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Cellulolytic fungi in geographically and edaphically different soils based on phylogenetic and functional gene markers

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ABSTRACT

Soil fungi are well known for their ability to degrade cellulose. However, the composition of cellulolytic fungi in soil and the enzymes they use in the degradation of cellulose remain unclear. The soil fungal communities that responded to cellulose and the immediate breakdown products of (hereafter referred to as cellulolytic) were identified in soil microcosms amended with ^{13}C-cellulose, using geographically and edaphically different soils. Active cellulolytic fungal communities were identified using DNA stable isotope probing followed by sequencing of the large subunit rRNA (LSU) and cellobiohydrolase I *(cbhI)* genes. Using either the LSU or *cbhI* gene sequences, the ^{13}C-cellulose enriched communities were typically less rich, less diverse, and distinct from the ^{12}C-non-enriched communities. Most of the ^{13}C-cellulose enriched operational taxonomic units (OTUs) were unique to a particular soil. Based on the LSU sequence libraries, the ^{13}C-cellulose enriched OTUs were identified as members of the *Trichocladium, Chaetomium, Dactylaria*, and *Arthrobotrys*. In addition, two novel clusters were identified in a longleaf pine soil. The *cbhI* sequence libraries mirrored these taxonomic descriptions, illustrating the utility of using either gene to assess distributional patterns of cellulolytic fungi in soils. The soil-specific nature of cellulolytic fungi responsive to ^{13}C-cellulose amendment indicates that multiple geochemical and plant-associated factors are likely to be important in shaping the composition of cellulolytic fungi in different soils.

INTRODUCTION

Cellulose is a major component of plant structural carbon (30, 32) and is a key component of the biosphere's carbon budget (3). Fungi have been described as the most important cellulose degraders in terrestrial environments (8). In addition, cellulolytic fungi contribute to the release of inorganic nutrients, turnover of soil organic matter soil (7), and stabilization of soil (8). The cellulolytic ability of fungi has been intensely studied at the process, enzyme and molecular levels in some members of the *Basidiomycota* (e.g. *Phanerochaete chrysosporium* and *Postia placenta* (35)) but little is known about the composition and activity of cellulolytic fungi in soils, and how chemical and physical soil properties may influence their distribution and activity.

Previous stable isotope studies that have examined cellulose and other complex plant carbon substrates focused only on characterization of the cellulolytic bacterial communities (4, 15, 28), not the cellulolytic fungi. Given the potential importance of cellulolytic fungi in soil carbon cycling processes, we sought to identify cellulolytic fungi in geographically and edaphically different soils using a stable isotope probing approach coupled with the use of phylogenetic and functional gene markers.

We hypothesized that the different physical and chemical factors inherent in soils would select for distinct cellulolytic fungal guilds in the presence of added cellulose substrate. We used a microcosm approach combined with stable isotope probing to identify soil fungi that actively incorporated ^{13}C-cellulose or immediate breakdown products of cellulose (hereafter referred to as cellulolytic) into their DNA in five geographically and edaphically distinct soils. The composition of cellulolytic fungi was assessed in ^{13}C labeled DNA from each of the soils by

sequencing the large subunit rRNA gene (LSU) as a taxonomic survey tool, and the cellobiohydrolase I gene (*cbh1*) as a marker for cellulolytic capability (11, 23). The GH7 cellobiohydrolase, an exocellulase that cleaves cellobiose or glucose from the nonreducing end of the cellulose molecule, acts in concert with endoglucanses to hydrolyze cellulose into glucose monomers (11, 23). This study identified the most dominant taxa that were enriched after thirty to thirty-five days in ^{13}C-cellulose amended microcosms, which will be targets for further physiological investigations.

MATERIALS and METHODS

Soil samples. Soils were collected from five geographic regions, and differed in dominant plant cover, physical and geochemical chemical characteristics. A detailed description can be found in Supplemental Material 1.

Soil microcosms. Triplicate, 240 ml-crimp-top sealed serum bottles, containing approximately five grams of soil, were either supplemented with 0.05 grams of ^{13}C-UL-maize cellulose (Isotec Sigma-Aldrich, Miamisburg, OH) or left as un-amended controls. To promote microbial activity in the soils, the water content of each soil was adjusted to generate moist conditions where soil particulates were aggregated, but not saturated (Eichorst and Kuske 201x). Microcosms were incubated at room temperature (ca. 23ºC), for 30-35 days, a similar incubation period utilized in previous work (15), and under low light conditions. An ambient air headspace was maintained in the microcosms by periodic venting.

DNA extraction, isopycnic centrifugation, fractionation at day 30. The soil microcosm DNA for isopycnic centrifugation and fractionation at day 30 was extracted with the MP Biomedicals FastDNA® Spin Kit (Solon, OH) from 8 x 0.25 gram aliquots from each of the three replicates of the ^{13}C-cellulose amended and un-amended microcosms.

Isopycnic gradients were set-up for two ^{13}C-cellulose amended and two un-amended microcosms per soil type as described by Eichorst and Kuske 201x. Briefly, a density gradient solution of 1.599 grams cesium chloride ml^{-1} of 1X TE buffer (pH=8.0) with ethidium bromide were used to separate ^{13}C-cellulose-enriched and ^{12}C-non-enriched DNA by centrifugation in a Beckman Optima Ultracentrifuge using a TLA 120.2 fixed-angle rotor for 69 hours at 57,000 RPM at 14°C. Fractions were collected and the refractive index was measured on a Baush and Lomb Abbe-3L Refractometer. Fractions containing the ^{13}C-cellulose-enriched and ^{12}C-non-enriched DNA were determined by the buoyant density and DNA concentration (Eichorst and Kuske 201x).

Generation of Sanger Libraries for time zero, ^{13}C-cellullose-enriched, and ^{12}C-non-enriched DNAs. Fragments of the LSU rRNA gene and catalytic domain of the *cbhI* gene were amplified from DNAs of the time zero (T0), ^{13}C-enriched (T30-^{13}C), and ^{12}C-non-enriched (T30-^{12}C) fractions.

(i) LSU PCR. A fragment of the LSU rRNA gene was PCR amplified in triplicate from T0, T30-^{13}C, and T30-^{12}C DNA fractions using the broadly inclusive fungal 25-28S rRNA gene primer set (LR0R (forward): 5' ACC CGC TGA ACT TAA GC 3') and (LR3 (reverse): 5' CCG TGT TTC AAG ACG GG 3') (http://www.biology.duke.edu/fungi/mycolab/primers.htm). Each

25 µl PCR reaction contained 1x PCR buffer, 1 mM $MgCl_2$, 0.03 mM of each dNTP, 0.2 µM each primer, and 5U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Reactions were run in triplicate. Thermal cycling consisted of the following steps: (1) 95ºC for 3 minutes; (2) 95ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 45 seconds (repeated 30X); (3) 72ºC for 10 minutes.

(ii) ***cbhI PCR.*** A fragment encoding a 166-173 amino acid-long fragment of the catalytic domain of *cbhI* was PCR amplified in triplicate from each aforementioned DNA in 50 µl reactions containing the following: 1.6 µM final concentration of cbh1F (5'ACC AAY TGC TAY ACI RGY AA3') and 1.6 µM final concentration cbh1R (5'GCY TCC CAI ATR TCC ATC 3') (11), 1X PCR Buffer (Applied Biosystems, Foster City, CA), 0.8 mM dNTPs, 5 µg Bovine Serum Albumin (Roche Diagnostics, Indianapolis, IN) and 2.5 U AmpliTaq Polymerase LD (Applied Biosystems, Foster City, CA). Thermal cycling consisted of the following steps: (1) 95ºC for 3 minutes; (2) 94ºC for 1 minute, 48ºC for 45 seconds, 72ºC for 1 minute (repeated 35X); (3) 72ºC for 10 minutes. Genomic DNA of *Fusarium poae* (Fusarium Research Center, Penn State University, College of Agricultural Science) was used as the positive control for the *cbhI* reactions.

