

Structure and resilience of fungal communities in Alaskan boreal forest soils¹

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Abstract: This paper outlines molecular analyses of soil fungi within the Bonanza Creek Long Term Ecological Research program. We examined community structure in three studies in mixed upland, black spruce (*Picea mariana* (Mill.) BSP), and white spruce (*Picea glauca* (Moench) Voss) forests and examined taxa involved in cellulose degradation at one upland site. We found that soil horizon was the factor by which fungal communities were most strongly structured and that predictable turnover in upland fungal species occurred through succession. Communities from consecutive summers were not significantly different, indicating that interannual variation was small in relation to differences between forest types and soil horizons, yet the community at a seasonal study site underwent significant changes within a year. In each study, mycorrhizal fungi dominated the community. Fungi rather than bacteria appeared to dominate [¹³C]cellulose degradation, with strongest growth in taxa that were not dominant members of the untreated community, including members of the genus *Sebacina*. Overall, our results point to considerable interannual resilience juxtaposed with narrow niche partitioning and the capacity of individual taxa in these hyperdiverse communities to respond strongly to resource inputs and changes in other abiotic environmental parameters such as temperature. Our data double the cumulative total of fungal sequences in GenBank and together achieve a better picture of fungal communities here than for any other ecosystem on earth at this time.

Résumé : Cet article décrit les analyses moléculaires effectuées sur les champignons du sol dans le cadre du programme de recherche à long terme en écologie de Bonanza Creek. Nous avons étudié la structure des communautés par le biais de trois études portant sur des forêts mixtes en milieu sec, d'épinette noire (*Picea mariana* (Mill.) BSP) et d'épinette blanche (*Picea glauca* (Moench) Voss) et nous avons étudié les taxons impliqués dans la dégradation de la cellulose dans une station sèche. Nous avons trouvé que l'horizon du sol était le facteur prépondérant dans la structuration des communautés fongiques et que le renouvellement prévisible des espèces fongiques en milieu sec se produisait par l'entremise de la succession. Les communautés présentes d'un été à l'autre n'étaient pas significativement différentes indiquant que la variation interannuelle était faible comparativement aux différences entre les types de forêt et les horizons du sol. Par contre, la communauté présente sur le site d'une étude saisonnière a subi des changements significatifs à l'intérieur d'une année. Dans chaque étude, les champignons mycorrhiziens dominaient la communauté. Les champignons plutôt que les bactéries semblaient dominer la dégradation de la [¹³C]cellulose; la plus forte croissance a été observée parmi les taxons qui n'étaient pas des membres dominants de la communauté non traitée, incluant des membres du genre *Sebacina*. Globalement, nos résultats font ressortir la grande résilience interannuelle juxtaposée à d'étroites niches écologiques ainsi que la capacité de taxons particuliers dans ces communautés hyper diversifiées de réagir fortement aux apports de ressources et à la variation d'autres paramètres environnementaux. Nos données viennent doubler le total cumulatif de séquences fongiques dans GenBank et ensemble donnent à ce jour une meilleure image des communautés fongiques ici que dans n'importe quel autre écosystème ailleurs dans le monde.

[Traduit par la Rédaction]

Introduction

Fungi play integral roles in terrestrial ecosystems and hence putatively influence the resilience of boreal systems in the midst of environmental changes such as global warming. Soil fungi impact net primary productivity through their

nourishment of plants as mycorrhizal symbionts. Because this symbiosis is supported by up to 20% of net primary productivity (Jakobsen and Rosendahl 1990; Erland et al. 1991), root symbiotic fungi also mediate soil respiration and carbon sequestration. Soil fungi are also the predominant decomposers in boreal systems where low pH, cold

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soils, and recalcitrant plant litter contribute to relatively slow turnover and the accumulation of a considerable soil organic carbon pool through succession. Through their roles as symbionts and decomposers, fungi play a central role in soil processes related to the turnover of nitrogen and phosphorus.

