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Responses of soil cellulolytic fungal communities to elevated atmospheric CO_2 are complex and variable across five ecosystems

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Summary

Elevated atmospheric CO_2 generally increases plant productivity and subsequently increases the availability of cellulose in soil to microbial decomposers. As key cellulose degraders, soil fungi are likely to be one of the most impacted and responsive microbial groups to elevated atmospheric CO_2. To investigate the impacts of ecosystem type and elevated atmospheric CO_2 on cellulolytic fungal communities, we sequenced 10 677 *cbhI* gene fragments encoding the catalytic subunit of cellobiohydrolase I, across five distinct terrestrial ecosystem experiments after a decade of exposure to elevated CO_2. The *cbhI* composition of each ecosystem was distinct, as supported by weighted Unifrac analyses (all *P*-values; < 0.001), with few operational taxonomic units (OTUs) being shared across ecosystems. Using a 114-member *cbhI* sequence database compiled from known fungi, less than 1% of the environmental sequences could be classified at the family level indicating that cellulolytic fungi *in situ* are likely dominated by novel fungi or known fungi that are not yet recognized as cellulose degraders. Shifts in fungal *cbhI* composition and richness that were correlated with elevated CO_2 exposure varied across the ecosystems. In aspen plantation and desert creosote bush soils, *cbhI* gene richness was significantly higher after exposure to elevated CO_2 (550 μmol mol^{-1}) than under ambient CO_2 (360 μmol mol^{-1} CO_2). In contrast, while the richness was not altered, the relative abundance of dominant OTUs in desert soil crusts was significantly shifted. This suggests that responses are complex, vary across different ecosystems and, in at least one case, are OTU-specific. Collectively, our results document the complexity of cellulolytic fungal communities in multiple terrestrial ecosystems and the variability of their responses to long-term exposure to elevated atmospheric CO_2.

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Introduction

Microbial decomposition of above- and below-ground plant litter is important in the carbon cycle, regenerating soil organic matter and releasing inorganic nutrients into the soil where they can be assimilated by plants. As atmospheric CO_2 increases, plant productivity generally increases and can alter the amount and quality of plant litter input to soil (e.g. Zak *et al.*, 1993; Couteaux *et al.*, 1995; Weatherly *et al.*, 2003; Hall *et al.*, 2005; Parsons *et al.*, 2008). Altered carbon input to soil may impact competitive interactions among soil microorganisms, thereby changing community composition and/or richness and potentially impacting rates of soil carbon cycling. Understanding the effects of elevated atmospheric CO_2 on microbial communities and the resulting changes to their functional characteristics is important to facilitate models of climate change consequences and feedbacks, and to determine if carbon derived from the increased net primary production in CO_2-enriched ecosystems is ultimately sequestered or released (e.g.

Hungate *et al*., 1997; Schlesinger and Andrews, 2000; Treseder, 2005).

Soil fungi are well known for their ability to degrade complex carbon (de Boer *et al*., 2005; Kubicek *et al*., 2010) and are likely a key component of microbial community responses to elevated CO_2. Studies examining fungal responses to elevated CO_2 have been largely restricted to use of metrics that quantify bulk properties of the fungal community (summarized in Treseder, 2005). For instance, previously noted impacts of elevated CO_2 on fungal communities include increased fungal biomass, increased sporocarp counts or root tip counts (Langley *et al*., 2003; Treseder, 2004; Lipson *et al*., 2005; Garcia *et al*., 2008; Andrew and Lilleskov, 2009), increased abundance as determined by qPCR and Sanger sequencing efforts (Lesaulnier *et al*., 2008; Castro *et al*., 2010), increased fungal colony-forming unit counts (Olszyk *et al*., 2001), increased arbuscular mycorrhizal hyphal counts (Treseder *et al*., 2003) and more rapid exoenzyme activities (Larson *et al*., 2002; Chung *et al*., 2006). However, changes in the abundance of saprotrophic fungi have also been mixed. Some studies have found an increase (Olszyk *et al*., 2001; Lesaulnier *et al*., 2008;) while others have found no change or a decrease (Klironomos *et al*., 1997; Kampichler *et al*., 1998; Insam *et al*., 1999). Responses of exoenzyme activities have also been mixed (Dhillion *et al*., 1996; Moorhead and Linkins, 1997; Kampichler *et al*., 1998; Carney *et al*., 2007; Kelley *et al*., 2011). Such general community characteristics integrate across ecological functional groups, which could be differentially responding to elevated CO_2. Application of molecular techniques that are specific for particular functional groups and allow discrimination of co-occurring changes of taxa within functional guilds of microorganisms are useful to gain insight into the mechanisms underlying overall community responses.

Although plant litter is chemically complex, its biochemical composition is dominated by cellulose (~60–70%; Kubicek *et al*., 2010). Cellulose metabolism requires the synergistic activities of three major groups of enzymes: (i) endoglucanses that cleave cellulose into smaller oligosaccharides, (ii) cellobiohydrolases that cleave cellobiose from the reducing and non-reducing ends of cellulose oligosaccharides, and (iii) β-glucosidases that cleave cellobiose into its glucose constituents (Kubicek *et al*., 2010). Understanding the synergistic functioning of the array of enzymes and organisms involved in the degradation of cellulose has been hampered by a lack of molecular tools for detecting and monitoring cellulolytic organisms. However, Edwards and colleagues (2008) recently designed a set of PCR primers that target the catalytic region of the fungal glycosyl hydrolase family 7 cellobiohydrolase I gene (*cbhI*) in *Ascomycota* and *Basidiomycota*. Although cellobiohydrolase I is only one of the enzymes involved in cellulose degradation, targeting this gene allows a representative group of cellulolytic fungi to be detected and monitored in soil ecosystems and has been shown to be a useful comparative functional gene marker for soil fungal communities (Edwards *et al*., 2008).

Using the *cbhI* gene fragment as a marker for cellulolytic fungal communities, we conducted a Sanger sequencing study to compare the richness and composition of cellulolytic fungal communities in replicate soil samples from five large-scale ecosystem experiments, to determine if they have changed after a decade or more of exposure to elevated atmospheric CO_2. The comparisons were conducted in soils from three Free-Air-Carbon Dioxide Enrichment (FACE) experiments and two open top chamber (OTC) experiments, in which the long-term effects of elevated atmospheric CO_2 and other environmental change factors have been investigated for many years (http://www.bnl.gov/face/faceProgram.asp, http://public.ornl.gov/face). Results presented here demonstrate that soil fungal *cbhI* composition is distinct in each of the ecosystems, and that the dominant *cbhI*-containing taxa are novel or known taxa that are not yet recognized as cellulose degraders. Statistically significant shifts in richness or relative abundance of specific operational taxonomic units (OTUs) were correlated with exposure to elevated CO_2 conditions in two of the ecosystems, while *cbhI*-containing taxa showed no apparent response to elevated CO_2 in the other three ecosystems examined.

Results

Characteristics of cbhI *sequence libraries*

In preliminary comparisons it was noted that the two habitats within the desert ecosystem, the cresosote bush root zone and desert crust, showed very different *cbhI* gene compositions and patterns of response to elevated CO_2. For that reason, they are treated as separate sites in the results presented here. A total of 10 677 high-quality *cbhI* gene fragments were obtained from creosote root zone (3513 across 17 libraries), desert crust (3074 across 17 libraries), aspen plantation (976 across six libraries), loblolly pine plantation (2239 across 12 libraries), scrub oak/palmetto (389 across six libraries) and marsh (486 across 10 libraries). Average numbers of gene sequences in replicate libraries at each of the sites were 206 (creosote root zone), 180 (crust), 162 (aspen plantation), 186 (loblolly pine plantation), 65 (scrub oak/palmetto) and 48 (marsh). Lower numbers of sequences in the scrub oak/palmetto and marsh soil libraries were a result of poorer sequence quality in these sequence libraries. Numbers of gene sequences from specific treatment and control plots at each site are presented in Table 1.

Table 1. Field sites, sampling regime and *cbhI* libraries.

Ecosystem	Location	CO_2 treatment	Additional factors	Field plots	No. of libraries	Total No. *cbhI* seq.
Desert shrubland	Southern NV, USA	Elevated	Crust	3	8	1645
	36 49′N, 115 55′W, 965–970 m elevation	Elevated	Creosote bush root zone	3	8	1876
		Ambient	Crust	3	9	1429
		Ambient	Creosote bush root zone	3	9	1637
Aspen plantation	Rhinelander, WI, USA	Elevated	None	3	3	422
	45 40.5′N, 89 37.5′E, 490 m elevation	Ambient	None	3	3	554
Loblolly pine	Durham, NC, USA	Elevated	Unfertilized	3	3	619
	35 58′N, 79 06′W, 130 m elevation	Elevated	N fertilized	3	3	531
		Ambient	Unfertilized	3	3	605
		Ambient	N fertilized	3	3	484
Scrub oak/palmetto	Cape Canaveral, FL, USA	Elevated	None	3	3	202
	28 38′N, 80 42′W, 0–3 m elevation	Ambient	None	3	3	187
Brackish tidal marsh	Edgewater, MD, USA	Elevated	None	5	5	247
	38 53′N, 76 33′W, sea level	Ambient	None	5	5	239

Additional information on each site is available through http://public.ornl.gov/face/.