Triplicate PCR products were pooled and separated on a 1% agarose gel in 0.5X tris-borate-EDTA (TBE) and visualized with ethidium bromide. PCR products were purified using either the Qiagen QIAquick PCR Purification Kit (Valencia, CA) (LSU rRNA gene) or Qiagen QIAquick Gel Extraction Kit (Valencia, CA) (*cbhI* gene). Purified PCR amplicons were cloned with the Invitrogen TOPO TA Cloning Kit for sequencing (Invitrogen Carlsbad, CA) as per the manufacturer's protocol, except the *cbhI* transformants were recovered for 2 hours instead of 1

hour at 37°C. Clones were bi-directionally sequenced using the M13 primers with Sanger technology at the LANL JGI Sequencing Facility.

Data analysis.

(i) Fungal large subunit rRNA gene analysis. Sequences were assembled using Fincon (unpublished software, courtesy of Cliff Han, Los Alamos National Laboratory). All fungal LSU rRNA gene sequences were aligned using the SILVA website (26). The position variability parsimony filter generated by SILVA (release, tree_LSURef_1900_slv_100) was used to generate a distance matrix in ARB (21) across all soils and treatments.

The Simpson index of diversity (transformed using the –lnD) and richness estimates based on rarefaction were assessed using the MOTHUR software (29) after binning the sequences based on sequence similarities of 99% (OTU_{99}), 97% (OTU_{97}), and 90% (OTU_{90}). Similar patterns in the data were observed across all of these OTUs definitions. For presentation clarity the data are presented only at the sequence similarity of 97% (OTU_{97}). Richness and diversity estimates in the time zero, T30-^{12}C, and T30-^{13}C libraries for each soil were analyzed for significant differences using an ANOVA with a Tukey's HSD mean separation using the JMP Statistical Discovery Software version 5.1 (SAS, Cary, NC). Beta-diversity was measured using the Bray-Curtis diversity index visualized and using agglomerative hierarchical clustering across these soils and treatments using the R program (27).

When describing the ^{13}C-cellulose enriched OTUs across the LSU rRNA gene clone libraries, the OTU had to be present in both, replicate libraries. Singleton OTUs were removed from the data set. When possible, the taxonomy was described at the family or genus level. This refined taxonomic dataset was classified with BLAST and MG-Rast (24).

The maximum likelihood algorithm (AxML) in ARB was used to generate the phylogenetic tree representing of sequences representing the dominant ^{13}C-cellulose enriched taxa across the soils. The position variability eukaryotic filter between positions 69,241 and 74,784 was used to ensure comparison of the same regions across the clone sequences and references in ARB (21). PAUP Version 4.0b10 was used for bootstrapping analysis (33).

(ii) *cbhI* gene analysis. Bidirectional reads from soil DNAs were assembled using Fincon (unpublished software, courtesy of Cliff Han, Los Alamos National Laboratory). Short sequences (< 470 bp) and sequences containing ambiguous bases were removed from the data set. Introns were predicted and excised from the sequences based on the Hidden Markov Model for glycosyl hydrolase family 7 (http://pfam.sanger.ac.uk/family?PF00840#tabview=tab5) using the program Genewise 2.2.0 (Birney et al. 2004). Intron-free sequences were translated using the batch translator on the Baylor College of Medicine Search Launcher (http://searchlauncher.bcm.tmc.edu/ seq-util/seq-util.html). Inferred amino acid sequences were aligned using Muscle (Edgar, 2004) and manually edited as necessary using ARB (Pruesse et al. 2007). Distance matrices were generated in ARB to determine the Simpson index of diversity (transformed using the –lnD) and richness estimates (based on rarefaction) using the MOTHUR software (29) at an amino acid sequence similarity of 90% (OTU_{90}). Beta-diversity across these soils and treatments was measured using the Bray-Curtis diversity index, which was calculated in the R program (version 2.11.1) (http://www.r-project.org/).

The maximum likelihood algorithm (ProML) in ARB (21) was used to generate the phylogenetic tree based on inferred amino acid sequences from representative sequences of the dominant ^{13}C-cellulose enriched OTUs across the soils. The phylogentic analysis included 171

deduced amino acid positions after filtering out the uneven lengths at the start and end of the fragments. Bootstrapping was completed in PAUP Version 4.0b10 (33).

The LSU and *cbhI* gene sequences have been deposited in Genbank with accession numbers ######## thru ##########.

RESULTS

Diversity and richness estimates of T0, T30-^{12}C, and T30-^{13}C for the LSU and *cbhI* libraries. A total of ca. 4,800 high quality, LSU gene sequences were generated with an average clone library size of 148 clones (ranging from 62 to 188). In parallel, a total of ca. 5,000 high quality, *cbhI* gene sequences were generated with an average clone library size of 180 clones (ranging from 105 to 347). Dataset details are provided in Supplemental Material 2.

For the dead piñon, grassland, and loblolly pine soils, the estimated LSU richness was ca. 3.3-fold lower (range 1.8 to 5.6-fold) in the T30-^{13}C libraries compared to the T30-^{12}C and ca. 4.3-fold less rich (range 2.5 to 5.4-fold) compared to the T0 libraries (Fig. 1). There was no measureable change in richness in the longleaf pine soil

Parallel *cbhI* clone libraries were analyzed at an amino acid sequence similarity of 90% (OTU_{90}) and mirrored the richness trends noted in the LSU clone libraries. The T30-^{13}C libraries were less rich than the T0 and T30-^{12}C libraries, except with the live piñon pine T30-^{13}C library (Fig. 1). The T30-^{13}C clone libraries were ca. 2.5-fold less rich (range 1.7 to 4.7-fold) than the T0 clone libraries and on average 2-fold less rich (range 1.1 to 2.4-fold) than the T30-^{12}C clone libraries. Similar patterns of the Simpson index of diversity were noted for both the LSU and *cbhI* clone libraries (see Supplemental Material 3).

ß-diversity among T0, T30-^{12}C, and T30-^{13}C LSU and *cbhI* libraries within a soil. Shared diversity was assessed among the T0, T30-^{12}C, and T30-^{13}C libraries for each soil using agglomerative hierarchical clustering based on the Bray-Curtis distance metric for the LSU and *cbhI* clone libraries. The replicates of T0 LSU clone libraries cluster together in all soils, whereas the replicate T30-^{12}C libraries only clustered together in the dead piñon pine soil. Except for the live piñon pine soil, the T30-^{13}C LSU clone libraries clustered together and were distinct from the T0 and T30-^{12}C libraries (Fig. 2, panel A).

The parallel *cbhI* clone libraries also illustrated enrichment (Fig. 2, panel B). The replicate enriched libraries for each soil clustered together in the dead piñon, managed grassland, and loblolly pine soils but not in the live piñon pine and longleaf pine soils. The replicate T0 and T30-^{12}C clone libraries clustered together in each soil except for the live piñon pine soil.

Shared diversity among the ^{13}C-cellulose enriched LSU and *cbhI* clone libraries was assessed using agglomerative hierarchical clustering based on the Bray-Curtis distance measure (Fig. 3). There were three distinct cellulolytic clusters observed in the LSU libraries: (1) longleaf pine soil; (2) the managed grassland and loblolly pine soils; and (3) the dead and live piñon pine soils (Fig. 3, panel A). The *cbhI* clone libraries shared this pattern with the exception of the managed grassland and loblolly pine soil cluster (Fig. 3, panel B). In these libraries, the managed grassland and loblolly pine each formed a distinct cluster.

OTUs in the LSU and *cbhI* ^{13}C-cellulose enriched libraries. The LSU ^{13}C-cellulose enriched clone libraries were typically dominated by less than eight OTUs which were consistent across both replicates: the dead piñon pine (OTUs 6, 44, 45, 135, 136, 141, and 143), live piñon pine

(OTU 42), managed grassland (OTUs 209, 210, 211, and 214), loblolly pine (OTUs 210, 211, and 380), and longleaf pine (OTUs 232, 233, 250, 222, 223, 235, 270, and 271) (Table 1). There was little overlap of these OTUs across the different soils; only OTUs 211 and 210 were present in the managed grassland and loblolly pine soils. When the dominant, replicated OTUs were summed for each soil (excluding the variable response from the live piñon pine soil), they represented ca. 67 to 95% of the total library.

