohlmeyer and Kohlmeyer [16], Kohlmeyer and Volkmann-Kohlmeyer [17], and Leuchtmann and Newell [21], using a 400 × Zeiss Standard 16 Research Microscope with interference contrast. The abaxial surface of each blade piece was also examined under the dissecting microscope to check for the presence of ascomata of species that were not recorded as having expelled ascospores.

Thirty-five fungal isolates were obtained from the decaying *S. alterniflora* for ITS analysis (see below). Mycelial ascomycetes were isolated by an ascospore-drop technique [22]. Wet blade pieces were positioned in a sterile plastic dish (60 × 15 mm) above dilute V8 agar (DV8; 2 ml V8 [Campbell Soup, Inc.], 20 g agar, in 1 L half-strength seawater [15 g salts L^{-1}]). Incubation was at 25°C in indirect sunlight under natural day/night lighting. Ascospores that had been expelled onto the agar surface were removed with a flame-sterilized, flattened-tip nichrome needle under the dissecting microscope and transferred to new plates of dilute V8 agar. A wet, autoclaved piece of senescent (yellow-green) blade of *S. alterniflora* was added to the surface of the isolation plate adjacent to the transferred ascospores to ensure the availability of natural substrate. Mitosporic fungi that were observed directly on the incubated blade pieces were brought into culture by micromanipulation of conidia, and ascospores of ascomycetes that did not eject spores in spore-drop plates were also isolated by nichrome-needle micromanipulation at the dissecting microscope using DV8 plates.

We obtained 3–5 isolates of each of the most common ascomycete species in order to determine within-species variation in ITS sequence. We also searched for rare species of ascomycetes within naturally decaying S. *alterniflora* blades, and we isolated one species (*Lachnum spartinae*) that is known in ascomatal form only from leaf sheaths. Finally, we obtained cultures of four ascomycetous yeasts isolated from *S. alterniflora* marshes in Louisiana that are potential contributors in the *S. alterniflora* decay system [19, 24]: *Debaryomyces hansenii, Kluyveromyces lactis,* and two strains of *Pichia spartinae* (Table 1).

The isolates used for DNA extraction (Table 1) were maintained in pre-sterilized sections of blades. For generation of mycelium, 5–10 small pieces (≅ 1 mm^3 volume each) of DV8 culture were incubated statically at 25°C in 20 ML sterile malt/yeast-extract medium (ME/YE; 20 g malt extract, 2 g yeast extract, in 1 L half-strength seawater). After a substantial quantity of mycelium had been formed after approximately 2 weeks, the liquid medium was withdrawn by sterile pipette in a laminar-flow cleanhood and the mycelium stored wet at room temperature until subsequent DNA extraction. Cultures of the isolates are maintained in the laboratory of S. Y. Newell, and representatives have been submitted to ATCC (*Phaeosphaeria spartinicola* SAP132, *P. spartinicola* SAP135, *Mycosphaerella* sp. 2 Group A SAP153, *Mycosphaerella* sp. 2 Group B SAP154, *Phaeosphaeria halima* SAP134, *Hydropisphaera erubescens* SAP145, and '4clt' SAP162).

DNA Extractions and ITS Amplification

Isolation of DNA from fungal isolates was carried out on either cultures scraped from plates (yeasts only) or mycelia using Soil DNA Extraction Kits (MoBio, Solana, CA). Isolation of DNA from decaying *S. alterniflora* blades was carried out on ten 3-cm leaf blades from each sample using Mega Size Soil DNA Kits (MoBio). The ITS region was amplified using the ascomycete specific ITS 1F (5′ CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS4A (5′ CGC CGT TAC TGG GGC AAT CCC TG) primers [20]. These primers amplify a product of ~600 bp including the ITS1, 5.8S, and ITS2 regions of the rRNA operon (Fig. 1). PCR was carried out with Ready-To-Go PCR Beads (Amersham Pharmacia, Piscataway, NJ) with 0.2 μM concentration of each primer and 50 ng of DNA. An initial 3 min at 95°C was followed by 35 cycles of 1 min at 95°C, 30 sec 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.

Construction of Environmental ITS Clone Libraries

To construct clone libraries from fungal ITS sequences associated with decaying *S. alterniflora* blades, amplified ITS products of the appropriate size were recovered from a 1% agarose gel using a QiaSpin Gel Extraction Kit (Qiagen, Valencia, CA). PCR products were cloned using a TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). ITS clone libraries were constructed for early-decay blades and late-decay blades from Plot 1 (32 clones and 20 clones, respectively).