Cross-site comparison of cbhI *richness and composition*

To compare *cbhI* richness across sites, data from treatment and control plots within each site were pooled. When rarefaction analyses on each of the six pooled libraries was normalized to 389 sequences, the following number of OTUs was collected for each of the respective ecosystems: 147 (aspen plantation), 108 (loblolly pine), 79 (scrub oak/palmetto), 77 (marsh), 69 (creosote root zone) and 53 (crust). This result suggests that aspen plantation and the desert environments are the most and least rich of the ecosystem types examined respectively. Further analysis of replicate sequence libraries representing each ecosystem is necessary to statistically compare richness across the ecosystems.

Non-metric multi-dimensional scaling (MDS) analyses based on OTU profiles generated from each replicate library, from all treatment and control plots demonstrate the distinct *cbhI* composition in each of the sites (Fig. 1). Using the binary Jaccard distance metric (Fig. 1A), which is based on the presence or absence of a particular OTU, the replicate samples from scrub oak/palmetto, estuary and desert ecosystems clustered together as replicates, and independently of each other and of the aspen/loblolly pine. The aspen plantation and loblolly pine plantation shared some of the OTUs but the ecosystems did not cluster together. Within the desert site, the creosote root zone and crust habitats shared presence of similar taxa. However, when relative abundance of each OTU is taken into account using the Bray-Curtis distance metric, MDS analysis demonstrates that each of these habitats is also distinct (Fig. 1B). Weighted and unweighted Unifrac and parsimony tests performed on sequence libraries pooled by site indicated that composition was statistically distinct (all P-values < 0.001).

The number of OTUs shared across sites was low. The 10 677 sequences obtained from all sites represented 658 OTUs, none of which was shared among all six sites (dominant taxa in Table 2). About 70% (462 OTUs) were unique to a given site (loblolly pine plantation = 138, aspen plantation = 107, creosote root zone = 70, scrub oak/palmetto = 53, marsh = 56, crust = 38).

Operational taxonomic units unique to a given site are among some of the most abundant at each site, indicating that it is not simply ‘rare types’ or singletons that differentiate composition among sites (Fig. S1 and Table 2). Of the 15 most abundant OTUs in each of the sites, the marsh libraries contained the highest number (11) of OTUs that were unique to that site (Table 2) and the scrub oak/palmetto site had the second highest number (8). Seven out of the 11 unique OTUs in the marsh site, seven out of the eight unique OTUs in the scrub oak/palmetto site, and three out of the seven unique OTUs in the aspen library had nearest BLAST hits to members of the Ascomy-

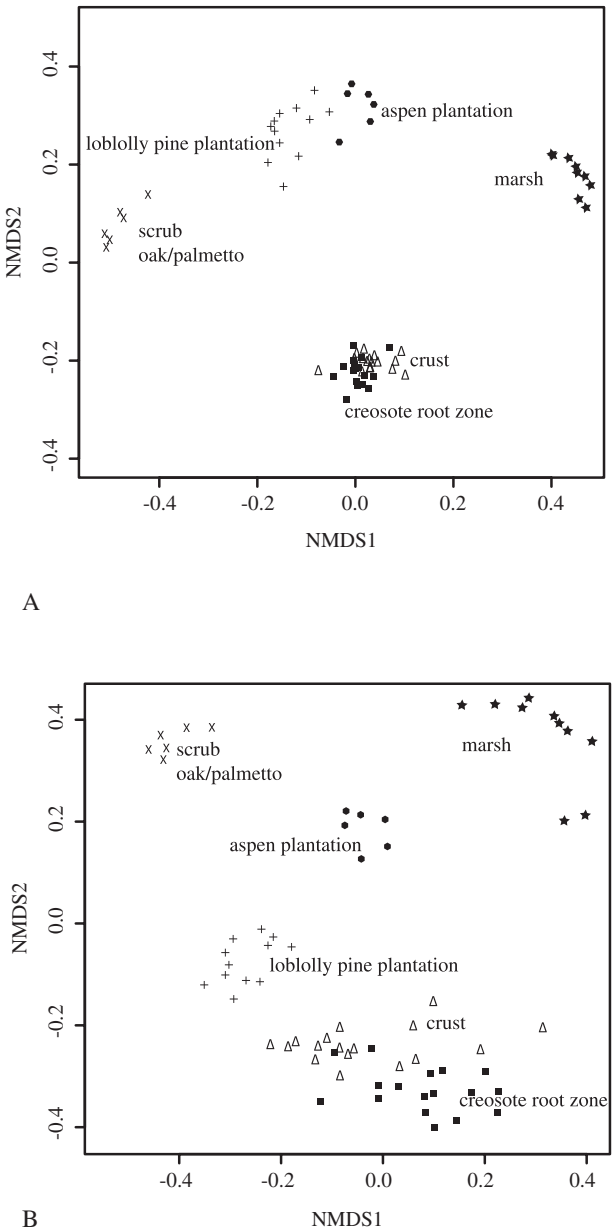

Fig. 1. Non-metric multi-dimensional scaling (MDS) analysis based on the Jaccard distance metric (A) and the Bray-Curtis distance (B) metric of OTU profiles (OTU = 0.10) for replicate libraries from treatment and control plots at each site. ■, creosote root zone; △, crust; ●, aspen plantation; +, loblolly pine plantation; ×, scrub oak/palmetto; ★, marsh.

cota (Table 2A), within the Sordariomycetes, Dothidiomycetes and Eurotiomycetes. Four of the seven unique OTUs in the aspen plantation and the one unique OTU present in the loblolly pine plantation had nearest BLAST hits to the Agaricales and Polyporales orders within the Basidiomycota, while the other three unique OTUs had nearest BLAST hits to members of the Ascomycota within the same classes as those in the scrub oak/palmetto and marsh. The 15 most abundant OTUs in the creosote root zone and crust were all shared between the two sites, but they collectively contained eight OTUs that were not found in the other ecosystems (Table 2). Four of these OTUs had nearest BLAST hits to the *Agaricales* within the *Basidiomycota* and four had nearest BLAST hits to the *Ascomycota* [*Pleosporales* (3), *Sordariales* (1)]. Collectively, these results indicate that *Ascomycota*-like *cbhI* sequences play a larger role in distinguishing the dominant members of the *cbhI*-containing community in the scrub oak/palmetto and marsh, while the opposite may be true for the aspen plantation. It is important to note, however, that BLAST hits only represent the closest sequences in the database; as the database is populated with more taxa, classification of these sequences, particular at finer levels of taxonomic resolution (e.g. family, class), may change.

Cross-treatment comparison of cbhI *richness and composition within each site*

Pairwise comparisons were performed on the average richness values for treatment and control libraries within each site (Fig. 2). In the creosote root zone and aspen plantation, OTU richness in the elevated CO_2 treatment was significantly higher than in the ambient control within a 90% confidence interval (one-tailed *t*-test, $P < 0.07$; Fig. 2). A similar trend was observed in the marsh libraries, but was not statistically significant (one-tailed *t*-test, $P < 0.21$). The opposite trend was observed in the loblolly pine and scrub oak/palmetto sites, wherein richness was lower in the elevated CO_2 treatment relative to the ambient control, but these trends were not statistically significant. Richness of *cbhI* genes was not statistically different for the elevated CO_2 treatment and ambient control libraries from the crust site (Fig. 2).

Within-site compositional variability among *cbhI* sequence libraries was substantial, thereby limiting our ability to discern potential shifts in community composition correlated with long-term exposure to elevated CO_2 (or N fertilization at the loblolly pine site). Hierarchical dendrograms based on the Bray-Curtis distance metric (with relative abundance), illustrated a lack of clustering among ambient control and treatment libraries (Fig. 3). Weighted and unweighted Unifrac as well as parsimony analyses supported this finding; *P*-values for comparisons of treatment and control libraries were highly variable ranging from less than 0.001 to 0.35. Such variability is reflected in the percentage of singletons in a given replicate library and the lack of overlap with other replicate libraries. For instance, in the richest site (aspen plantation), an average of 57% of the OTUs in a given replicate library were singletons, whereas in the site with the lowest richness (crust), an average of 39% of the OTUs in each library were singletons. The compositional overlap of singletons

Table 2. Distribution of the 15 most abundant OTUs in each site that are most closely related to *Ascomycota* (A) and *Basidiomycota* (B) by BLAST analysis.