There were seven or fewer ^{13}C-cellulose enriched, replicated OTUs for *cbhI* clone libraries (OTU_{90}): the dead piñon pine (OTUs 143, 261, 267, 268, 297, 266, and 298), live piñon pine (OTUs 143, 162, 197, 267, 291, and 268), managed grassland (OTUs 338, 339, 314, and 341), loblolly pine (OTUs 164, 163, 266, 142, and 269), and longleaf pine (OTUs 55, 117, 137, 145, 148, 215, and 238) (Table 2). Collectively, these OTUs comprised 75 to 95% of the clone libraries from each soil. The dead and live piñon pine soils harbored the most overlap, sharing representation in OTUs 143, 267, and 268. The dead piñon pine and loblolly pine shared OTU 266. Some of the ^{13}C-cellulose enriched OTUs were also identified in the T0 and T30-^{12}C libraries (Supplemental Material 4).

Taxonomic identification of the dominant ^{13}C-cellulose enriched clusters.

(i) LSU clone libraries. The phylogeny of the sequence representatives from the dominant enriched LSU OTUs (n=21) is represented in Figure 4. Across the five soils the *Basidiomycetes* were prevalent in the T0 clone libraries with relative proportions ranging from ca. 35 to 97% (Supplemental Material 5). However members of the *Ascomycota* were enriched in each soil with addition of ^{13}C-cellulose.

The identity of the dominant OTUs, determined by nearest BLAST sequence match, is shown in Figure 4. Eight ^{13}C-cellulose enriched OTUs were most closely related *Chaetomium*, *Trichocladium*, and *Dactylaria* reference sequences with sequence similarities ranging from ca. 80% to 99%. Although these OTUs are being taxonomically described as a "*Chaetomium*/*Trichocladium*/*Dactylaria*-like cluster", some OTUs were unique to a particular soil. Two OTUs enriched in the managed grassland (16% of the libraries) and loblolly pine (82% of the libraries) soils, were most similar to *Chaetomium globosum* CBS 148.51: OTU 210 (ca. 80% sequence similarity) and OTU 211 (ca. 93% sequence similarity). OTUs 214 and 209 were only enriched in the managed grassland soil. They represented 76% of the total sequences and had 95% sequence similarity to *Chaetomium globosum* CBS 148.51. OTU 235 was unique to the longleaf pine soil and was 91% similar to *Dactylaria hyalotunicata.* OTUs 44 and 6 were unique to the dead piñon pine soil and 98% similar to *Trichocladium asperum*. OTU 271 was unique to the longleaf pine representing 16% of the clone libraries and was 88% similar to *Trichocladium pyriforme*.

OTUs 45, 135, and 136 were unique to the dead piñon pine soil and were most similar to *Arthrobotrys sp.* ATCC MYA-4125 with an average sequence similarity of 94%. Together, these OTUs representing an average 40% of the dead piñon pine clone libraries.

Three of the ^{13}C-cellulose enriched OTUs are most similar to members of the *Chytridiomycota*. OTU 380 was most similar to *Nowakowskiella sp*. JEL 127 (89% sequence similarity) and was only enriched in the loblolly pine soil (4% of the libraries). OTU 141 was only enriched in the dead piñon pine soil (3% of the libraries) and was 96% similar to *Rhizophlyctis rosea*. OTU 143 was also distantly related to *Rhizophlyctis rosea* (83% sequence similarity) and only enriched in the dead piñon pine soil (3% of the libraries).

OTU 250 was most similar to a *Dinoflagellate*. It was only enriched in the loblolly pine soil and was 92% similar to *Prorocentrum micans*. OTU 250 is being described as "novel dinoflagellate-like group". OTU 42 was most similar to a *Cladophialophora chaetospira* (98% sequence similarity) and was only enriched in the live piñon pine soil. Both of these OTUs represented on average less than 6% of their respective clone library.

There were two novel clusters harboring ^{13}C-cellulose enriched OTUs that had low sequence similarity to reference sequences (Fig. 4, depicted as "novel longleaf pine soil cluster 1 and novel longleaf pine soil cluster 2"), which were unique to the longleaf pine soil. OTUs 222 and 223 were distantly related (average sequence similarity of 84%) to *Cladophialophora chaetospira*. OTUs 233, 232, and 270 were distantly related to *Naemacyclus minor* (average sequence similarity of 88%). These novel groups typically represented on average 18% of the longleaf pine clone libraries.

(ii) *cbhI* clones libraries. The phylogeny of the *cbhI* sequences representing dominant OTUs in the T30-^{13}C libraries for all five soils (n=25; Table 2) is in Figure 5. Almost half of the OTUs appear to fall into clades within the *Ascomycota* containing members of the genera *Chaetomium* (*Chaetomium*-like cluster) or *Trichocladium* (*Trichocladium*-like cluster).

Eight of the OTUs are within the "*Chaetomium*-like cluster". Even though these OTUs are taxonomically described as "*Chaetomium*-like" they appear to be unique to a particular soil, except for OTU 143. OTU 143 was present in both the dead (6% of the clone libraries) and live piñon pine (44% of the libraries) soils and were 87% similar to *Chaetomium globosum*. OTUs 164, 163, and 142 were unique to the loblolly pine soil and together comprised 74% of the clone libraries. OTUs 163 and 164 were most closely related to *Chaetomium* mg128 with an average

sequence similarity of 84%. OTUs 137, 145, and 148 were unique to the longleaf pine soil and together represented an average of 24% in the clone libraries. OTU 148 was 83% similar to *Acremonium thermophilum*, whereas OTUs 145 and 137 were on average 82% similar to *Chaetomium globosum* CBS 148.51 and *Chaetomium* mg 128 b10. OTU 162 was unique to the live piñon pine soil, representing ca. 6% of the clone libraries, and most similar to *Chaetomium* mg128 (86% sequence similarity).

Four of the OTUs enriched across the dead piñon, live piñon, and loblolly pine soils are within the "*Trichocladium*-like cluster". OTUs 267 and 268 were common between the dead (39% of the libraries) and live piñon (33% of the libraries) pine soils. OTU 266 was common between the loblolly pine (3% of the libraries) and dead piñon pine soil (1% of the libraries). OTU 269 was unique to the loblolly pine soil and comprised 10% of the clone libraries. These OTUs had an average sequence similarity of 95% to *Trichocladium asperum* IHEM2884.

The remaining thirteen OTUs were not within the *Chaetomium*-like or the *Trichocladium*-like clusters. Interestingly, these OTUs appear to be exclusive to a particular soil: longleaf pine soil (OTUs 238, 117, 55, 215), managed grassland soil (OTUs 314, 338, 339, 341), dead piñon pine soil (OTUs 261, 298, 297), and live piñon pine soil (OTUs 291, 197).

The longleaf pine OTUs 55 and 215 were distantly related to *Botryotinia fuckeliana* (average sequence similarity of 72%), whereas OTUs 238 and 117 were distantly related to *Sclerotinia sclerotiorum* 1980 (average sequence similarity of 80%). Three of the managed grassland OTUs (341, 338, and 339) was distinct and not closely related to any reference sequences. OTU 314 was distantly related to *Botryotinia fuckeliana* (average sequence similarity 74%). The dead pinon pine soil OTUs 261, 298, and 297 formed their own distinct clade and were not closely related to any reference sequences. The live pinon pine soil OTU 291

did not cluster with any reference sequences, whereas OTU 197 was distantly related to *Botryotinia fuckeliana* (71% sequence similarity).

Correlations between the ^{13}C-cellulose enriched LSU and *cbhI* clone libraries. Both the ^{13}C-cellulose enriched LSU and *cbhI* gene clone libraries were dominated by sequences most closely related to either members of the *Trichocladium* or *Chaetomium* genus (Table 3). Eight and twelve of the OTUs were *Trichocladium/Chaetomium*-like in the LSU and *cbhI* gene clone libraries, respectively. The relative percentage of *Trichocladium/Chaetomium*-like sequences ranged from 34 to 95% (LSU clone libraries) and 19 to 93% (*cbhI* gene clone libraries) (Table 3). However, none of the managed grassland soil *cbhI* sequences grouped with *Trichocladium/Chaetomium*-like reference sequences, whereas 92% (average of two replicates) of the LSU sequences grouped with *Trichocladium/Chaetomium*-like reference sequences. Three *cbhI* OTUs (341, 338 and 339) only found in the T30-^{13}C libraries from the managed grassland soil grouped together and were distinct from the *Trichocladium/Chaetomium cbhI* reference sequences (Fig. 5).