Sequencing and Phylogenetic Analysis

Approximately 400 bp of sequence information was obtained for the ITS regions of fungal isolates and environmental clones by either directly sequencing the PCR product following purification with an Ultra Clean PCR Clean-Up Kit (MoBio) (isolates) or sequencing purified plasmid DNA (clones) using the ITS1F primer on an ABI 310 Prism (Applied Biosystems, Foster City, CA). In some cases, full sequences were obtained by using the ITS4A primer in a second sequencing reaction. Sequences were analyzed using the Genetics Computer Group program package 10.0 (Wisconsin Package Version, Madison, WI). Coverage of amplicon diversity in the ITS libraries was determined by the method of Good [10] using the equation $1-(n/N)] \times 100$, where n = unique clones and N = total clones. Sequences are deposited in GenBank under accession numbers AF422939 to AF423029.

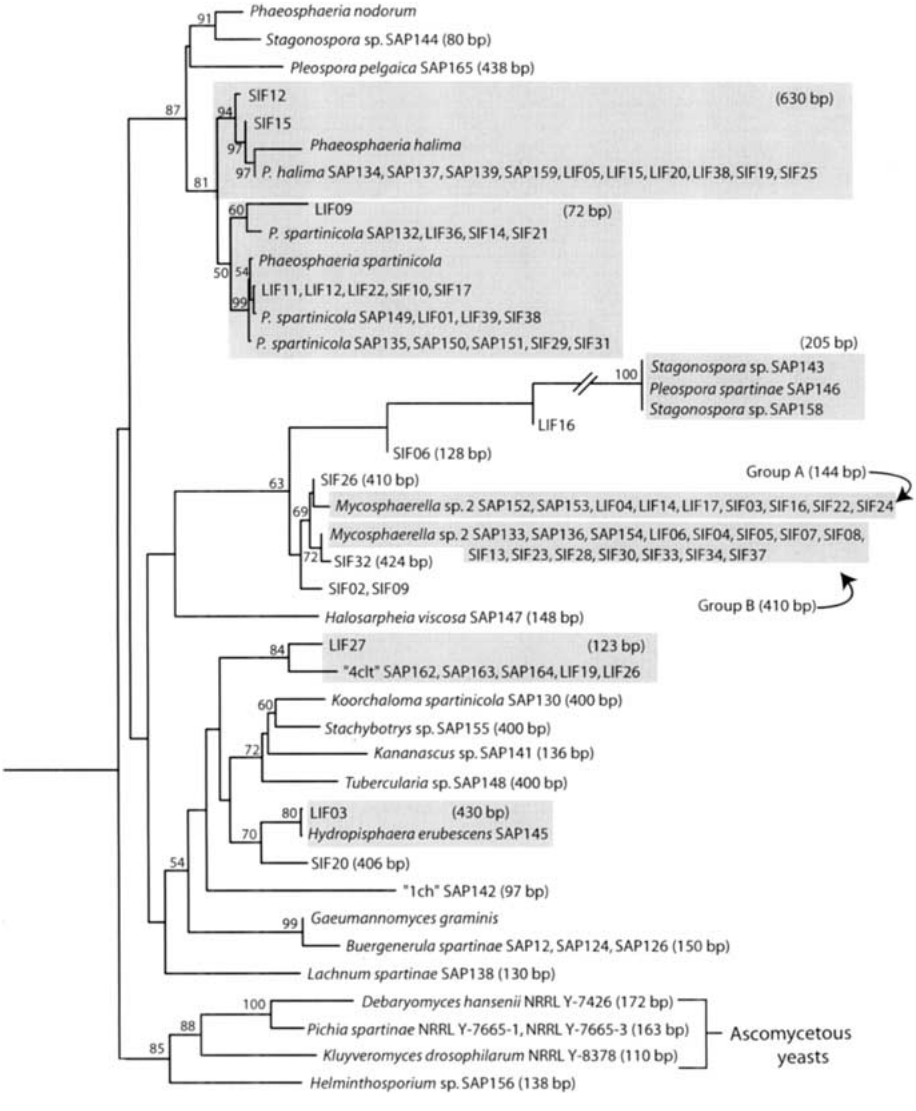

Fig. 2. Phylogenetic tree of ascomycete ITS sequences obtained from cultured strains and PCR amplicons from decaying *S. alterniflora* blades. Sequence labels are as follows: "SAP" = isolates from Sapelo Island; "NRRL" = yeast isolates from the National Center for Agricultural Utilization Research; "SIF" = ITS amplicons from early-decay blades from Sapelo Island; "LIF" = ITS amplicons from late-decay blades from Sapelo Island. The tree was constructed from 350 positions (ITS1, 5.8S rRNA gene, and ~30 bp of ITS2) using the Phylip program with Kimura distances with *Scutellospora castanea,* a zygomycete, as the outgroup. Bootstrap values greater than 50% are indicated at branch nodes. Groups of sequences with identical terminal restriction fragment size following digestion with *Hae*III are identified by shading; the size of the terminal restriction fragment is given in parentheses. GenBank accession numbers are as follows: *P. spartinicola,* AF181713; *P. halima,* AF181711; *P. nodorum,* AF246946; *G. graminis,* AJ010032. The *P. spartinicola* sequence is misnamed as *P. typharum* in GenBank (see [21]).