OTU No.	Desert creosote	Desert crust	Aspen	Loblolly	Scrub oak/palmetto	Tidal marsh	Fungal order (nearest BLAST hit)	*e*-value
A. *Ascomycota*								
41	10.4	4.5	0.1	0.2	0	0	*Pleosporales* (*Leptosphaeria* mg157 d8 JF522242)	1.00E-69
23	9.8	7.8	0	0.1	0	0	*Pleosporales* (*Phaeosphaeria nodorum* SN15 gbEAT838241)	8.00E-82
24	6	0.7	0	0	0	0	*Sordariales* (*Chaetomium thermophilum* gbAAW64926.1)	1.00E-75
12	5.2	1.8	0	0	0	0	*Pleosporales* (*Alternaria alternata* gbAAF05699)	1.00E-91
16	3.9	5.7	0	0	0	0.2	*Sordariales* (*Chaetomium thermophilum* gbAAW64926.1)	1.00E-75
13	3.2	21.7	0	35.1	0	0	*Pleosporales* (*Alternaria alternata* gbAAF05699)	2.00E-75
19	2.3	1.4	0.1	0	0	0	*Pleosporales* (*Leptosphaeria* mg157 b8 JF694944)	1.00E-94
14	2	0.4	0	0	0	0	*Pleosporales* (*Phaeosphaeria nodorum* SN15 gbEAT838241)	9.00E-71
15	1.7	3.9	0	0	0	0	*Pleosporales* (*Leptosphaeria* mg157 d8 JF522242)	3.00E-83
75	0.1	1.1	0.1	0	0	0	*Pleosporales* (*Alternaria alternata* gbAAF05699)	6.00E-93
189	0	0	0.9	0.1	18.8	0	*Eurotiales* (*Penicillium janthinellum* embCAA41780.1)	4.00E-85
191	0	0	0.3	0	1.3	0	*Hypocreales* (*Acremonium thermophilum* embCAM98445.1)	4.00E-87
197	0	0	0.1	0	0	4.3	*Pleosporales* (*Alternaria alternata* gbAAF05699)	4.00E-82
204	0	0	1.6	0	0	0	*Pleosporales* (*Alternaria alternata* gbAAF05699)	3.00E-79
209	0	0	1.5	0.1	0	0	*Hypocreales* (*Gliocladium roseum* DSMZ1165 d1 JF694949)	1.00E-89
234	0	0	2.9	0	0	0.4	*Helotiales* (*Botryotinia fuckeliana* B05.10 XM001550657.1)	9.00E-74
241	0	0	5.2	0	0	0.8	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	7.00E-78
251	0	0	2.3	0.3	0	0	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	1.00E-81
265	0	0	2.3	0	0	0	*Sordariales* (*Chaetomium thermophilum* gbAAW64926.1)	2.00E-77
267	0	0	0.1	0.1	10.3	0	*Eurotiales* (*Talaromyces emersonii* AAL895531)	2.00E-76
279	0	0	3.7	0	0	0	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	6.00E-83
290	0	0	0.3	2.1	0	0	*Eurotiales* (*Aspergillus* mg175 h3 JF694961)	5.00E-74
332	0	0	0.1	0	0	2.3	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	1.00E-75
397	0	0	0	0	1.3	0	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	2.00E-85
400	0	0	0	0.3	6.4	0	*Eurotiales* (*Aspergillus* mg175 h3 JF694961)	7.00E-80
402	0	0	0	0	1.3	0	*Hypocreales* (*Acremonium thermophilum* embCAM98445.1)	5.00E-86
406	0	0	0	0	1.5	0	*Eurotiales* (*Aspergillus terreus* NIH2624 NT165930)	1.00E-74
422	0	0	0	0	18.8	0	*Pleosporales* (*Preussia* mg156 f3 JF694978)	1.00E-82
425	0	0	0	0	3.1	0	*Eurotiales* (*Penicillium funiculosum* embCAC85737)	1.00E-80
445	0	0	0	0	1.3	0	*Diaporthales* (*Cryphonectria parasitica* gbAAB00479.1)	3.00E-75
462	0	0	0	0	0	4.7	*Sordariales* (*Humicola grisea* var. *thermoidea* dbjBAA09785)	2.00E-87
470	0	0	0	0	0	2.1	*Pleosporales* (*Alternaria alternata* gbAAF05699)	8.00E-92
474	0	0	0	0	0	9.9	*Eurotiales* (*Thermoascus aurantiacus* embCAM98447.1)	1.00E-76
476	0	0	0	0	0	4.3	*Pleosporales* (*Alternaria alternata* gbAAF05699)	7.00E-91
486	0	0	0	0	0	3.5	*Hypocreales* (*Acremonium thermophilum* embCAM98445.1)	4.00E-81
492	0	0	0	0	0	2.3	*Sordariales* (*Chaetomium globosum* h11 JF694968)	7.00E-75
509	0	0	0	0	0	2.5	*Sordariales* (*Chaetomium* mg128 b10 JF522246)	2.00E-78
534	0	0	0	1.5	0	0	*Pleosporales* (*Leptosphaeria* mg157 b8 JF694944)	6.00E-82
B. *Basidiomycota*								
8	2.3	1.1	0.1	0	0	0	*Agaricales* (*Nidularia pulvinata* a6 JF694971)	4.00E-79
20	1.5	1.1	0	0	0	0	*Agaricales* (*Hebeloma* PajaritoNM JF694959)	1.00E-81
25	1.9	0.2	0.1	0	0	0	*Polyporales* (*Phanerochaete chrysosporium* gbAAB46373.1)	1.00E-77
29	14.3	4.6	0.2	0	0	0	*Polyporales* (*Trametes* L d5 JF522236)	3.00E-71
36	0.3	1.7	0	0	0	0.2	*Agaricales* (*Hebeloma* PajaritoNM JF694959)	6.00E-88
44	0.9	6.4	0.1	0.1	0	0	*Polyporales* (*Trametes* L f1 JF522239)	5.00E-80
47	1.8	0.1	0.1	0	0	0	*Polyporales* (*Trametes* H e11 JF694946)	2.00E-79

51	1.4	11.7	0.3	0	0	0	*Polyporales* (*Trametes* L f1 JF522239)	8.00E-80
54	2.7	0.2	0.2	0	0	0	*Agaricales* (*Coprinus cinereus* 619 g6 JF522245)	1.00E-84
57	2.3	0.3	0	0	0	0	*Agaricales* (*Coprinus cinereus* 619 a7 JF694983)	1.00E-83
67	1.7	8.4	0	0	0	0	*Agaricales* (*Amanita manicata* 1 JF694948)	4.00E-61
190	0	0	1.4	0.1	0	0	*Agaricales* (*Lycoperdon* IL 640 a6 JF694966)	9.00E-92
193	0	0	1.6	0	0	0	*Agaricales* (*Lycoperdon* IL 640 a6 JF694966)	1.00E-92
194	0	0	0.2	0	1.8	0	*Agaricales* (*Lycoperdon* IL 640 a6 JF694966)	2.00E-92
198	0	0	1.7	0	0	0	*Polyporales* (*Trametes* H e11 JF694946)	2.00E-93
207	0	0	0.9	3.1	0	0	Agaricales (*Lentinula edodes* AF411250.i)	2.00E-76
211	0	0	0.6	2.7	2.1	1.2	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	6.00E-80
212	0	0	1	1.5	0	0	*Agaricales* (*Amanita manicata* 1 JF694948)	6.00E-84
217	0	0	5.1	0	0	0	*Polyporales* (*Ganoderma* E640 d10 JF694956)	3.00E-83
220	0	0	0.2	0	0	2.1	*Boletales* (*Coniophora putanea* AB501101.1)	2.00E-68
221	0	0	1.2	1.7	0	0	*Agaricales* (*Amanita manicata* 1 JF694948)	2.00E-73
222	0	0	1.8	0.1	0.3	0.4	*Agaricales* (*Melanoleuca rasilis* 1 JF694949)	3.00E-71
227	0	0	2	0	0	0	*Agaricales* (*Nidularia pulvinata* a6 JF694971)	5.00E-81
233	0	0	0.9	1.7	0	0	*Polyporales* (*Trametes* L f1 JF522239)	5.00E-77
235	0	0	1.9	0	0	0.2	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	2.00E-79
259	0	0	2.5	0.1	0.3	0	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	1.00E-80
264	0	0	1.1	2.9	0	0	*Agaricales* (*Lentinula edodes* AF411250.i)	3.00E-84
291	0	0	0.7	1.6	0	0	*Agaricales* (*Amanita manicata* 1 JF694948)	9.00E-87
292	0	0	0.1	1.2	1	0	*Agaricales* (*Nidularia pulvinata* a6 JF694971)	2.00E-82
300	0	0	0.9	2.5	0	0	*Agaricales* (*Lentinula edodes* AF411250.i)	2.00E-77
303	0	0	0.1	0	0	3.9	*Agaricales* (*Hebeloma* PajaritoNM JF694959)	2.00E-89
314	0	0	0.9	1.4	0	0	*Agaricales* (*Lentinula edodes* AF411250.i)	3.00E-83
333	0	0	0.6	1	0	0	*Agaricales* (*Amanita manicata* 1 JF694948)	3.00E-80
433	0	0	0	0.4	3.3	0	*Agaricales* (*Amanita manicata* 1 JF694948)	3.00E-81
434	0	0	0	0	1.8	0	*Eurotiales* (*Thermoascus aurantiacus* embCAM98447.1)	5.00E-68
450	0	0	0	0	1.3	0	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	4.00E-72
463	0	0	0	0	0	10.5	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	2.00E-79
478	0	0	0	0	0	4.3	*Agaricales* (*Coprinus* IL f12 JF694963)	1.00E-78
507	0	0	0	0	0	7.4	*Polyporales* (*Phanerochaete chrysosporium* gbAAB46373.1)	3.00E-78
515	0	0	0	0	0	2.3	*Agaricales* (*Nidularia pulvinata* d7 JF694965)	3.00E-75
525	0	0	0	1.1	0	0	*Agaricales* (*Cyathus stercoreus* a4 JF694967)	2.00E-81