The dead piñon pine and longleaf pine soils were the least enriched with *Trichocladium/Chaetomium*-like sequences for both *cbhI* and LSU gene libraries. Based on the LSU clone libraries, the dead piñon pine soil harbored 3 OTUs that were most closely related to members of the *Arthrobotrys* genus (Fig. 4). These OTUs were only enriched in the dead piñon pine soil and represented an average of 40% of the dead piñon pine soil clone libraries. At this time, there are no readily available reference sequences for the *Arthrobotrys' cbhI* gene. Interestingly, there were 3 OTUs in the *cbhI* gene clone libraries that were only enriched in the

dead piñon pine soil (OTUs 261, 298, and 297), which formed a distinct clade in the *cbhI* gene amino acid-based tree (Fig. 5).

The longleaf pine soil was enriched with OTUs 55, 117, 215, and 238 (*cbhI* gene) and OTUs 380 and 235 (LSU gene). These LSU OTUs are *Trichocladium/Chaetomium*-like, but constituted a lower proportion of the libraries. The remaining clones were distributed across six additional OTUs. These remaining OTUs are most similar to each other, forming two novel, distinct clades (Fig.4). The *cbhI* gene OTUs 117, 215, and 55 also are most similar to each other and are also not closely related to any of the available *cbhI* sequences from named taxa (Fig. 5). The *cbhI* gene OTU 235 is most closely related to *Sclerotinia sclerotiorum*.

DISCUSSION

Fungi are known to be essential in the deconstruction of plant biomass, but the taxa that dominate that process in different soils has not been explored. Toward that goal, we document the ability of phylogenetic (LSU) and functional (*cbhI*) gene markers to describe the active fungi that become enriched in ^{13}C-cellulose amended microcosms from five different soils. Collectively, our results illustrate that (1) the LSU and *cbhI* genes are useful gene markers that provide parallel results in assessment of compositional shifts in cellulolytic fungi in response to ^{13}C-cellulose amendment; (2) the ^{13}C-enriched DNAs contained cellulolytic fungi based on the similar compositional patterns between the LSU and *cbhI* gene clone libraries; and (3) the ^{13}C-cellulose enriched fungal communities were unique to a particular soil, demonstrating the influence that the combination of soil and plant-associated properties has on the cellulolytic fungal community.

We observed remarkable similarities in the diversity, richness, phylogenetic clustering patterns, and taxonomic identification in the LSU and *cbhI* gene clone libraries, with most of the ^{13}C-cellulose enriched libraries being less rich than the T0 and T30-^{12}C libraries for both genes (Fig. 1). The T30-^{13}C fungal communities were distinct from the T0 and T30-^{12}C libraries for the both genes illustrating a clear selection for cellulose-degrading communities across most of the soils (Fig. 2). Although we cannot determine the metabolic contributions of each OTU in the cellulose degradation process, the parallel phylogenies of the LSU and *cbhI* gene libraries indicate that the dominant, cellulolytic fungi in these microcosms likely harbor the GH7 cellobiohydrolase. This suggests that the *cbhI* gene is an appropriate functional gene marker to identify compositional and distribution patterns of a responsive subset of cellulolytic soil fungi. ALTERNATE SENTENCE: The common response patterns and similarities in identified OTUs between the two marker genes indicates that either could be used to survey for cellulolytic fungi where stable isotope labeled substrates are used, and that the cbh1 gene is an appropriate indicator of cellulolytic populations in soil surveys with natural or unlabeled substrates.

Three distinct LSU clusters were identified in the LSU clone libraries from ^{13}C-cellulose enriched DNA fractions across the soils: (1) longleaf pine; (2) managed grassland and loblolly pine; and (3) the dead and live piñon pine soils based on the dendrograms (Fig. 3, panel A). The *cbhI* gene libraries mirrored these clustering patterns, although the managed grassland and loblolly pine soil did not form a distinct cluster (Fig. 3, panel B). Of the limited soil properties examined in this study, soil pH appeared to best correlate with this clustering pattern. The managed grassland and loblolly pine soil had a starting pH of 5.4 and 5.2, respectively. The dead and live piñon pine soil had a starting pH of 6.5 and 6.4, respectively, and the longleaf pine was the apparent outlier with a starting soil pH of 3.8. Soil pH has been documented previously to

influence bacterial diversity patterns across geographically different soils (12, 17) and cellulolytic bacteria (Eichorst and Kuske 201x).

Although the fungal community in each soil contained multiple members of the *Basidiomycota*, our soil microcosms enriched primarily for members of the *Ascomycota*. Many *Basidiomycetes* have extensive hyphal networks allowing them to act independently of locally available nutrients and carbon and form mycorrhizal associations with plants (5, 13, 19). Such networks and associations are highly susceptible to physical disturbance (6, 14, 20), which may have contributed to the decline of *Basidiomycetes* and ultimate dominance of opportunistic *Ascomycota* in our microcosms.

Based on the LSU gene clone libraries, there were distinct cellulolytic fungi present in the ^{13}C-enriched DNA extracted from each of the different soils. The enriched OTUs were tentatively described as members of the following genera: *Trichocladium/Chaetomium* (dead piñon pine, managed grassland, loblolly pine, and longleaf pine soils), *Dactylaria* (longleaf pine soil), *Arthrobotrys* (dead piñon pine soil), and two novel clusters (longleaf pine soil). This suggests that cellulose as well as other factors such as the past history and/or soil chemistry influenced this distribution.

A unique fungal community was enriched in ^{13}C-cellulose microcosms in each of the different soils (Figure xx). In each soil, a dominant component of this community contained OTUs belonging to the *Trichocladium* or *Chaetomium* genera (Table 3), but the OTUs were not the same in each soil. This suggests that different species within these two genera are active in the different soils. The genus *Trichocladium* is a saprotrophic soil fungus known for its celluloytic activity and may be useful biomass deconstruction of feedstocks (10). For example, when grown under microaerophilic conditions, certain *Trichocladium* species can convert ca. 90

to 96% of available cellulose to ethanol (10). Members of the genus *Chaetomium* are known to degrade lignocellulosic biomass and various carbohydrates (31).

Sequences in the LSU libraries from the dead piñon pine soil were most similar to members of the *Arthrobotrys* genus, which are known as "nematode destroying fungi". Members of the genus *Arthrobotrys* are natural enemies of plant parasitic nematodes (25) trapping them by producing adhesive branches (36) and immobilizing them with toxins (22). The nematode-trapping phenotype is believed to be an evolutionary response of cellulolytic or lignin-degrading fungi to nutrient limitation in nitrogen-limiting environments (1, 2, 9). It is unclear if arthrobotrys-like sequences were enriched in the *cbhI* gene clone libraries since there are no readily available reference sequences for comparison. Nevertheless, there was a *cbhI* gene cluster ("dead piñon pine soil cluster") containing three OTUs that were only enriched in the dead piñon pine soil (Fig. 5) that did not cluster closely with any of the known reference sequences and may represent this genus.

Sequences most similar to members of the *Chytridiomycota* were enriched with ^{13}C-cellulose in the loblolly pine and dead piñon pine soils, typically representing less than 6% of the clone library based on the LSU rRNA gene clone libraries. Chytrids are a ubiquitous group of fungi found in both aquatic and terrestrial ecosystems, characterized as saprotrophs, capable of degrading pollen, chitin, karatin, and cellulose (16). Two of the OTUs in the LSU gene clone libraries (141 and 143) were distantly related to the genus *Rhizophlyctis*, a previously identified cellulose-degrading chytrid (18). It is unclear if chytrid-like sequences were enriched in the *cbhI* gene clone libraries since there are no readily available reference sequences. Cellobiohydrolase genes of chytrids might not be captured by our primer set which was designed solely from

Ascomycota and *Basidiomycota* (11), or they could harbor alternative cellulose degrading enzymes.