From numerous laboratory and microcosm studies, it is now clear that species of symbiotic and decomposer fungi vary widely in their traits of ecological interest, such as the range of polymers their enzymes are adapted to attack, edaphic preferences, hyphal foraging strategies, host specificity, and dispersal and root colonization strategies. These species-specific traits suggest that functional resilience should be tied to community structure, although the relationships may be complex (Botton et al. 2006). Chapin et al. (2010) provide the following definition “‘Resilience’ is the capacity of the system to sustain its fundamental function, structure, and feedbacks when confronted with perturbations such as unprecedented warming.” More specifically, ecological resilience is often measured by the amount of stress or change that can be absorbed before a system moves to a new state. Whether a system is considered to be resilient will depend on the system variable of interest, the temporal and spatial scales over which responses are measured, and the precision of the measurements. It is important to point out that community composition is but one system variable and that it can respond quite differently from functional variables such as the presence or rate of a particular biogeochemical process (Botton et al. 2006). In fact, dramatic changes in community composition may be required to maintain particular ecosystem functions in the face of perturbations.

We argue that an essential first step in elucidating the resilience of soil fungi, from both functional and community composition perspectives, is to understand how communities are structured in space and time and how composition changes in response to predictable drivers such as seasonality. Yet current progress in cataloging fungal diversity and community structure is barely on a par with characterization of plant communities in the 18th and 19th centuries (Sachs 2006). The difficulty in simply surveying fungal communities arises from their immense diversity and the small size and cryptic, belowground abode of fungal individuals. Molecular methods are capable of relatively precise taxonomic identification of fungi in the environment. However, due to the labor and expense of these methods, it has been impractical until recently to characterize fungal communities across spans of space and time that would appear trivial to a botanist.

Despite these constraints, over the last 6 years of the Bonanza Creek Long Term Ecological Research LTER program, we have achieved an initial characterization of the structure of fungal communities across plant successional stages in the uplands and across diverse black spruce (*Picea mariana* (Mill.) BSP) community types. We have also carried out detailed studies of seasonal dynamics at a single white spruce (*Picea glauca* (Moench) Voss) site. Our efforts have doubled the cumulative total of fungal data in GenBank and, at present, we have more information about fungal communities in Bonanza Creek than for any other ecosystem on earth. In this paper, we present a synthesis and integration of selected studies stemming from these fun-

gal data sets. Our primary focus will be on fungal communities in successional sequences (post-fire upland and black spruce lowland), seasonal dynamics (four seasons across 1 year), and interannual variability (summer across 2 years). We have also spent considerable effort phylogenetically identifying the fungi and characterizing spatial patchiness of these communities, but these studies will be considered only in passing here. While some progress has been made in recent years (Courty et al. 2005, 2006), little is known concerning resilience in the function of fungal communities from a species perspective and so will not be emphasized in this synthesis. Studies of microbial function in the boreal forest from a black box perspective, i.e., without consideration of microbial species, have been reviewed elsewhere (e.g., Schimel and Chapin 2006). However, exciting new molecular methods that can link species to functions are beginning to be employed at Bonanza Creek and will be described here in a section on cellulose degradation.

In the last decade, numerous studies have applied molecular methods to the identification of mycorrhizal fungi colonizing plant roots (see reviews in Dahlberg 2001; Horton and Bruns 2001; Anderson and Cairney 2007). Fewer, but noteworthy, studies have encompassed the total communities of fungi in soil (Schadt et al. 2003; O’Brien et al. 2005; Lynch and Thorn 2006; Lindahl et al. 2007; Buée et al. 2009). Studies of fungal communities of highest relevance to forest ecosystem resilience have dealt with several anthropogenic disturbances, especially logging, increased CO₂ concentrations, and nitrogen pollution. Other studies have investigated natural dynamics that may be altered by climate change, including temporal community shifts and recovery following fire. In such studies, it has often proven difficult to attribute differences in fungal community composition to the factor of interest simply because nearly every sample proved different from every other (Jonsson et al. 1999; Walker et al. 2008). In other words, the diversity and patchiness of fungal communities lead to tremendous intersample variance, making it difficult or impossible to detect statistically significant trends (Horton and Bruns 2001; Taylor 2002). Nevertheless, there is reasonable evidence that all of the drivers listed above cause shifts in fungal community composition in at least some studies and under certain conditions, e.g., fire (Smith et al. 2004), logging (reviewed in Jones et al. 2003), nitrogen deposition (Lilleskov et al. 2001), elevated CO₂ (Parrent et al. 2006), and soil moisture (Swaty et al. 2004). Several studies have addressed seasonal dynamics of fungal communities. The landmark study of Schadt et al. (2003) reported statistically significant shifts in the overall community in high alpine soils in comparisons of summer, winter, and spring. Several studies of communities on ectomycorrhizal root tips in other ecosystems have noted changes in the abundances of particular taxa but no statistically significant shifts in total communities across seasons (Walker et al. 2008). In contrast, Courty et al. (2008) found that certain dominant ectomycorrhizal symbionts of oak in northeastern France were present year-round, while several others were statistically more abundant in winter, spring, or summer.