ITS T-RFLP Analysis

For T-RFLP analysis of ITS regions, PCR amplification was carried out as described above with the exception that the ITS1F primer was fluorescently labeled on the 5′ end with FAM (Applied Biosystems). Products were recovered from a 1.0% agarose gel with the QiaSpin Gel Extraction Kit (Qiagen). Restriction digests were carried out in a 10 μL total volume containing either 100 ng (community) or 10 ng (isolate/clone) purified PCR product and 10 U *Hae*III (Roche, Indianapolis, IN). Digests were carried out at 37°C for 3 h, after which samples were precipitated in ethanol and suspended in 12 μL of deionized formamide with 1 μL of DNA fragment length standard GeneScan-2500 (TAMRA; Applied Biosystems). The terminal restriction fragment 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 (R. Stepanauskas, personal communication). Peaks comprising <1% of total chromatogram area were excluded from analysis.

Results

Reference ITS Sequence Database

The ITS regions sequenced from a collection of ascomycetes isolated from the Dean Creek plots (15 strains) and other marsh sites on Sapelo Island (13 strains) represented 18 morphologically distinct species. In addition, ITS regions were sequenced from 4 ascomycetous yeasts (representing 3 species) isolated previously from *S. alterniflora* salt marshes in Louisiana (see Methods) (Table 1). These sequences were used to establish a reference ITS database for identifying fungal sequences amplified directly from decaying *Spartina alterniflora* blades.

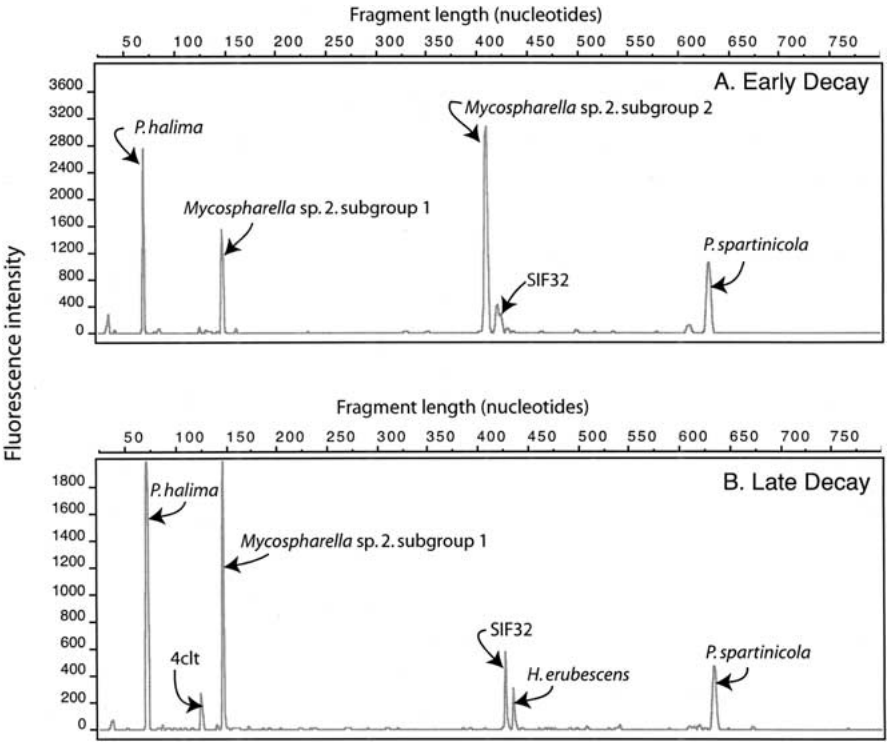

Fig. 3. T-RFLP chromatograms of amplified ITS regions from the ascomycete community associated with early (A) and late (B) stage decay *S. alterniflora* blades from plot 1. Peak identifications were based on terminal restriction fragment lengths generated by *Hae*III digests of cultured isolates and/or clones.