We identified three novel clusters of cellulose-degrading fungi in our LSU clone libraries that had low sequence similarities to reference sequences. The "novel dinoflagellate-like group" containing OTU 250, that was enriched in the loblolly pine soil, was more similar to a dinoflagellate, *Prorocentrum micans*. "Novel longleaf pine soil cluster 1", consisting of OTUs 223 and 222, was distantly related to the *Ascomycota, Cladophialophora chaetospira* (84% sequence similarity). Finally "novel longleaf pine soil cluster 2" (OTUs 270, 233, and 232) was distantly related to *Naemacyclus* with an average sequence similarity of 88%. Across our *cbhI* gene clone libraries, we identified two clusters ("longleaf pine soil cluster" and "managed grassland soil cluster") that were distinct from readily available reference sequences. The use of our microcosm approach has identified new targets that demand further characterization.

ALTERNATE PARAGRAPH: Three novel OTU clusters that had low sequence similarities to reference sequences were detected. These include an LSU OTU (#250) that clustered with a dinoflagellate, and an OTU cluster distantly related to the genus *Cladophialophora.* Currently nothing is known about the cellulolytic abilities of either of these groups, and they represent interesting targets for study of the diversity of this trait in the Fungi.

In comparison of the dead and live piñon soils, the decaying piñon biomass appeared to affect the resident fungal community and response to ^{13}C-cellulose in the microcosms . Cellulose addition to the dead piñon pine soil microcosm significantly decreased richness of the T30-^{13}C community relative to the T30-^{12}C and T0 communities in the both gene libraries. In contrast, cellulose addition to the live piñon pine soil microcosms did not significantly decrease the richness of the T30-^{13}C LSU rRNA gene libraries, but did decrease richness of the T30-^{13}C *cbhI*

gene community. Although the live and dead piñon pine soils appear to contain similar cellulolytic communities based on the clustering pattern for both the LSU gene and *cbhI* gene (Fig. 3), some of the enriched OTUs were unique to either the dead or live soil (Table 1 and 2). At this time it is difficult to identify these OTUs to a fungal taxon with our current databases.

Members of the bacteria and fungi are essential for degrading cellulose in terrestrial ecosystems (8). Our study identified a variety of previously described and novel fungi capable of degrading cellulose. Some of these fungi were unique to a particular soil suggesting the importance of past history and soil properties. Our accompanying paper identified cellulolytic bacteria in these soils. Since cellulose decomposition is believed to be multiphasic, with fungi dominating the first phase (1 to 3 weeks) and bacteria dominating the second phase (34), future work is needed to determine the importance of these identified bacterial and fungal taxa in a time series microcosm experiment.

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Figures:

Figure 1. Average richness estimates (± standard deviation) for the LSU rRNA gene libraries at OTU_{97} (panel A) and *cbhI* gene libraries at OTU_{90} (panel B) for time zero (black bars), day 30 ^{12}C non-enriched (gray bars) and day 30 ^{13}C-cellulose enriched libraries (light gray bars). P-value from ANOVA analysis with a Tukey's HSD mean separation is depicted below the x-axis. Letters indicate the similarity pattern among the treatments for a given soil (nsd=no significant difference).

Figure 2. Agglomerative hierarchical cluster dendrograms of community similarity of time zero (■), day 30 ^{12}C-non-enriched (●), and day 30 ^{13}C-cellulose enriched (★) fungal communities based on the LSU rRNA gene (OTU_{97}, panel A) and *cbhI* gene (OTU_{90}, panel B). Scale bar indicates the related similarity of the communities.

Figure 3. Agglomerative hierarchical cluster dendrograms of community similarity of the ^{13}C-cellulose enriched fungal communities based on the LSU rRNA gene ($OTU_{97,}$ panel A) and *cbhI* gene ($OTU_{90,}$ panel B). Replicate libraries are depicted as "R1" and "R2". Scale bar indicates the related similarity of the communities.

Figure 4. Maximum-likelihood tree of the sequence representatives from the dominant ^{13}C-cellulose enriched OTUs (bolded with respective soil) based on the large subunit rRNA gene using sequences obtained from cultivated representatives. *Sulfolobus acidocaldarius* (CP000077) was used as the outgroup (not shown). Taxonomic classifications of the distinct clusters are indicated to the right of the tree. Internal nodes supported by a bootstrap value of >95% are indicated with a filled circle (•) and of >70% with an open circle (o). The scale bar indicates 0.01 changes per nucleotide.

Figure 5. Maximum-likelihood tree of the sequence representatives from the dominant ^{13}C-cellulose enriched OTUs (bolded with respective soil) based on the deduced amino acid sequences of the *cbhI* gene using sequences obtained from cultivated representatives. An endoglucase from *Aspergillus oryzae* (BAE66197) within the glycosyl hydrolase family 7 was used as the outgroup (not shown). Taxonomic classifications of the distinct clusters are indicated to the right of the tree. Internal nodes supported by a bootstrap value of >95% are indicated with a filled circle (•) and of >70% with an open circle (o). The scale bar indicates 0.10 changes per amino acids

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Table 1. Dominant OTUs from ^{13}C-cellulose enriched libraries clustering at OTU_{97} for the LSU rRNA gene. The depicted OTUs contained ≥3% of the sequences in any one of the soil libraries. The shaded OTUs were present in both technical replicates for a given soil. The summed library proportion represented these replicated OTUs. Cluster numbers were generated by analysis in the MOTHUR software package.

	Dead piñon pine soil		***Live piñon pine soil***		***Managed grassland soil***		***Loblolly pine soil***		***Longleaf pine soil***	
OTU_{97}	1	2	1	2	1	2	1	2	1	2
136	26.37	27.78	-	-	-	-	-	-	-	-
135	7.69	8.89	-	-	-	-	-	-	-	-
143	4.40	1.11	-	-	-	-	-	-	-	-
141	3.30	2.22	-	-	-	-	-	-	-	-
6	35.16	33.33	-	22.58	-	-	-	-	-	-
44	1.10	1.11	-	4.30	-	-	-	-	-	-
45	5.49	4.44	-	2.15	-	-	-	-	-	-
150	-	3.33	-	-	-	-	-	-	-	-
42	-	-	10.59	1.08	-	-	-	-	-	-
2	-	-	7.06	-	-	-	-	-	-	-
66	-	-	4.71	-	-	-	-	-	-	-
17	-	-	3.53	-	-	-	-	-	-	-
19	-	-	3.53	-	-	-	-	-	-	-
28	-	-	3.53	-	-	-	-	-	-	-
32	-	-	3.53	-	-	-	-	-	-	-
60	-	-	3.53	-	-	-	-	-	-	-
72	-	-	3.53	-	-	-	-	-	-	-
41	-	-	-	50.54	-	-	-	-	-	-
49	-	1.11	-	3.23	-	-	-	-	-	-
43	-	-	-	3.23	-	-	-	-	-	-
209	-	-	-	-	70.13	77.05	-	2.20	-	-
214	-	-	-	-	1.30	3.28	-	-	-	-
211	-	-	-	-	11.69	8.20	69.27	75.27	-	-
210	-	-	-	-	6.49	6.56	13.41	6.59	1.11	-
380	-	-	-	-	-	-	2.79	6.59	-	-
271	-	-	-	-	-	-	5.03	-	11.11	21.62
235	-	-	-	-	-	-	-	-	23.33	49.19
270	-	-	-	-	-	-	-	-	6.67	3.78
232	-	-	-	-	-	-	-	-	6.67	1.62
233	-	-	-	-	-	-	-	-	6.67	1.62
250	-	-	-	-	-	-	-	-	4.44	1.08
222	-	-	-	-	-	-	-	-	4.44	0.54
223	-	-	-	-	-	-	-	-	3.33	1.62
236	-	-	-	-	-	-	-	-	4.44	-
303	-	-	-	-	-	-	0.56	-	-	3.78
622	-	-	-	-	-	-	-	3.30	-	-
Prop. Lib	83.5	78.9	Variable	Variable	89.6	95.1	85.5	88.5	66.7	81.1

Table 2. Dominant OTUs from ^{13}C-cellulose enriched libraries clustering at OTU_{90} for the *cbhI* gene. The depicted OTUs contained ≥3% of the sequences in any one of the soil libraries. The shaded OTUs were present in both technical replicates for a given soil. The summed library proportion represented these replicated OTUs. Cluster numbers were generated by analysis in the MOTHUR software package.