Values are the per cent representation of an OTU within a given library.

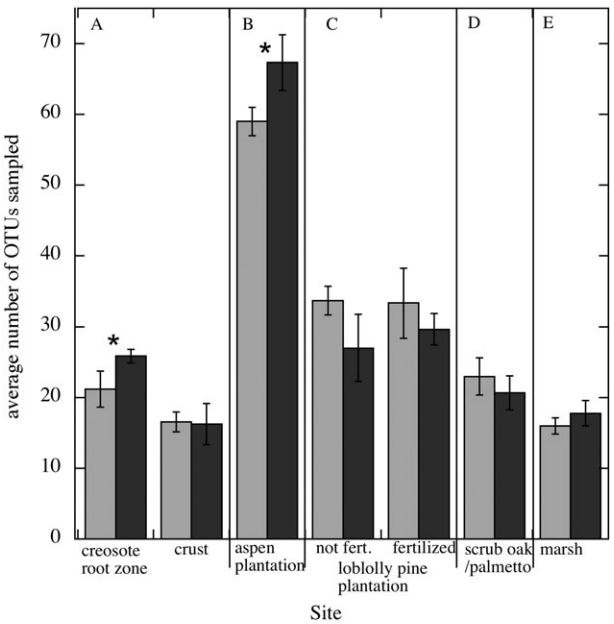

Fig. 2. Average richness (OTU = 0.10) ± SE of replicate libraries within treatment and control plots in (A) creosote root zone and crust, (B) aspen plantation, (C) loblolly pine plantation, (D) scrub oak/palmetto and (E) marsh. Richness was normalized for the greatest common number of sequences collected in a replicate library at a given site. Greatest common numbers of sequences = 116 (creosote root zone), 90 (crust), 133 (aspen plantation), 101 (loblolly pine plantation), 59 (scrub oak/palmetto) and 38 (marsh). Numbers of replicate libraries averaged for each treatment or control at each site were 3 (aspen plantation, scrub oak/palmetto, loblolly pine plantation), 5 (marsh), 8 (elevated CO_2 for crust and creosote root zone), 9 (ambient CO_2 for crust and creosote root zone). The asterisk '*' indicates potentially significant difference based on a one-tailed Student's *t*-test with *P*-value < 0.07 (creosote root zone and aspen plantation).

varied substantially across treatment and control replicates. In the aspen plantation libraries, on average, only 55% of the singletons present in any given library were present in another library. In the crust, 84% of the singletons on average were shared among two or more replicate libraries.

Despite the variability in *cbhI* sequence composition, rank abundance analyses indicated that the dominant OTUs were consistently present in spatially separated samples within each field site (Fig. S1). Given the lack of obvious compositional shifts at the community level, we explored shifts in relative abundance of the dominant OTUs at each of the sites that might correlate with exposure to elevated CO_2 and/or to fertilization (loblolly pine plantation only). Evidence of a complex but significant shift the treatment libraries was only observed in the most abundant OTUs in the crust (Fig. 4). The relative abundance of the six most abundant OTUs in the crust differed significantly between the elevated CO_2 treatment and ambient control libraries (Fig. 4). Four of these OTUs significantly increased in relative abundance under elevated CO_2 conditions (OTU #13, 51, 23, 44; one-tailed pairwise *t*-tests, $P < 0.05$–0.09), while two of them significantly decreased in abundance (OTU #67, 16; one-tailed pairwise *t*-tests, $P < 0.07$–0.11, Fig. 4). Collectively, these results provide evidence for a complex and OTU-specific shift correlated with exposure to elevated CO_2 in the crust. Two of the OTUs that increased in relative abundance had nearest BLAST hits to members of *polysporales* within the Basidiomycota, while the other two OTUs had nearest BLAST hits in the Pleosporales within the Ascomycota. One of the OTUs that decreased in relative abundance had a nearest BLAST hit to *Amanita manicata* and the other OTU had a nearest BLAST hit to *Chaetomium thermophilum* (Table 2A and B).

Taxonomic classification of FACE cbhI *sequences using an OTU-based approach and* BLAST *analysis*

All 10 677 sequences were binned into OTUs with the 114-member *cbhI* reference sequence database compiled in this study (Table S1). Only 97 (0.9%) of the environmental sequences clustered into OTUs with any of the 114-member *cbhI* database sequences. The 97 environmental sequences clustered into 20 of the 658 OTUs identified across the 10 677-sequence data set, which clustered with 26 (23%) of the reference sequences (Table S2). Fifteen of the 20 OTUs clustering with reference sequences were classified as Ascomycota. Most of the OTUs that contained reference sequences (17 out of 20) contained less than 10 of the environmental sequences indicating that the current database, derived largely from cultured fungi, is only relevant for classifying a minor fraction of the *cbhI*-containing taxa *in situ,* and needs to be substantially expanded to provide insight into the family level classification of over 99% of the environmental sequences.

Sequences from three phyla in the database (Ascomycota, Basidiomycota, Oomycota) formed distinct clusters in the phylogeny and the affinity of an environmental sequence to BLAST to a sequence from one of these three phyla in the *cbhI* sequence database likely serves as a strong indicator of phylum identity. Using a BLAST analysis against the *cbhI* sequence database to tentatively classify all 10 677 FACE site sequences at the phylum level (Table S3) indicated that Ascomycota-like *cbhI* sequences comprised the majority of sequences from five of the sites [crust (55.3%), creosote root zone (54.3%), marsh (59.5%), scrub oak/palmetto (83.5%) and loblolly pine plantation (54.4%)], while Basidiomycota-like sequences comprised the majority of the aspen plantation. Sequences most similar to *Pythium intermedium,* an Oomycete, were most abundant in the marsh (1.9%), which is consistent with the large subunit gene profiles of these sites (C.R. Kuske *et al.*, unpubl. data). Sequences