Variation in ITS sequences readily distinguished strains at the species level (Fig. 2). For example, all four strains *of Phaeosphaeria halima* clustered with 98.3% similarity, along with a previously sequenced *P. halima* strain isolated from decaying wood (83.5% similarity). Similarly, the three strains of *Buergenerula spartinae* clustered (99.4% similarity), as did all yeast strains (47.8% similarity). ITS sequence analysis showed that the *Mycosphaerella* strains isolated from the Sapelo Island marshes formed two phylogenetic subgroups (86% sequence similarity). Likewise, the *Phaeosphaeria spartinicola* strains formed two subgroups (77.2% similarity).

A subset of Sapelo ITS sequences were aligned in order to identify the restriction enzyme that would be most useful for discriminating among species during T-RFLP analysis. *Hae*III was predicted to give the greatest variability in length of the terminal restriction fragment among the cultured ascomycetes. We then empirically determined the actual size of the *Hae*III terminal restriction fragment for all isolates, and found they produced 20 different fragment sizes (Fig. 2).

The size of the terminal restriction fragment was conserved among strains of the same species, yet was sufficiently variable to discriminate between species, or in some cases between groups within a morphological species (Fig. 2). The two phylogenetic groups of *Mycosphaerella* evident from ITS sequence analysis had different terminal restriction fragment lengths (Group A = 144 bp and Group B = 410 bp), although the groups of *P. spartinicola* did not (72 bp for both). Three mitosporic species that formed a phylogenetic cluster (*Stachybotrys* sp., *Koorchaloma spartinicola,* and *Tubercularia* sp.) also shared a terminal restriction fragment size (400–402 bp).

In two cases, terminal restriction fragments of unrelated species were too similar to be confidently distinguished in complex chromatograms. *Helminthosporium* sp. (SAP 156) and *Kananascus* sp. (SAP 141) had terminal restriction fragment lengths of 138 and 136 bp, respectively. *B. spartinae* (SAP12, SAP124, and SAP26) and *Halosarpheia viscosa* (SAP147) had *Hae*III terminal fragment lengths of 150 and 148 bp.

ITS Sequences from Decaying S. Alterniflora Blades

Partial ITS sequences were obtained for 32 clones from the early-decay library and 20 clones from the late-decay library. Coverage of amplicon diversity in the ITS libraries was 56% (early-decay library) and 45% (late-decay library).

Many of the ITS sequences amplified from decaying *S. alerniflora* blades matched sequences from the fungal isolate collection (Fig. 2). Nineteen of 52 sequences