There is an increasingly convincing body of evidence that individual fungal taxa respond differentially to changes in their environment, yet relatively few studies have docu-

mented convincing and reproducible changes in overall community structure, likely due to difficulties in achieving adequate sampling and replication. Furthermore, while responses to major disturbances such as stand-replacing fire have been shown with certainty, we have scant information on resilience of fungal communities with respect to milder, more frequent drivers such as seasonal temperature regimes or interannual climate variability.

Methods

Overview of studies

Successional and interannual studies

In 2003, we initiated a research program of extensive and intensive studies of soil fungal diversity in interior Alaska within Bonanza Creek LTER. Our primary tools were polymerase chain reaction (PCR), clone libraries, and high-throughput sequencing technologies. Beginning in 2004, at each sampling site and date, we collected a total of 50 soil cores at 10 m intervals arrayed along four parallel transects. Cores were 1.8 cm in diameter and collected to roughly 20 cm depth. The cores were returned to the laboratory in sterile plastic sleeves on wet ice and approximately 1 g subsamples of the Oi and A horizons (Soil Survey Division Staff 1993) excised from each core within 4 days of collection. These subsamples were pooled for each site by horizon. Soils were frozen at -80°C and then lyophilized.

We sampled each of the three replicate sites for the three major upland successional stages that have been established as core monitoring sites within the Bonanza Creek LTER. The early successional sites (UP1A, UP1B, and UP1C) are now roughly 23–25 years into post-fire recovery and are dominated by Alaska paper birch (*Betula neoalaskana* Sarg.) and trembling aspen (*Populus tremuloides* Michx.) with some understory white spruce. The midsuccessional sites (UP2A, UP2B, and UP2C) have a similar species composition (one is aspen dominated, one is birch dominated, and one is mixed), but these sites are 93–98 years post-fire. The mature sites (UP3A, UP3B, and UP3C) are dominated by large white spruce trees and have occasional birch and the stands are 225–230 years old. The 12 black spruce sites are spread over a larger geographical area. One site is within Bonanza Creek LTER proper, one is within the Caribou Poker Creek Research Watershed, and the others are located on the road system from Fairbanks to Delta Junction (~160 km southeast of Fairbanks). The black spruce sites encompass three representatives of each of four vegetation/edaphic categories: (1) dry/acidic, (2) dry/nonacidic, (3) wet/acidic, and (4) wet/nonacidic. Note that we use “dry” only in a relative sense; these sites are all wetter than the upland deciduous sites. Brief site descriptions are provided in Table 1. Detailed information about the black spruce sites can be found in Hollingsworth et al. (2006), while detailed information for the upland sites is available on the Bonanza Creek web site at www.lter.uaf.edu/.

Sites were sampled in two consecutive years to assess temporal stability of these fungal communities: 2004 was the driest year on record in interior Alaska and fires consumed greater acreage than ever recorded (Kasischke et al. 2010), while 2005 was also a dry year but more closely ap-

proximated a normal year (it had above-average precipitation in May and June but below-average precipitation in July and August; www.lter.uaf.edu/.) Hence, our study happened to coincide with unusual climatic conditions and probable drought stress.

Seasonal studies

We are also interested in understanding intraannual seasonal community dynamics in boreal forest fungal communities. Toward this goal, we conducted spatiotemporally intensive surveys focused on a single spruce stand on the North Campus area of the University of Alaska Fairbanks (I.C. Herriott, N. Lennon, C. Nusbaum, and D.L. Taylor, unpublished data). Beginning in 2004, we have maintained long-term plots and have sampled during fall, winter, spring, and summer at three discrete locations in the soil profile: Oi, Oa, and A (Soil Survey Division Staff 1993). Here, we outline results of the first year of this study, fall 2004 through summer 2005, and of a second study conducted in late spring 2007. We established this long-term seasonal site by setting up 20 replicate plots in a stratified, randomized spatial design in 100 m \times 100 m of a mixed white and black spruce forest in the North Campus area. Each plot consists of a snow exclusion subplot and adjacent control subplot. The purpose of the snow exclusion treatment subplots is to test for effects of changes in snow regime of relevance to climate change. These plots employ a small open-sided chamber onto which a roof is placed during the snow season. Automated data loggers at each subplot measure and record soil temperature. Soils were collected in fall (October), winter (February), spring (May), and summer (August). Replicate subplot samples were pooled and DNA was isolated from each soil pool, as described below. In the second study at this site, we conducted a comparison of the resident (DNA) versus active (RNA-based) community in a single humic sample collected in spring (May) (I.C. Herriott, J. McFarland, N. Lennon, C. Nusbaum, and D.L. Taylor, unpublished data).