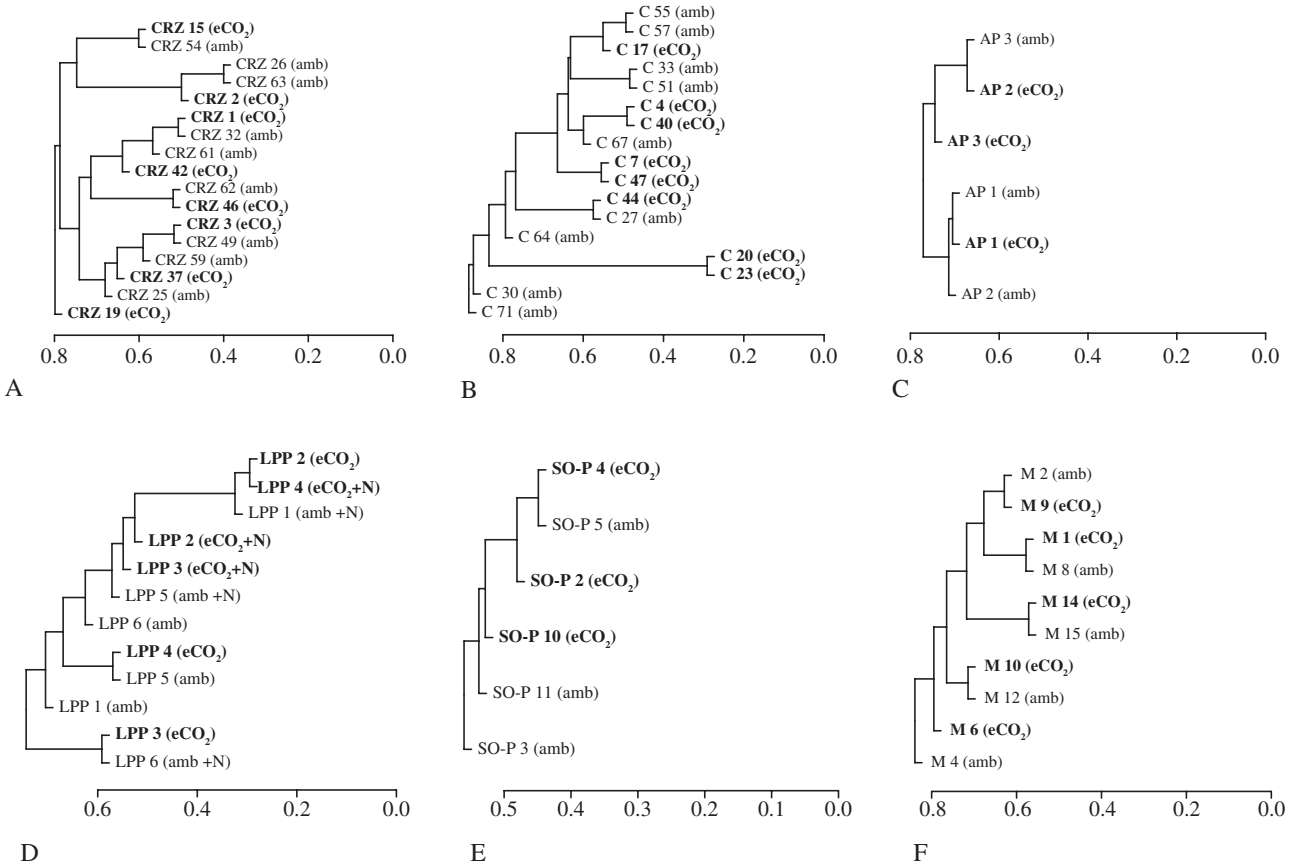

Fig. 3. Bray-Curtis dendrograms of replicate libraries from ambient and elevated CO_2 plots as well as fertilization treatments (loblolly pine plantation only) for (A) creosote root zone (CRZ), (B) crust (C), (C) aspen plantation (AP), (D) loblolly pine plantation (LPP), (E) scrub oak/palmetto (SO-P) and (F) marsh (M).

most closely related to *Mucor heimalis,* a member of the Mucoromycotina, comprised less than 1% of the sequences at three of the sites [aspen plantation (0.1%), loblolly pine plantation (0.3%), scrub oak/palmetto (0.8%)].

Across all of the sites, about 18% of all sequences (1897 sequences) belonged to OTUs that were unique to a given site (Table S4). We performed a BLAST analysis of these unique sequences to determine if they were dominated by a particular phylum and thus maybe driving compositional differences among ecosystems. BLAST analysis of these sequences revealed that 44.6% had nearest hits in the Basidiomycota, 53.7% in the Ascomycota, 1.5% in the Oomycota and 0.1% in the Mucoromycotina, which mirrored the composition of each site (Table S4). The unique sequences in the loblolly pine plantation and crust libraries were dominated by Basidiomycota-like sequences (58% and 57% respectively; Table S4). For all other sites, Ascomycota-like sequences dominated the unique sequences. Thirty-two of the sequences of the 10 677-sequence data set had closest BLAST hits to the only Oomycete in the database. Of these 32 sequences, 29 were unique to a given site indicating that *cbhI*-containing non-fungal organisms may have distinct patterns of distribution across ecosystem types.

Discussion

Fungi have long been known to degrade cellulose, but their composition and richness in soil is not well understood and even less is known regarding their responses to changes in plant litter production under elevated atmospheric CO_2. We conducted a large-scale sequencing effort of a *cbhI* gene fragment to compare the richness and composition of cellulose-degrading fungi across and within five different ecosystems exposed to elevated CO_2. We also generated a 114-member *cbhI* phylogeny to use as a scaffold for taxonomic classification of environmental sequences. Results of this study demonstrate (i) the distinct composition of *cbhI*-containing fungal assemblages in five terrestrial ecosystems as well as in two desert environments (Fig. 1 and Table 2), (ii) the dominance of novel cellulose degraders or known fungi that

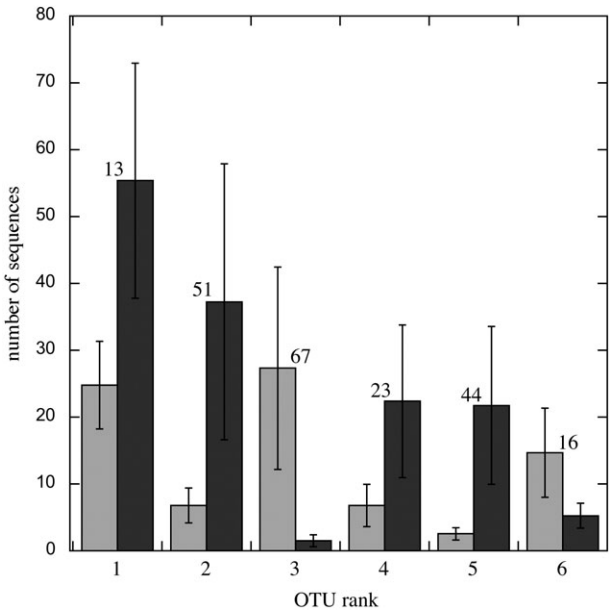

Fig. 4. Relative abundance of sequences from elevated CO_2 plots (black bars) and ambient plots (grey bars) for the six most abundant crust OTUs. Bars represent the average of eight replicate libraries for elevated CO_2 ± SE and the average of nine replicate libraries for ambient CO_2 ± SE Numbers above the bars are OTU identifier numbers assigned arbitrarily in Mothur. Prior to averaging, richness was normalized for the greatest common number of sequences sampled in each of the replicate libraries (116). One-tailed pairwise *t*-tests for OTU #13, 51, 23 and 44 had *P*-values < 0.05–0.09). One-tailed pairwise *t*-tests for OTU #67 and 16 had *P*-values < 0.07–0.11).

are not yet recognized as cellulose degraders at the sites investigated, and (iii) the complexity and variability of the response of cellulose-degrading fungi to elevated CO_2 (Figs 2 and 4). Collectively, our data illustrate that cellulose-degrading fungi potentially respond in disparate and sometimes complex ways across terrestrial ecosystems, that cellulose-degrading fungi *in situ* are highly diverse, and that more extensive sequence databases will be necessary to allow us to link function, taxonomy and ecology of soil fungi.

Comparison of *cbhI* sequences from replicate soil samples, using multiple analyses, clearly illustrated that each ecosystem as well as the creosote root zone and crust soils within the desert ecosystem harboured a distinct community of cellulolytic fungi. These differences were driven primarily by the dominant taxa at each site, which were consistently represented in these relatively small (average range of sequence: 48–206) Sanger clone libraries. If the taxa representing these dominant OTUs play major roles in soil carbon cycling in each of these ecosystems, this may have implications on community function and is a topic worthy of future research.

Although this study was not designed to specifically test the impacts of soil properties and plant cover on the composition of *cbhI*-containing soil fungi, our results suggest that they have an effect. For instance, MDS and rank abundance analyses indicate that the marsh is one of the most compositionally unique sites with 11 out of 15 of the most abundant OTUs being unique to that site (Table 2 and Fig. S1). The marsh soil is also the most distinct in terms of its physical and chemical properties (e.g. soil moisture, pH, organic matter, NO_3, P, K^+, Mg^{+2}, Ca^{+2}; Table S5). The aspen and loblolly pine sites are much closer compositionally, which also corresponds with the more similar soil chemical properties of these sites (Table S5). Compositional data from the crust and creosote root zones provide evidence that plants may have a strong influence on the compositional uniqueness of the sites. Although the same *cbhI* OTUs dominate both the creosote root zone and the crust (Table 2 and Fig. S1) and both sites were sequenced to a similar sampling depth of coverage (Table 1), the creosote root zone contained 70 OTUs that were unique to that environment, while the crust only contained 38 OTUs that were unique to that environment. In addition, normalized richness was slightly higher in the creosote root zone (69 OTUs) than in the crust (53 OTUs). This indicates that plants likely create rhizosphere niches that can accommodate a greater richness of *cbhI*-containing fungi.