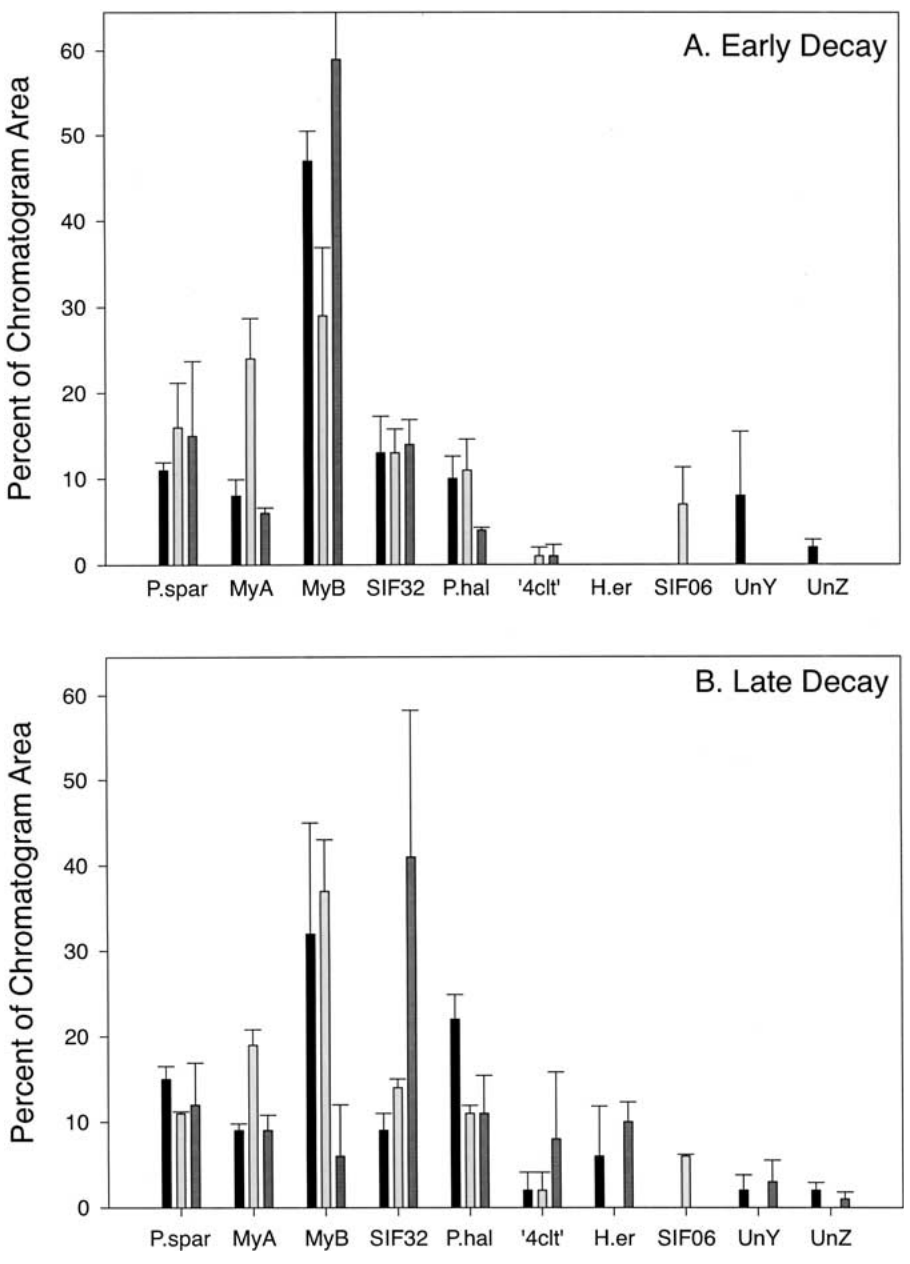

Fig. 4. Spatial variability in the pool of ascomycete ITS amplicons obtained from early (A) and late (B) stage decay *S. alterniflora* blades. Each cluster of bars represents T-RFLP analyses in plots 1–3 for a single taxon. Error bars (±1 standard error) show variability in triplicate analysis of a single DNA sample. Species codes are as follows: P.spar = *P. spartinicola*; MyA = *Mycosphaerella* sp. 2, Group A; MyB = *Mycosphaerella* sp. 2, Group B; SIF32 = clone from the early decay library clustering with *Mycosphaerella* strains; P.hal = *P. halima*; "4clt" = undescribed species nicknamed 4clt; H.er = *Hydropisphaera erubescens*; SIF06 = clone from early decay library; UnY = unidentified taxon with a 159 bp terminal fragment; UnZ = unidentified taxon with a 619 bp terminal fragment.

were identical to isolates representing one or the other of the two groups of *Mycosphaerella* sp. 2, six were identical to *P. halima* sequences, and eight were identical to one or the other of the *P. spartinicola* strains. Other amplified ITS sequences had one or more mismatches with isolate ITS sequences, but nonetheless clustered with known species (Fig. 2). None of the cloned ITS sequences appeared related to *B. spartinae,* an organism identified by direct microscopy and ascospore expulsion assays to be an important component of the *S. alterniflora* decay system [9, 15, 28]. Similarly, no ITS sequences clustered with those of the ascomycetous yeasts.

T-RFLP Analysis of Fungal Community ITS Sequences

To determine analytical variability associated with the T-RFLP analysis, we subjected each of the six DNA samples (i.e., from two decay stages in each of three plots) to three separate analyses, including PCR amplification, *Hae*III digestion, and fragment analysis. These triplicate analyses showed strong within-sample consistency with regard to the presence/absence and size-calling of ITS restriction fragments (not shown). The relative abundance of the fragment expressed as percent of the total T-RFLP chromatogram area was more variable, however, with the coefficient of variation for the triplicate

analyses of the same DNA sample ranging from 3% to >100%.

The database of terminal restriction fragment lengths assembled from the cultured ascomycetes and the *S. alterniflora* ITS clones was helpful in interpreting the community T-RFLP patterns, in many cases allowing us to identify taxa responsible for specific fragments (Fig. 3). For early-decay blades, sequences related to *Mycosphaerella* sp. 2 were the most common ITS amplicons; *Mycosphaerella* sp. 2 Group B accounted for 45% of the chromatogram area (average of plots 1, 2, and 3), *Mycosphaerella* sp. 2 group A accounted for 13% of the area, and a clone grouping with *Mycosphaerella* sp. 2 group B based on ITS sequence analysis (SIF32) accounted for an additional 13% (Fig. 4A). Two other taxa well represented in the T-RFLP analysis were *P. spartinicola* (14%) and *P. halima* (9%). Three taxa present but at low and variable frequency were '4clt' (an undescribed species of ascomycete; 1%), SIF06 (a clone from the early decay library without a match to a cultured ascomycete; 2%), Unknown Y (a 159 bp fragment with no match in the culture or clone libraries; 3%), and Unknown Z (a 619 bp fragment; 1%) (Fig. 4A).