Stable isotope probing of cellulose degradation

In a recent study, we applied stable isotope probing (SIP) to identify cellulose utilizers in organic horizon soil. SIP was performed by the addition of ^{13}C -labeled cellulose to soil microcosms constructed using organic horizon soil collected from a midsuccessional upland site (Bonanza Creek LTER UP2A), homogenized, wetted to 37% moisture, and incubated over a time course of 0, 7, 14, 21, and 28 days (hereafter T0, T7, T14, T21, and T28, respectively). $^{13}\text{CO}_2$ samples were analyzed to confirm [^{13}C]cellulose degradation using isotope ratio mass spectrometry after which the soil was harvested and DNA extracted. [^{13}C]DNA was separated from unlabeled DNA using density gradient centrifugation and then was subjected to microbial community fingerprinting, cloning, and sequence analyses to determine the taxonomic affiliation of microorganisms that derived carbon from cellulose (K.E. Stone, S. Runck, D. Valentine, D.L. Taylor, and M.B. Leigh, unpublished data). SIP results were compared with analyses of microbes colonizing birch tongue depressors buried in soils near the SIP sampling location for 1 year prior to analysis (Runck 2008; K.E. Stone, S. Runck,

Table 1. Sites, soil DNA extracts, clone libraries, and sequences.

Study, site description	Site codes	Latitude, longitude	Collection year(s)	Soil horizons	No. of soil cores	No. of DNAs	Clones*
White spruce, seasonal study, DNA	UAF		2004, 2005	Oi, Oa, A	160	20	9 216
White spruce, seasonal study, RNA and DNA	UAF		2007	Oa	1	2	4 224
Early-succession upland mixed forest	UP1A	64.73473541, -148.2976791	2004, 2005	Oi, A	300	12	18 048
	UP1B	64.73195762, -148.2974016					
	UP1C	64.73195762, -148.2974016					
Midsuccession upland mixed forest	UP2A	64.68945831, -148.3599027	2004, 2005	Oi, A	300	12	12 288
	UP2B	64.69390269, -148.3537914					
	UP2C	64.68862521, -148.3790685					
Late-succession upland mixed forest	UP3A	64.7666796, -148.2740661	2004, 2005	Oi, A	300	12	12 288
	UP3B	64.75973477, -148.2446238					
	UP3C	64.72445795, -148.324901					
Dry acidic black spruce	TKN0012	65.16721667, -147.8941333	2004, 2005	Oi, A	300	4	9 216
Dry acidic black spruce	TKN0122	63.90620584, -145.3711972	2004, 2005	Oi, A	300	4	9 216
Dry acidic black spruce	TKN0001	64.76572442, -148.295527	2004, 2005	Oi, A	300	4	9 216
Moist acidic black spruce	TKN0015	65.15451667, -147.8631667	2004, 2005	Oi, A	300	4	9 216
Moist acidic black spruce	TKN0022	64.99653333, -147.65305	2004, 2005	Oi, A	300	4	9 216
Moist acidic black spruce	TKN0109	65.16166012, -147.4878514	2004, 2005	Oi, A	300	4	9 216
Dry nonacidic black spruce	TKN0039	64.91003773, -147.8218001	2004, 2005	Oi, A	300	4	9 216
Dry nonacidic black spruce	TKN0123	63.92459496, -145.3167362	2004, 2005	Oi, A	300	4	9 216
Dry nonacidic black spruce	TKN0126	63.84655372, -145.7208714	2004, 2005	Oi, A	300	4	9 216
Moist nonacidic black spruce	TKN0051	64.86588323, -147.8736745	2004, 2005	Oi, A	300	4	9 216
Moist nonacidic black spruce	TKN0119	63.81379184, -144.9532331	2004, 2005	Oi, A	300	4	9 216
Moist nonacidic black spruce	TKN0040	64.9148493, -147.8297945	2004, 2005	Oi, A	300	4	9 216
Total					2160	90	16 2432

*This column shows the total number of colonies that were picked and sequenced. Many fewer sequences were used in the analyses due to our stringent quality checks.