This *cbhI* gene survey represents the most comprehensive cross-ecosystem comparison of a fungal functional gene conducted to date. Based on prior cultivation studies, we expected to detect previously studied cellulolytic fungal taxa in these soil samples. On the contrary, the vast majority of the dominant OTUs were not identifiable with high confidence using current databases (BLAST) or our 114-member *cbhI* gene data set, indicating that many of the dominant cellulolytic fungi in soil are not the prototypical cellulose degraders that have been much studied (e.g. *Trichoderma, Chaetomium, Phanerochaete*; Webster and Weber, 2007). Rather, they represent novel taxa or known taxa, in which cellulose-degrading capabilities have not yet been recognized.

From our analysis of *cbhI* genes in cultured fungi and sporocarps (Table S1, Fig. 5) plus the soil sequences, it is clear that presence of this gene (and inferred cellulolytic capability) is widespread among the Ascomycota and Basidiomycota. Diverse Ascomycota sequences from soils included members of the Pleosporales, Diaporthales, Eurotiales, Hypocreales and Sordariales (Table 2). Many genera within these orders are species rich (e.g. 80–100 species), cosmopolitan and opportunistic saprotrophs (Webster and Weber, 2007). Many of the *cbhI* genes from members of these orders (e.g. *Aspergillus*) do not form monophyletic clades. Because the *cbhI* phylogeny does not always parallel that of ribosomal gene sequences and many isolates possess multiple and different copies of *cbhI*, it is difficult to discern taxonomic

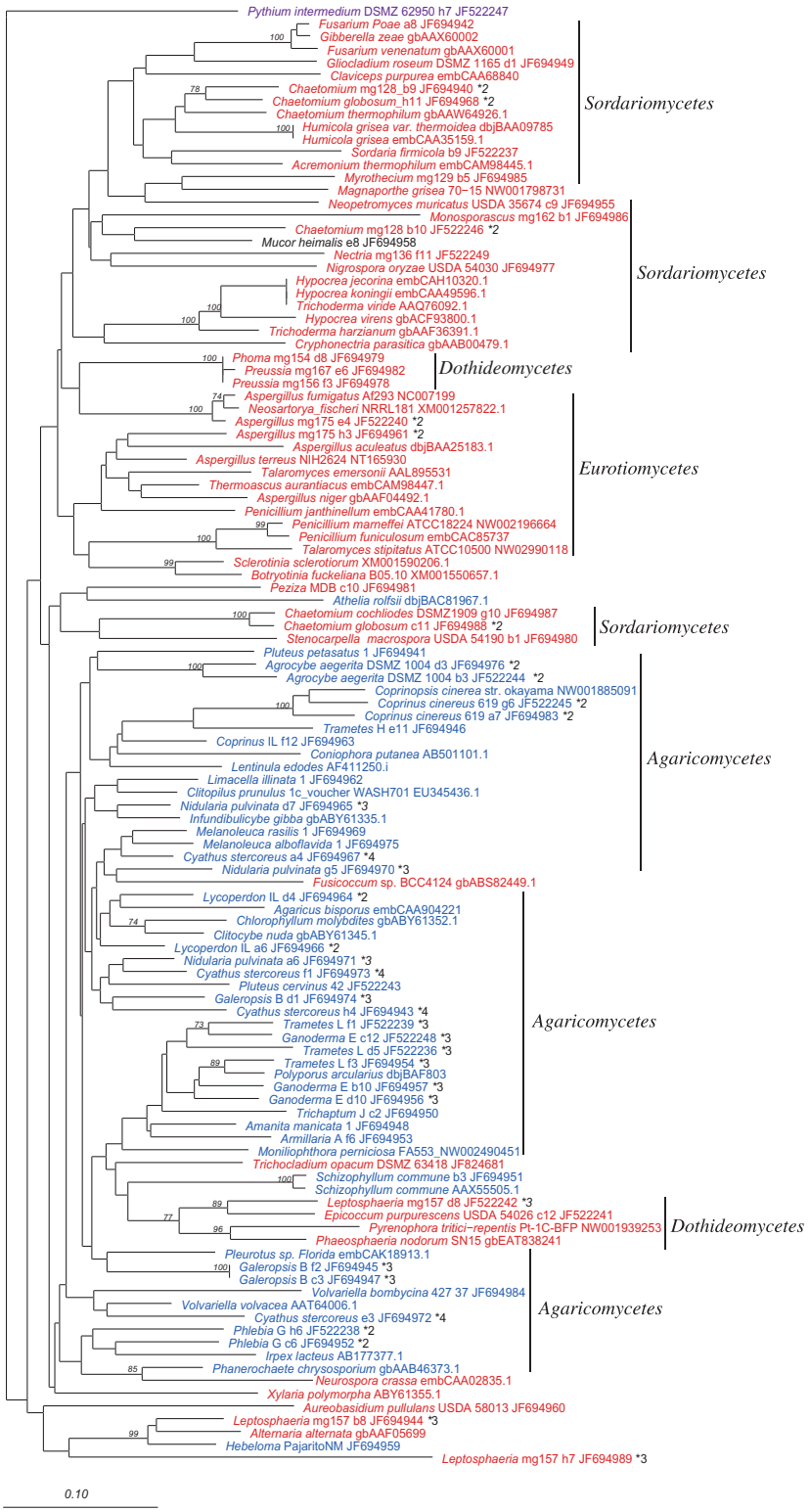

Fig. 5. Neighbour-joining tree of *cbhI* gene fragments (inferred amino acid sequences) from named fungal cultures and sporocarps generated using 1000 bootstrap replicates. Only bootstrap values greater than 70 are displayed. The tree was rooted with an endoglucanse from *Aspergillus oryzae* (BAE66197) within the glycosyl hydrolase 7 Family. Sequences are coloured by phylum: red = Ascomycota, blue = Basidiomycota, black = Mucoromycotina and purple = Chromista. The asterisk '*' followed by a number indicates cultures or sporocarps in which more than one gene *cbhI* gene sequence was found and the number of sequences found.

identity of an environmental *cbhI* sequence from its nearest BLAST hit or position within the phylogeny. Basidiomycota OTUs were also widely distributed across the phylum (Table 2), with many sequences having nearest hits to members of the Agaricales, Polyporales and Boletales, but again, these hits are a function of the database composition and some of the some of the sequences may very well be members of phylogenetically neighbouring orders.

We detected responses of the *cbhI*-containing fungal community to elevated CO_2 in the aspen plantation site and both habitats (crust and creosote root zone) in the desert site. In these two ecosystems, the nature of the response appears complex and is probably controlled by different mechanisms in each site (Table 3). Our study did not detect effects of elevated CO_2 on the *cbhI*-containing fungal community. Whether this result indicates 'no response' to elevated CO_2, or the inability of our sampling and study design to detect differences remains inconclusive. Although this sequence survey was replicated ($n = 3$ to 9 per treatment in different sites) and sequencing depth of coverage was sufficient to repeatedly sample the dominant OTUs (Fig. S1), spatial heterogeneity within each site was high, making it difficult to reliably discern treatment differences with 95% confidence.

Despite this variability, *cbhI* richness was found to increase in the aspen plantation site. This finding is consistent with previous results based on fungal ribosomal gene surveys at this site that identify shifts in fungal community composition, particularly among mycorrhizal taxa (Lesaulnier *et al*., 2008; Andrew and Lilleskov, 2009; Edwards and Zak, 2011). Lesaulnier and colleagues (2008) found that relative abundance of several taxa was altered in elevated CO_2 plots (i.e. increased abundance of the mycorrhizal genus, *Inocybe*; declination of the genus *Cazia* while the genus *Pachyphloeus* became dominant). Andrew and Lilleskov (2009) also noted increased abundance of ectomycorrhizal sporocarps under elevated CO_2 at this site. Edwards and Zak (2011) noted that *Agaricomycete* community composition in the forest floor shifted in response to elevated CO_2, with an increased relative abundance of *Sistotrema.* Although mycorrhizal fungi may not be primary participants in cellulose degradation, higher exoenzyme activity rates have been noted in mycorrhizal mats suggesting that they can enhance and alter saprotrophic activities, and perhaps richness, in the surrounding community (Entry *et al*., 1991; Kluber *et al*., 2010).

Responses to elevated CO_2 at the desert site were dependent on the specific location in the patchy landscape (creosote root zone or crust). Richness was found to increase in response to elevated CO_2 in the creosote root zone, but not the soil crusts. Using $^{13}CO_2$-fed plants at this site, Jin and Evans (2010) were able to document that ^{13}C-labelled PLFA had higher fungal to bacterial ratios under elevated CO_2 than under ambient conditions. This result coupled with our data suggests that under elevated CO_2, plants may exude a variety of carbon sources that can be consumed by *cbhI*-containing fungi, thus creating more niches for them to occupy and promoting increased *cbhI* richness. In contrast to the creosote bush root zone, the relative abundance of dominant OTUs recovered from the desert crusts was significantly altered under elevated CO_2 conditions. The crust has the lowest cellulose inputs of all the sites examined due to minimal vascular plants. However, they are dominated by cyanobacteria, which may be an important source of soil carbon in this ecosystem. In addition, in these plant interspaces Jin and Evans (2007) document that microbial biomass N in plant interspace soils at this site were lower under elevated CO_2 which may also alter the composition of *cbhI*-containing fungi.