For late-decay blades, the three *Mycosphaerella* groups also dominated the ITS amplicons, averaging 25% of chromatogram area for *Mycosphaerella* sp. 2 group B subgroup 2, 12% for group A, and 21% for SIF32 (Fig. 4B). Other abundant taxa among the amplified ITS sequences were *P. spartinicola* (12%), *P. halima* (15%), and *Hydropisphaera erubescens* (5%). Present in lower abundance were '4clt' (4%), SIF06 (2%), Unknown Y (2%), and Unknown Z (1%).

The ascomycete community associated with decaying *S. alterniflora* blades was spatially homogeneous in Dean Creek marsh. Most of the same fragments were present in the T-RFLP chromatograms for all three plots, and the relative peak area for the fragments i.e., representation of the sequence in the ITS amplicon pool, was also similar (Fig. 4). The low-abundance taxa showed more variability than the major groups. For example, Unknown Y was present in plots 1 and 3 but not plot 2, and SIF06 was present only in plot 2.

Direct Observations of Fungal Communities

In the early-decay community, *P. spartinicola* was most prolific in expelling ascospores in all three plots, with *Mycosphaerella* sp. 2 ranking second (Table 2). No other ascomycete spores were recorded from early-decay blades. In the later-decay community, five ascomycete species expelled spores: *P. spartinicola, P. halima, Mycosphaerella* sp. 2, '4clt,' and *B. spartinae*. Both *P. spartinicola* and *Mycosphaerella* sp. 2 were found in all three plots for both decay stages.

The species nicknamed '4clt' is an undescribed species (J. Kohlmeyer, personal communication) discovered in this study that is probably the same as the species independently found in naturally decaying *S. alterniflora* and nicknamed "asco7" (by J. and B. Kohlmeyer). A capsulized description of "4clt" follows: ascomata pale brown in the approximately 80×90 μm basal portion, with a 50×75 μm neck, blending from pale brown toward hyaline at the tip, ascospores hyaline, 4-celled, constricted at the septa, approximately 23×7 μm, without a sheath; ascomatal necks often protrude as white tufts through clay-film layer on older decaying blades.

Discussion

Using ITS Regions for Fungal Community Analysis

We initially focused our community analysis efforts on the 18S rRNA gene, but a screen of sequences from Sapelo Island fungal isolates indicated that the gene was too highly conserved to fully distinguish species in this ecosystem. For example, isolates of *P. spartinicola* and *P. halima* had 99.4% sequence identity over ~1100 bp of their 18S rRNA genes. In contrast, sequences of the ITS regions of the rRNA operon were sufficiently variable for characterizing natural fungal communities at the species and strain level. One limitation of the ITS region, however, is that the level of sequence variation prevents the design of universal fungal primers [reviewed in 4]. Because previous intensive culturing and microscopy had indicated that ascomycetes were the dominant fungal species in the salt marsh [27, 28], we chose to focus on this taxon through the use of ascomycete-specific ITS primers [20].

We established an ITS sequence database for salt marsh fungal isolates that would allow identification of sequences amplified directly from the *S. alterniflora* decay system. The isolate collection contained representatives of ascomycetes most commonly observed by direct microscopy in decaying *S. alterniflora* blades from the Sapelo Island marshes [27, 28], along with species of ascomycetous yeasts typically found in ecologically similar salt marsh systems [24]. In our search for isolates (beginning July

2000) we did not encounter all of the rare species that are known to be present in the *S. alterniflora* decay system (see [28]). Thus it is likely that one or more of the clones and T-RFLP peaks that we could not match to our current culture set (Table 1) represent rarer species, rather than species not detectable by microscopy or culturing.

Because rRNA operons in fungi are often found as tandem repeats of up to 100 copies [14, 23], the possibility exists of significant interspecies differences in ITS copy number. Such differences in template number would potentially act as an additional source of PCR bias, ultimately affecting the composition of the ITS amplicon pool. Intraorganismal heterogeneity among ITS regions of the multiple rRNA operons has also been documented in some fungi [2, 13, 35]. We found no evidence for this here, however, since all amplified ITS regions could be sequenced directly from the PCR products, i.e., heterogeneous ITS sequences in a single isolate would have required cloning prior to sequencing.

Comparison of Molecular and Traditional Methods

There was general consistency between the ITS-based and the microscopy-based methods in describing the major components of the ascomycete community. All three methods (ITS sequencing, ITS T-RFLP analysis, and ascospore expulsion) identified *Mycosphaerella* sp. 2 and *P. spartinicola* as dominant colonizers in both decay stages of *S. alterniflora* blades, and '4clt' on late-decay blades. Instances in which the methods differed were: (i) *P. halima* ascospores were not found on early-decay blades despite the abundance of sequences from this organism in the early ITS clone library and T-RFLP analysis; (ii) *H. erubescens* ascospores [36] were not found on late-decay blades despite the presence of sequences from this organism in the ITS clone library and T-RFLP analysis; and (iii) *B. spartinae* ascospores were found on late-decay blades despite the absence of sequences from this organism in both ITS-based analyses.