	Dead piñon pine soil		***Live piñon pine soil***		***Managed grassland soil***		***Loblolly pine soil***		***Longleaf pine soil***	
OTU_{90}	1	2	1	2	1	2	1	2	1	2
26	5.49	-	-	-	-	-	-	-	-	-
296	4.76	-	-	-	-	-	-	-	-	-
297	5.86	16.07	-	-	-	-	-	-	-	-
298	7.69	13.10	-	-	-	-	-	-	-	-
261	11.36	18.45	-	-	-	-	-	-	-	-
266	1.10	1.79	0.55	-	-	-	2.31	3.95	1.17	-
267	6.59	1.79	21.43	11.60	-	-	-	-	-	-
268	31.14	38.69	19.23	14.36	-	-	-	-	-	-
143	11.72	0.60	44.51	42.54	-	-	0.86	-	-	-
162	0.73	-	8.24	3.87	-	0.61	-	-	-	-
197	-	-	0.55	13.26	-	-	-	-	-	-
291	-	-	0.55	3.87	-	-	-	-	-	-
196	-	-	-	5.52	-	-	-	-	-	-
10	-	-	-	3.87	-	-	-	-	-	-
338	-	-	-	-	27.44	20.00	-	-	-	-
339	-	-	-	-	50.61	55.76	-	-	-	-
341	-	-	-	-	14.63	11.52	-	-	-	-
314	-	-	-	-	3.05	2.42	-	-	-	-
164	-	-	-	-	-	2.42	70.03	68.39	-	-
269	-	-	-	-	-	-	10.09	10.94	-	-
163	-	-	-	-	-	-	3.17	1.22	-	-
142	-	-	-	-	-	0.61	1.73	3.95	-	-
175	-	-	-	-	-	-	5.48	-	-	-
55	-	-	-	-	-	-	-	-	18.13	2.58
117	-	-	-	-	-	-	-	-	25.15	58.71
137	-	-	-	-	-	-	-	-	5.26	12.26
145	-	-	-	-	-	-	-	-	9.36	10.97
148	-	-	-	-	-	-	-	-	4.09	5.81
215	-	-	-	-	-	-	-	-	12.28	1.29
238	-	-	-	-	-	-	-	-	15.79	3.23
Prop. Lib	75.5	90.5	94.5	89.5	95.7	89.7	87.3	88.4	90.1	94.8

Table 3. *Trichocladium/Chaetomium*-like sequences identified in the LSU rRNA and *cbhI* gene libraries. The OTUs are listed and the summed relative proportions of those OTUs are given for the two replicate clone libraries. "NI" – not identified.

	***Trichocladium/Chaetomium*-like Sequences**			
	LSU gene clone libraries		*cbhI* gene clone libraries	
	OTU numbers	Rel. prop. library	OTU numbers	Rel. prop. library
Dead piñon pine soil	6, 44	36.3%, 34.4%	143, 267, 268, 266	50.5%, 42.9%
Live piñon pine soil	Variable		143, 162, 267, 268,	93.4%, 72.4%
Managed grassland soil	211, 210, 209, 214	89.6%, 95.1%	NI	NI
Loblolly pine soil	211, 210,	82.7%, 81.9%	164, 269, 163, 266, 142	87.3%, 88.4%
Longleaf pine soil	271, 235	34.4%, 70.8%	137, 145, 148	18.7%, 29%

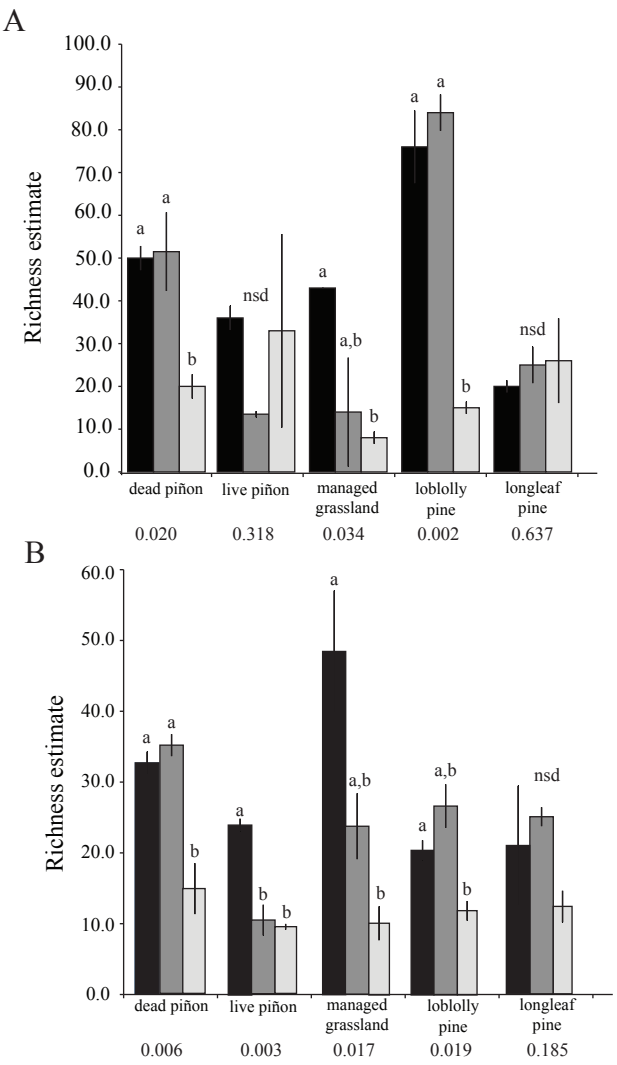
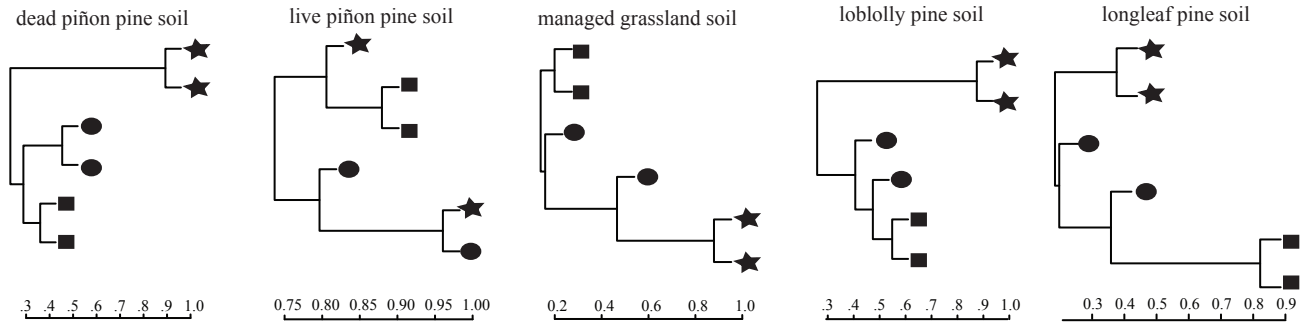

A
Richness estimate
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
a
b
nsd
a
a,b
b
a
a
b
nsd
dead piñon
live piñon
managed grassland
loblolly pine
longleaf pine
0.020
0.318
0.034
0.002
0.637
B
Richness estimate
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
a
b
a
b
b
a
a,b
b
a
a,b
b
nsd
dead piñon
live piñon
managed grassland
loblolly pine
longleaf pine
0.006
0.003
0.017
0.019
0.185

Figure 1.