It is also remarkable to note that the same taxa dominated both the creosote root zone and the crust, but this shift was not observed in the creosote root zone. This suggests that mechanisms controlling the response of cellulolytic fungi to elevated CO_2 are spatially variable within the same ecosystem. In this ecosystem, there is little litter deposition or organic matter retained in the soils, and the observed trends may be due to other physiological traits of *cbhI*-containing fungal species. However, mechanistic understanding of such a response is beyond the scope of the current study and requires further investigation.

This study was conducted at one time point, in summer or early fall, at each of the experimental field sites, often

Table 3. Summary of richness and compositional responses to elevated CO_2.

Field site	Richness response	Compositional response
Aspen plantation	↑ ($P < 0.07$)	None detected
Desert creosote	↑ ($P < 0.07$)	None detected
Marsh	↑	None detected
Desert crust	No response	Significantly altered relative abundance of six most dominant OTUs (Fig. 4)
Loblolly pine (N fertilized)	↓	None detected
Loblolly pine (unfertilized)	↓ ($P < 0.13$)	None detected
Scrub oak/palmetto	↓	None detected

associated with final destruction of that field site. Seasonal and year-to-year trends have been found to influence observed ecosystem responses at these field sites, and we may have missed responses that were manifest in other seasons. For example, in a recent meta-analysis of exoenzyme activities, Kelley and colleagues (2011) found that season may in part explain the mixed results across studies that have used exoenzymatic assays to examine microbial responses to elevated CO_2. Edwards and Zak (2011) also note temporal changes in cellobiohydrolase activity at the aspen plantation site, which were measured over the course of a decade; they noted that elevated CO_2 stimulation of cellobiohydrolase activity has decreased in magnitude over the years.

Environmental surveys such as this one represent an important first step to identifying key microbes involved in a particular process. However, it is important to remember that soil fungi harbouring *cbhI* genes almost certainly play multiple roles in soil carbon cycles, and it is possible that the observed community shifts attributed here to *cbhI* are linked to other functions carried by *cbhI*-containing fungi. Inclusion of gene expression assays in soil surveys to examine the response of active fungi to elevated CO_2 *in situ*, as well as studies that link *cbhI* genes to fungal taxonomy and ecological function is necessary to understand shifts in richness and composition of *cbhI*-containing fungal communities observed in this study.

In summary, this study demonstrates that ecosystem type and association with plants have greater influence on the composition of *cbhI*-containing soil fungal communities than elevated CO_2 (or nitrogen fertilization in a loblolly pine plantation). Greater than 99% of the environmental sequences could not be taxonomically classified with confidence indicating that many cellulose degraders *in situ* remain to be discovered or recognized. Compositional responses to elevated CO_2 were difficult to discern and interpret given the high degree of spatial heterogeneity and inability to classify them taxonomically. Nonetheless, richness or relative abundance of specific OTUs was found to respond in three sites. To understand the mechanisms behind these responses to elevated CO_2 and what they mean functionally, future studies need to focus on linking environmental sequences with taxonomy, function and ultimately, ecology.

Experimental procedures

Description of field sites and soil sampling

Soils were collected from three US Department of Energy FACE experiments and two OTC experiments located in different regions of the USA (Table 1). Detailed information on each site is available at http://public.ornl.gov/face/, and the individual FACE or OTC websites, accessible from this website. Field site locations, experimental designs and samples are summarized in Table 1, and discussed in more detail below. Soil chemistry and other characteristics are presented in Table S5.

Soil at the desert site is a calcareous aridisol. The vegetation is patchily distributed and dominated by *Larrea tridentate* (creosote bush), *Lycium* spp. and *Ambrosia dumosa.* Continuous CO_2 fumigation began on 28 April 1997 in three 25-m-diameter circular FACE rings (550 μmol mol^{-1}). Three similar rings were fumigated with ambient air (360 μmol mol^{-1} CO_2; Hamerlynck *et al.*, 2000). Due to the patchiness in the vegetation, samples were collected from ambient and elevated CO_2 rings in both the creosote bush root zone soil (referred to as 'creosote root zone') and biological soil crusts (referred to as 'crust') dominating the plant interspace (Belnap *et al.*, 2001). Each sample was a composite of soil collected from four compass points around the dripline of an individual creosote plant at a 0–5 cm depth (rock below), or was a composite of soil collected from four places within a 1 m^2 area in the plant interspace at a depth of 0–2 cm. In July 2007, three creosote root zone and three crust samples were collected in each ring, resulting in a total of six samples from each plot (36 samples in total across all six plots) (Table 1).

The aspen plantation site in Northern Wisconsin consists of six 30-m-diameter FACE rings that were planted with *Populus tremuloides* Michx. (trembling aspen), *Betula papyrifera* Marsh. (paper birch) and *Acer saccharum* Marsh. (sugar maple). The soil at the site is a Padus Sandy Loam. Factorial CO_2 and O_3 treatments were arranged in a randomized complete block design with three replicates. During the growing season, plots were fumigated with elevated (397.4 $\pm$ 14.7 ppm) or ambient CO_2 (84.0 $\pm$ 16.6 ppm) during daylight hours (http://public.ornl.gov/face/ASPEN_home.shtml, accessed June 2010). In July 2007, one composite soil sample was collected from the aspen community in each of three elevated CO_2 rings and three ambient CO_2 rings; each sample was a composite of five 2.5 $\times$ 5 cm cores collected at the 0–10 cm depth interval after removing the Oi horizon.

The *Pinus taeda* L. (loblolly pine) plantation was established in the Blackwood Division of the Duke Forest (North Carolina) in 1983 (McCarthy *et al.*, 2010). The soil is an acidic clay loam of the Enon series and described as being of moderately low fertility (McCarthy *et al.*, 2010). The six 30-m-diameter FACE rings were established in late August 1996; three rings have been fumigated with elevated CO_2 (571 ppm or about 200 ppm above ambient) and three rings have been fumigated with ambient CO_2 (McCarthy *et al.*, 2010). Ambient and elevated rings were quartered in 2005 and fertilization treatment in the form of pellet ammonium nitrate began in two of the quarters (11.2 g N m^{-2}; http://face.env.duke.edu/fertilization.cfm; accessed in June 2010). In June 2007, three 0–15 cm soil cores were randomly collected from the each of the quadrants (two fertilized and two unfertilized) in each elevated CO_2 and each ambient CO_2 ring. Triplicate 15 cm cores from each quadrant were composited to create one sample (four samples from each of the six rings). After DNA extraction of the 24 samples (below), equimolar quantities of DNA from each sample were combined for a given treatment in a plot, which resulted in 12-pooled DNA extracts from this site.

The scrub oak/palmetto site is located in the Merritt Island Wildlife Refuge near Cape Canaveral, Florida. The plant community is dominated by *Quercus myrtifolia* (76%), but

also contains *Quercus geminata* (15%), *Q. chapmannii*, *Serenoa repense* and *Lyonia ferreginea* (7%) (Dijkstra *et al*., 2002; Brown *et al*., 2007; Seiler *et al*., 2009). The soils are Poalo and Pomello Sand. Sixteen open-top chambers were set up and fumigation with elevated CO_2 (350 ppm above ambient or 2× ambient) began in May 1996. The octagonal chambers are 3.5 m in diameter and 2.5 m high (Dijkstra *et al*., 2002; Brown *et al*., 2007; Seiler *et al*., 2009). A total of six soil samples were used for the analyses presented here, three from ambient chambers and three from elevated chambers. Each sample is a composite of ten 10-cm-deep cores collected in July 2007.

The brackish tidal marsh soils were collected from an OTC experiment located in an infrequently flooded marsh on the Rhode Island River, a sub-estuary of the Chesapeake Bay. The marsh has a patchy distribution of plants, which are dominated by *Spartina patens* (Ait.) Muhl., *Scirpus olneyi* Grey, *Distichlis spicata* (L.) Greene, *Typha angustifolia* L. or *Iva fructescens* L. Open-top chambers sampled in this study were dominated by *S. olneyi,* and consisted of five replicate chambers fumigated with elevated CO_2 [686 ± 30 μl l^{-1} (s.d.)] and five replicate chambers fumigated with ambient CO_2 [350 ± 22 μl l^{-1}(s.d.)] from sunrise to sunset since 1986. The 10 plots were assigned in a randomized block design (Curtis *et al*., 1989). Each sample (1 per chamber or 10 total) collected in September 2008 was a composite of three 0–10 cm soil cores that were collected from different compass points within each chamber.