A more quantitative comparison of the methods is possible for plot 1, for which subsamples of the same blades were used in clone library construction, T-RFLP analysis, and ascospore expulsion surveys. The ITS-based methods together found 7 ascomycete taxa on early-decay blades and 8 taxa on late-decay blades in this plot (Table 3). By comparison, the ascospore method found 2 and 5 taxa on the early- and late-decay blades, respectively. The relative abundance of ascospores did not correlate well with the relative numbers of ITS amplicons for a given species. For example, *Mycosphaerella* sp. 2 sequences clearly dominated the ITS amplicon pools in both decay stages, but ranked second and third in ascospore expulsion (Table 3).

Quantitative matches between the molecular- and microscopy-based methods are not necessarily expected, however, given the well-recognized biases in both approaches. Ascospore analysis can only identify species induced to expel ascospores during laboratory incubations [28]. We scanned the blades for evidence of vegetative growth of other fungi and did not find any, however, suggesting that the method did not miss any colonizers that could be identified by visual inspection. The quantity of ascospores expelled is also not necessarily correlated with species biomass. Although it may be reasonable to expect that ascospore expulsion is at least roughly correlated with total mycelial production or state of fungal maturity for a given species, this has not yet been tested.

Meyers et al. [25] concluded from culturing fungi from living shoots in Louisiana salt marshes that *Pichia spartinae* was a common endophyte of the grass, and they found other yeasts to be common in the *S. alterniflora* decay system as well [24]. We did not find these species of yeasts as ITS amplicons (Fig. 2). One explanation of this situation might be that *P. spartinae* is not an effective competitor in the *S. alterniflora* tissue after the mycelial ascomycetes have begun pervasion and digestion of the dying and dead leaf blades [33].

Buergenerula spartinae is the only mycelial ascomycete among those recognized by direct microscopy to be a part of the *S. alterniflora* fungal-decay community [9, 28] that did not appear among the ITS amplicons from our sam-

Table 2. Ascospore expulsion from early and late decay *S. alterniflora* blades[a]

	Early-decay blades	Late-decay blades
Plot 1	*Phaeosphaeria spartinicola* *Mycosphaerella* sp. 2	*Phaeosphaeria halima* *Phaeosphaeria spartinicola* *Mycosphaerella sp.* '4clt' *Buergenerula spartinae*
Plot 2	*Phaeosphaeria spartinicola* *Mycosphaerella* sp. 2	*Phaeosphaeria spartinicola* *Mycosphaerella* sp. 2 *Buergenerula spartinae*
Plot 3	*Phaeosphaeria spartinicola* *Mycosphaerella* sp. 2	*Phaeosphaeria spartinicola* *Mycosphaerella* sp. 2 *Phaeosphaeria halima*

[a] Ascomycete species found in each plot are listed in rank order of ascospore abundance, from most to least abundant.

Table 3. Comparison of methods for describing fungal community composition for plot 1

Species/phylotype	Early decay			Late decay		
	Clones (%)	T-RFLP (%)	Spores (rank)	Clones (%)	T-RFLP (%)	Spores (rank)
Mycosphaerella sp. 2[a]	59	68 ± 14	2	20	50 ± 25	3
P. spartinicola[b]	22	11 ± 2	1	35	15 ± 3	2
P. halima	13	10 ± 5	np	20	22 ± 5	1
'4clt'	np	np	np	15	2 ± 4	4
H. erubescens	np	np	np	5	6 ± 10	np
B. spartinae	np	np	np	np	np	5
SIF06	3	np	np	np	np	np
LIF16	np	np	np	5	np	np
SIF20	3	np	np	np	np	np
Unknown Y (159 bp)	np	8 ± 13	np	np	2 ± 3	np
Unknown Z (619 bp)	np	2 ± 2	np	np	2 ± 2	np

[a] *Mycosphaerella* subgroups cannot be distinguished based on ascospore morphology and therefore are grouped together for all methods.
[b] *P. spartinicola* subgroups cannot be distinguished based on ascospore morphology or T-RFLP fragment size and therefore are grouped together for all methods.
Clone library data are based on sequence analysis of randomly selected ITS clones (32 early decay and 20 late decay). T-RFLP data are based on the percent of total chromatogram area assigned to a specific taxon (average of 3 replicate analyses ± 1 s.d.). Ascospore data are species ranks based on relative abundance of expelled ascospores. np = not present.