A. Large subunit rRNA gene clone libraries



B. Cellobiohydrolase *cbhI* gene clone libraries

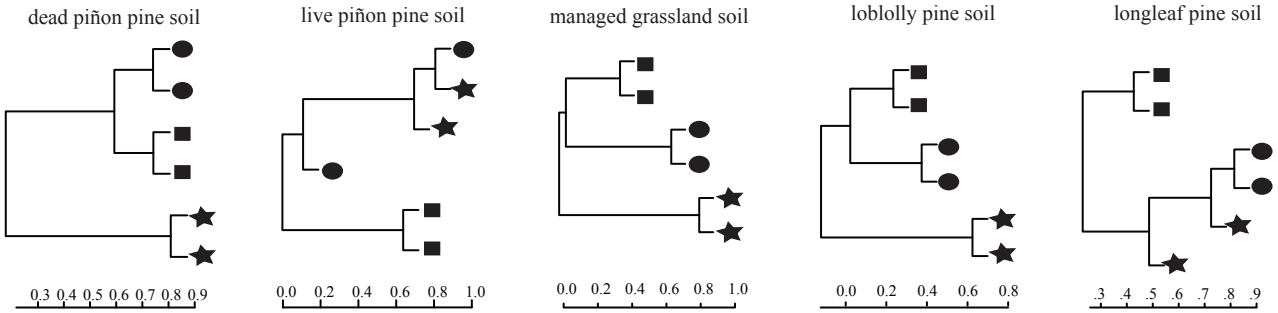


Figure 2.

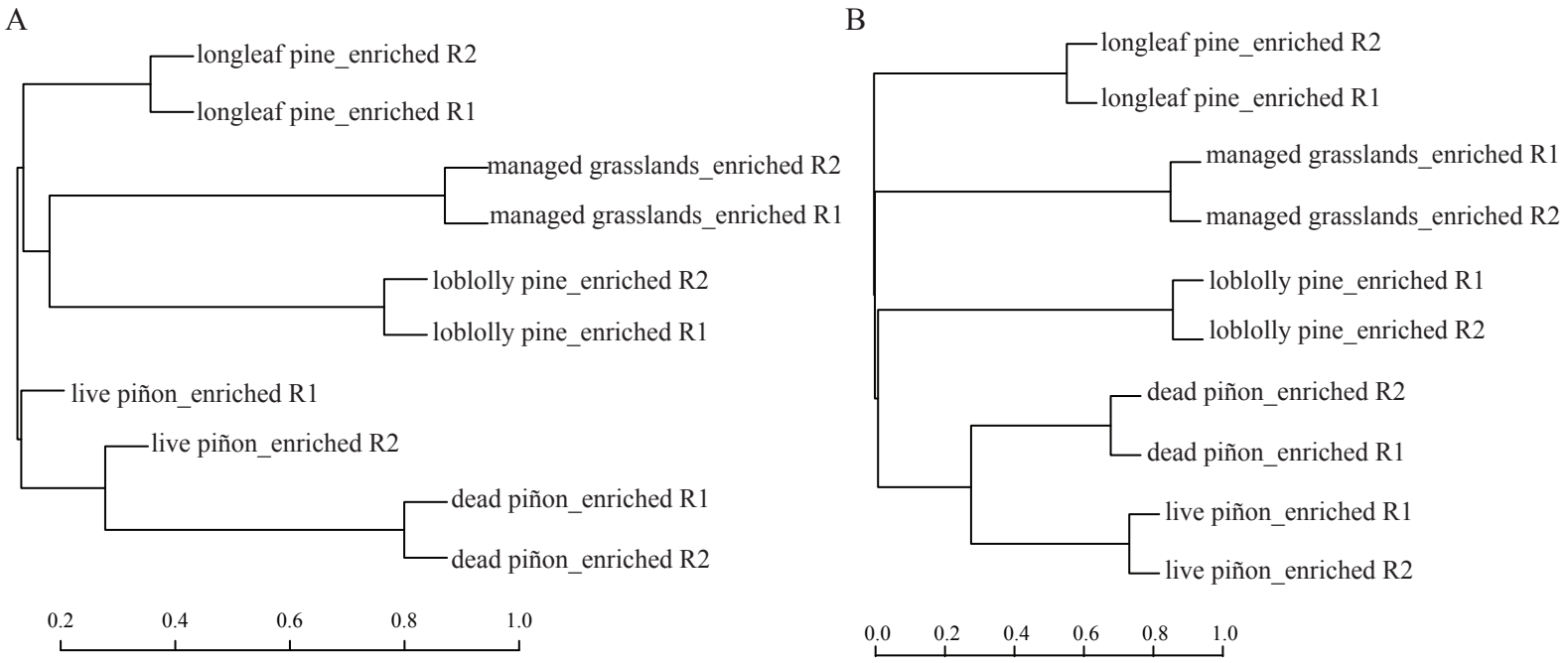

A
longleaf pine_enriched R2
longleaf pine_enriched R1
managed grasslands_enriched R2
managed grasslands_enriched R1
loblolly pine_enriched R2
loblolly pine_enriched R1
live piñon_enriched R1
live piñon_enriched R2
dead piñon_enriched R1
dead piñon_enriched R2
0.2
0.4
0.6
0.8
1.0
B
longleaf pine_enriched R2
longleaf pine_enriched R1
managed grasslands_enriched R1
managed grasslands_enriched R2
loblolly pine_enriched R1
loblolly pine_enriched R2
dead piñon_enriched R2
dead piñon_enriched R1
live piñon_enriched R1
live piñon_enriched R2
0.0
0.2
0.4
0.6
0.8
1.0

Figure 3.