Soil DNA extractions and PCR amplification of cbhI

DNA was extracted from 0.5 g of each soil sample using the FastDNA SPIN Kit (MP Biomedicals, Solon, OH). A fragment encoding a 166- to 173-amino-acid-long fragment of the catalytic domain of *cbhI* was PCR-amplified from each soil DNA extract (in triplicate) in 50 μl of reactions containing the following: 1.6 μM final concentration of cbh1F (5′-ACC AA[C,T] TGC TA [C,T] ACI [A,G]G[C,T] AA-3′) and 1.6 μM final concentration cbh1R (5′-GC[C,T] TCC CAI AT[A,G] TCC ATC-3′) (Edwards *et al*., 2008), 1× PCR Buffer (Applied Biosystems, Foster City, CA), 0.8 mM dNTPs, 5 μg of Bovine Serum Albumin (Roche Diagnostics, Indianapolis, IN) and 2.5 U of AmpliTaq Polymerase LD (Applied Biosystems, Foster City, CA). Thermal cycling was carried out in an MJ research PTC-200 with the following cycles: initial denaturation at 95°C for 3 min, 35 cycles of 94°C for 1 min, 48°C for 45 s and 72°C for 1 min followed by a final extension at 72°C for 10 min. Triplicate PCR products from soil were pooled and visualized on 1.2% TBE agarose stained with ethidium bromide. Products were gel excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA). From each sample that was PCR-amplified, 192–384 clones were selected and bidirectionally sequenced using Sanger technology.

Sequence assembly and analysis

Bidirectional reads from soil DNA were assembled using Fincon (Los Alamos National Laboratory internal program, courtesy of Cliff Han). Short sequences (< 470 bp) and sequences containing ambiguous bases were removed from the data set. Introns were predicted and excised from the sequences using Genewise 2.2.0 (Birney *et al*., 2004) based on the Hidden Markov Model for glycosyl hydrolase family 7 (http://pfam.sanger.ac.uk/family?PF00840#tabview=tab5). 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 CLUSTAL X 2.0.10 and manually edited as necessary using ARB (Ludwig *et al*., 2004). Positions in the sequences where a gap occurred were filtered out (170 amino acid positions included in the analysis) prior to making distance matrices in ARB; distance matrices were then imported into Mothur (Schloss *et al*., 2009) and sequences were clustered into OTUs using default parameters and an OTU definition of 0.10. This OTU definition is based on OTU analyses conducted using *cbhI* sequences from named fungal cultures and sporocarps (see *Generating a* cbhI *database from named fungal cultures and sporocarps* below). Significant difference in replicate library composition was examined using hypothesis testing approaches (weighted and unweighted Unifrac and parsimony) in Mothur (Schloss *et al*., 2009). Rarefaction analysis was also completed for each replicate library in Mothur with a sampling frequency of one sequence. Prior to comparing *cbhI* richness across sites or treatments, all rarefaction analyses were normalized to the greatest common number of sequences present across libraries.

Hypothesis-testing approaches (weighted Unifrac and parsimony) were used to detect statistically significant differences among replicate libraries from each of the five distinct ecosystems or the plant and crust locations in the desert environment (each subsequently referred to as 'sites', $n = 6$), and among the libraries generated from control and treatment plots within each site. Cross-site differences were also examined using non-metric MDS analyses. MDS plots were generated based on the Jaccard and Bray-Curtis distance measures calculated between the OTU profiles for each soil sample. MDS plots were generated in R (version 2.11.1; http://www.r-project.org). Dendrograms based on the Bray-Curtis distance metric were generated in R to examine cross-treatment differences within each ecosystem or sub ecosystem environment. The 10 677-sequence data set was also clustered into OTUs with 114 *cbhI* sequences from named taxa in order to tentatively classify the OTUs. A BLASTP was also performed for all 10 677 sequences against a 114-member *cbhI* sequence database described in more detail below.

Generating a cbhI *database from named fungal cultures and sporocarps*

Fifty-three fungal cultures and sporocarps from validated culture or private collections were obtained to PCR-screen for the presence of the glycosyl hydrolase family 7 cellobiohydrolase I (*cbhI*) gene as described above. Taxonomic classification of the cultures and sporocarps screened were further verified at the genus level based on BLAST analysis of an ITS gene fragment. Seventy percent (37/53) of the cultures and sporocarps PCR-screened had the *cbhI* gene and 26% (14/53) had two to four sequence types, resulting in a total of 58

cbhI sequences (Table S1). These sequences combined with 49 sequences from NCBI and seven from Benjamin Wolfe (Harvard University) comprised our 114-member *cbhI* database.

DNA was extracted and purified from *c*. 1 mg of fungal tissue that had been bead-milled with CTAB extraction buffer (5% w/v CTAB, 350 mM NaCl, 16 mM NaH_2PO_4, 220 mM Na_2HPO_4), using a phenol:chloroform extraction followed by a sodium acetate-isopropanol precipitation and ethanol wash. The *cbhI* gene fragment was PCR-amplified from fungal DNA extracts as described above for the soil DNA extracts. The *cbhI* gene fragments were purified using the QIAquick PCR cleanup Kit (Qiagen, Valencia, CA). For each isolate, eight to 12 clones were sequenced and processed as described above for the soil sequences, prior to translation, alignment and phylogenetic analyses.

Phylogenetic analysis of aligned database sequences (inferred amino acids) was carried out using a neighbour-joining analysis and 1000 bootstrap replicates in PAUP version 4.0b (Sinauer Associates; Sunderland, MA). Positions containing missing and/or ambiguous characters were excluded from the analysis. The phylogenetic trees were rooted with an endoglucanase from *Aspergillus oryzae* within the glycosyl hydrolase family 7. The majority of the *cbhI* sequences from *Ascomycetes* clustered together and separately from the *Basidiomycetes*, albeit with low bootstrap support (< 70) and a few exceptions (Fig. 5), and provided a scaffold for classifying environmental sequences.

To determine an OTU definition for binning environmental *cbhI* sequences in a taxonomically cohesive fashion, a distance matrix generated in ARB, as described above, was used as input to Mothur (Schloss *et al*., 2009) to examine OTU binning at distances ranging from unique to 0.10. Multiple sequence copies that were present within single isolates of sporocarps did not always cluster together phylogenetically and prevented binning gene fragments at the species or genus level. However, when using an OTU definition of 0.10, almost all of the bins contained sequences from within the same fungal family and 0.10 was the definition chosen for binning the soil *cbhI* sequences.

Nucleotide accession numbers

The *cbhI* sequences from named fungal taxa obtained in this study and from soils of the five terrestrial ecosystems were deposited in GenBank under Accession No.: JF694940–JF694989 (*cbhI* from named fungal taxa), JF348774–JF355360 (desert), JF347798–JF348773 (aspen plantation), JF355361–JF357599 (loblolly pine plantation), JF346923–JF347311 (scrub oak/palmetto), JF347312–JF347797 (marsh).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The average abundance of the 15 most abundant OTUs (binned at a distance of 0.10) present in the replicate *cbhI* libraries for each of the six sites surveyed (± SE). Number of replicate libraries averaged = 17 (creosote root zone), 17 (crust), 6 (aspen plantation), 12 (loblolly pine plantation), 6 (scrub oak/palmetto) and 10 (marsh). Number above the bars are OTU identifiers assigned in Mothur. Note difference in scale on the *y*-axes.

Table S1. Fungal isolates and sporocarps PCR screened for the presence of the *cbhI* gene. Sources of cultures are as follows: [A], Andrea Porras-Alfaro, Western Illinois University, Macomb, IL; [F], Fusarium Research Center, Department of Plant Pathology, Pennsylvania State University, State College, PA; [C], Carolina Biological; [T], Terri Porter, McMaster University, Hamilton, Ontario, Canada; USDA, United States Department of Agriculture; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; IHEM, Belgian Co-ordinated collection of Microorganisms; NM, sporocarp collected in New Mexico; IL, sporocarp collected in Northern Illinois.

Table S2. OTU-based classification of soil sequences based on clustering with reference *cbhI* sequences from named taxa.

Table S3. Per cent composition based on phylum top BLAST hit of all 10 677 soil sequences.

Table S4. Phylum level classification (by top BLAST hit) of sequences that are unique to a given site.

Table S5. Soil properties in each of the treatment and control plots at the FACE and OTC sites surveyed in this study. Values represent an average of two replicates. Phosphorus (P) was $NaHCO_3$ extracted.

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