ples. It has been suggested that *B. spartinae* is a member of the community of fungi that decompose leaf sheaths of *S. alterniflora* [28]. When it appears in leaf blades, the ascomata are embedded in darkly melanized patches within the unmelanized areas of occupation by the *Phaeosphaeria/Mycosphaerella* complex. The blackened areas, with probable high contents of polyphenols, may not be susceptible to facile DNA extraction. Alternatively, the melanization may be a sign of competition with the surrounding *Phaeosphaeria/Mycosphaerella* complex [32]; perhaps the competition results in early loss of much of the hyphal mass and DNA of *B. spartinae*.

Biases in ITS-based methods may arise from the interspecies variability in DNA extraction efficiency, primer binding, PCR amplification kinetics, and cloning efficiency [38, 39]. Thus the ITS amplicon mixture used in clone library construction and T-RFLP analysis does not necessarily represent the composition of the original templates. Nonetheless, ITS analysis proved particularly valuable for identifying the presence of less abundant members of the fungal community that were not evident from microscopic surveys.

ITS analysis was also sensitive enough to identify intraspecific and intrageneric variation that was not apparent from morphological analysis. As has been reported previously [7, 34, 35, 37, 43], fungal ITS sequence analysis may prove to be extremely useful for unraveling the systematics and phylogeny of the dominant saltmarsh fungi. At least two groups of *Mycosphaerella* sp. 2 that are not readily distinguishable based on mycelia and ascospore morphology were differentiated by ITS analysis (Fig. 1). Likewise, ITS sequence analysis indicated the presence of two cryptic groups of *Phaeosphaeria spartinicola* (Fig. 2; the greatest sequence variability for these groups is in ITS2, a region not used in tree construction). ITS analysis also provided information on the phylogenetic affiliations of the two mitosporic *Stagonospora* species, 4-celled and 8-celled, which are considered asexual forms of ascomycetes. Based on sequence analysis, the two *Stagonospora* species are not closely related. The 4-celled form (SAP 144) is likely to be the pycnoconidial form of a *Phaeosphaeria* other than *P. spartinicola* or *P. halima* (as suggested by [21]), and the 8-celled forms (SAP143, SAP158) are likely to be *Pleospora spartinae* [17] (Fig. 2). The similarity of the ITS sequence of *Buergenerula spartinae* to that of *Gaeumannomyces graminis* (Fig. 2), the pathogen that causes take-all disease of cereal grasses, is in agreement with the shared morphological features in these fungi including vibrioid microconidia, dark-brown, lobed hyphopodia associated with runner hyphae [41]. Finally, the recovery of an ITS sequence that could not be linked to known ascomycetes motivated an intensive reexamination of ascospore identities and led to the discovery of a new species nicknamed '4clt'.

Ascomycete Community Dynamics

In a typical summer, *S. alterniflora* blades in southeastern U.S. saltmarshes progress from the early-decay category to the late-decay category in ~10 weeks (estimated from data in [29, 30]). The major ascomycete colonizers apparently do not change during this time period: *P. spartinicola, P. halima,* and subgroups of *Mycosphaerella* sp. 2 dominate both early and late blades. However, both ITS-based and microscopy-based analyses suggest a more diverse community on the late-decay blades: six sequence clusters were present in the late ITS clone library vs 5 in the early (despite that fact that there was poorer coverage of the late clone library); 11 vs 9 major T-RFLP fragments were found for late vs early blades; and 5 vs 2 species expelled ascospores from late vs. early blades. '4clt' and *H. erubescens* are tentatively identified here as late-stage decay specialists.

Plots 1–3 were located in the ecological zone referred to as 'low marsh,' region regularly flooded by tidal waters that supports a tall form of *S. alterniflora.* The ITS T-RFLP chromatograms were similar for all three plots (Fig. 4), suggesting a spatially homogeneous ascomycete community within this zone. The ascospore expulsion data, however, were more variable (Table 2), indicating either that ascospore expulsion is not strongly linked to fungal biomass (although it may be linked to fungal production) or that the PCR amplification of ITS sequences is biased toward specific taxa, driving the composition to appear homogeneous regardless of starting templates. A previous survey of ascospore expulsion from U.S. Atlantic coast saltmarshes over a latitude range from 29° to 43°N latitude identified four ascomycete species on decaying *S. alterniflora* blades at all sites: *P. spartinicola, Mycosphaerella* sp. 2, *P. halima,* and *B. spartinae* [31]. The first three of these species were confirmed by ITS sequence analysis to dominate the Sapelo Island marshes in the summer of 2000.

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