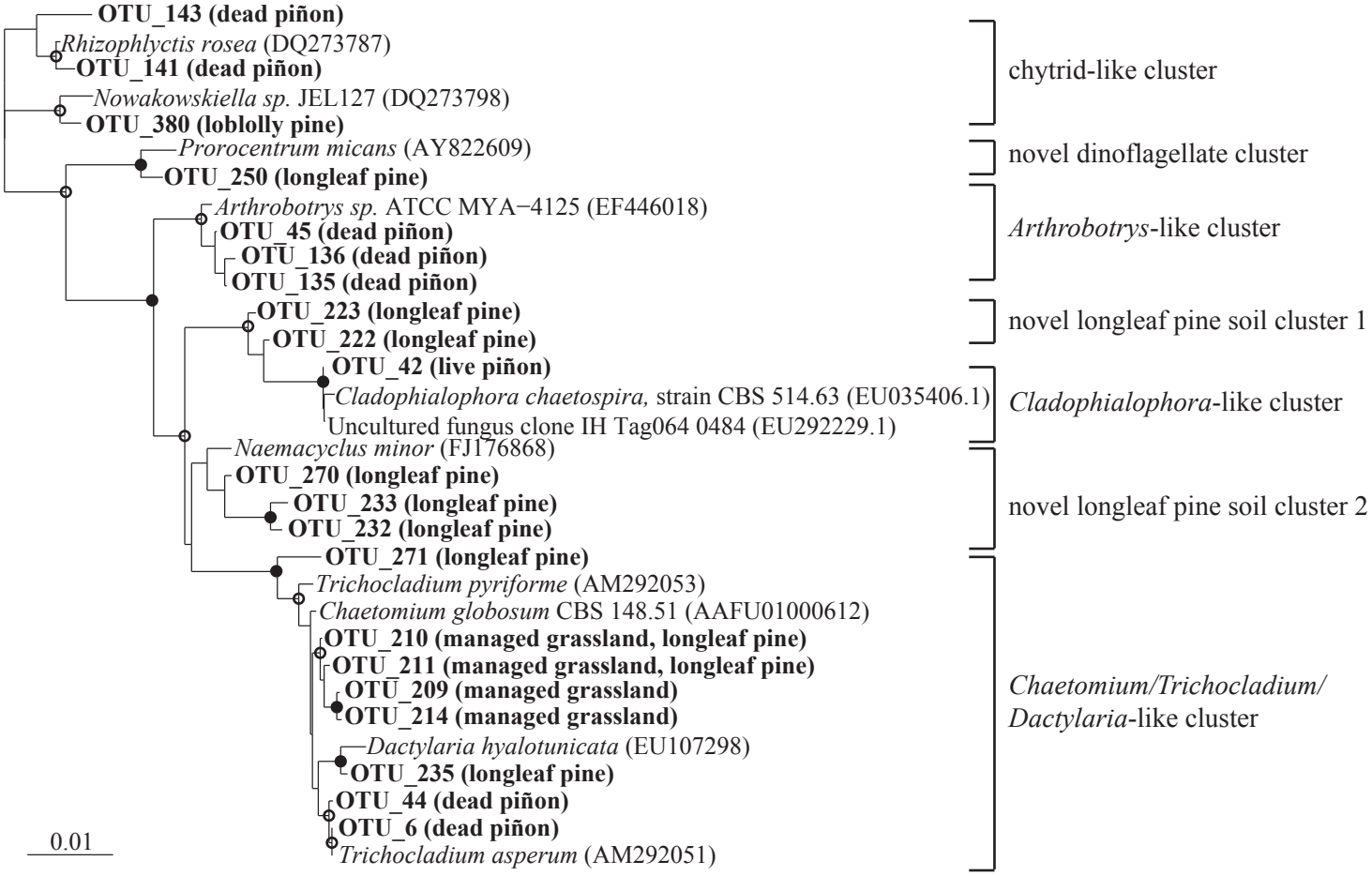

OTU_143 (dead piñon)
Rhizophlyctis rosea (DQ273787)
OTU_141 (dead piñon)
Nowakowskiella sp. JEL127 (DQ273798)
OTU_380 (loblolly pine)
Prorocentrum micans (AY822609)
OTU_250 (longleaf pine)
Arthrobotrys sp. ATCC MYA−4125 (EF446018)
OTU_45 (dead piñon)
OTU_136 (dead piñon)
OTU_135 (dead piñon)
OTU_223 (longleaf pine)
OTU_222 (longleaf pine)
OTU_42 (live piñon)
Cladophialophora chaetospira, strain CBS 514.63 (EU035406.1)
Uncultured fungus clone IH Tag064 0484 (EU292229.1)
Naemacyclus minor (FJ176868)
OTU_270 (longleaf pine)
OTU_233 (longleaf pine)
OTU_232 (longleaf pine)
OTU_271 (longleaf pine)
Trichocladium pyriforme (AM292053)
Chaetomium globosum CBS 148.51 (AAFU01000612)
OTU_210 (managed grassland, longleaf pine)
OTU_211 (managed grassland, longleaf pine)
OTU_209 (managed grassland)
OTU_214 (managed grassland)
Dactylaria hyalotunicata (EU107298)
OTU_235 (longleaf pine)
OTU_44 (dead piñon)
OTU_6 (dead piñon)
Trichocladium asperum (AM292051)
0.01
chytrid-like cluster
novel dinoflagellate cluster
Arthrobotrys-like cluster
novel longleaf pine soil cluster 1
Cladophialophora-like cluster
novel longleaf pine soil cluster 2
Chaetomium/Trichocladium/ Dactylaria-like cluster

Figure 4.

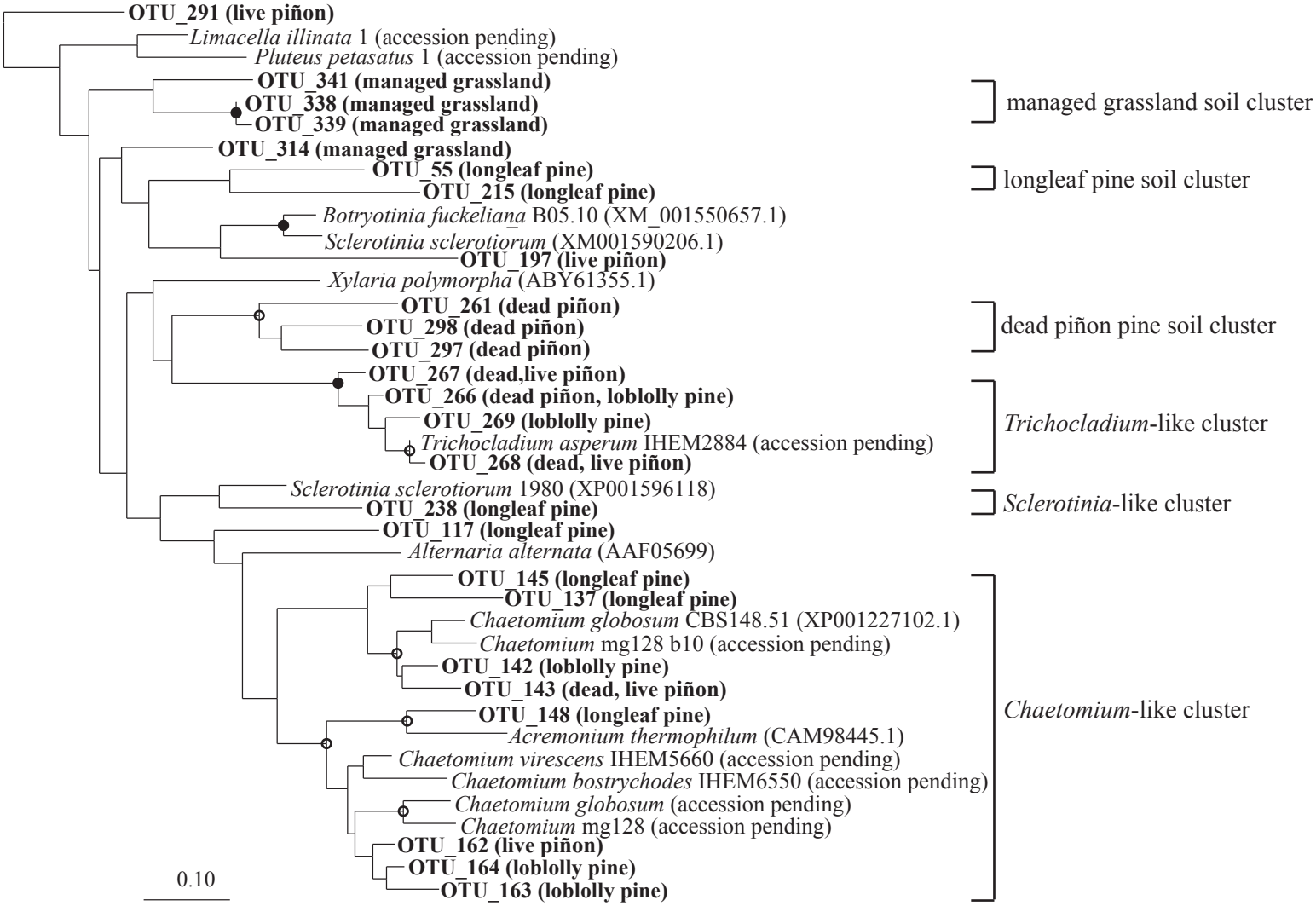

OTU_291 (live piñon)
Limacella illinata 1 (accession pending)
Pluteus petasatus 1 (accession pending)
OTU_341 (managed grassland)
OTU_338 (managed grassland)
OTU_339 (managed grassland)
OTU_314 (managed grassland)
OTU_55 (longleaf pine)
OTU_215 (longleaf pine)
Botryotinia fuckeliana B05.10 (XM_001550657.1)
Sclerotinia sclerotiorum (XM001590206.1)
OTU_197 (live piñon)
Xylaria polymorpha (ABY61355.1)
OTU_261 (dead piñon)
OTU_298 (dead piñon)
OTU_297 (dead piñon)
OTU_267 (dead,live piñon)
OTU_266 (dead piñon, loblolly pine)
OTU_269 (loblolly pine)
Trichocladium asperum IHEM2884 (accession pending)
OTU_268 (dead, live piñon)
Sclerotinia sclerotiorum 1980 (XP001596118)
OTU_238 (longleaf pine)
OTU_117 (longleaf pine)
Alternaria alternata (AAF05699)
OTU_145 (longleaf pine)
OTU_137 (longleaf pine)
Chaetomium globosum CBS148.51 (XP001227102.1)
Chaetomium mg128 b10 (accession pending)
OTU_142 (loblolly pine)
OTU_143 (dead, live piñon)
OTU_148 (longleaf pine)
Acremonium thermophilum (CAM98445.1)
Chaetomium virescens IHEM5660 (accession pending)
Chaetomium bostrychodes IHEM6550 (accession pending)
Chaetomium globosum (accession pending)
Chaetomium mg128 (accession pending)
OTU_162 (live piñon)
OTU_164 (loblolly pine)
OTU_163 (loblolly pine)
0.10
managed grassland soil cluster
longleaf pine soil cluster
dead piñon pine soil cluster
Trichocladium-like cluster
Sclerotinia-like cluster
Chaetomium-like cluster

Figure 5.