D. Valentine, D.L. Taylor, and M.B. Leigh, unpublished data).

Molecular methods and bioinformatics

Our molecular methods have been described in detail elsewhere (Taylor et al. 2007, 2008; Geml et al. 2008, 2009). In brief, we have utilized PCR amplification of a fragment spanning the internal transcribed spacer (ITS) plus approximately 700 bp of the large subunit, cloning into the Invitrogen TOPO TA vector, and high-throughput robotic colony picking and sequencing. Cloning and sequencing efforts are summarized in Table 1. The RNA analyses utilized the ITS1 region via cDNA amplification with ITS1-F and ITS2* after first strand synthesis with random hexamers.

The molecular methods applied to SIP studies will be detailed elsewhere (K.E. Stone, S. Runck, D. Valentine, D.L. Taylor, and M.B. Leigh, unpublished data). Briefly, [¹³C]DNA was isolated using isopycnic centrifugation. Gradients were fractionated and then fractions containing [¹³C]DNA were identified based on quantitation of bacterial 16S rRNA genes and fungal ITS in each fraction using quantitative PCR (q-PCR) by identifying high-density fractions with elevated quantities of DNA relative to equivalent gradient fractions from T0. Microbial community analyses were performed on [¹³C]DNA (T0–T28), and total soil community and birch tongue depressor DNA using fungal automated ribosomal intergenic spacer analyses (F-ARISA), bacterial terminal-restriction fragment length polymorphism (T-RFLP), and clone library (16S rRNA and ITS) sequence analyses.

Our approaches to sequence bioinformatics have also been described in detail elsewhere. In brief, we mask low-quality base calls and correct clone orientations using in-house Perl scripts, isolate the ITS region of each clone, and then group sequences into operational taxonomic units (OTUs) at 97% sequence identity using Cap3 (Huang and Madan 1999).

In the SIP study, fungal sequences were analyzed as described above and bacterial sequences were analyzed using Ribosomal Database Project (RDP) II (rdp.cme.msu.edu/), including base calling with PHRED (cutoff of 20) and quality trimming by LUCY via the RDP pipeline followed by taxonomic identification using RDP Classifier and Sequence Match functions. F-ARISA peaks were identified by performing F-ARISA on clones that had been sequenced and taxonomically identified. Peaks were identified for T-RFLP through *in silico* digestion of 16S rRNA clone library sequences.

Statistical analyses

To visualize the variation in fungal community composition across sites and years, we carried out ordination using nonmetric multidimensional scaling (NMS) with the quantitative version of Sorenson similarity (= Bray–Curtis index) in PC-Ord 5.0 (McCune and Grace 2002). Horizon and successional stage were included in a site environmental factor matrix as categorical variables (0 = early succession UP1 sites, 1 = midsuccession UP2 sites, and 3 = late-succession UP3 sites), while tree abundances (Alaska paper birch, trembling aspen, and black and white spruce) were described on simple ordinal scales (0 = 0–10 trees per 300 m², 1 = moderate densities of 11–60 trees per 300 m², and 2 = 60+ trees

per 300 m²). All trees above 0.3 diameter at breast height were included in these counts; the data were downloaded from the Bonanza Creel LTER server at www.lter.uaf.edu/data_detail.cfm?datafile_pkey=320. Following NMS ordination of sites, we examined the Pearson correlation values between the primary community ordination axes and these environmental variables. We also tested whether fungal communities across years and habitat types were statistically different using the multiple response permutation procedure (MRPP) and examined the responses of individual species to environmental gradients using indicator species analyses, also in PC-Ord. For the indicator species analyses, the ordinal variables described above were reduced to categorical presence–absence groupings. In all of these analyses, OTUs occurring in fewer than two sites were excluded and abundances were relativized to the maximum number of clones for all sites. In vegetation studies, it is more common to normalize by species sum totals across sites rather than site totals. We felt that normalizing by site totals is preferable in our case because variation in total numbers of clones across libraries is driven largely by laboratory processes. We first conducted analyses with samples separated by year (2004 and 2005) and horizon (organic and mineral) for all sites and separately for upland and black spruce sites. Because MRPP indicated that fungal communities did not differ significantly by year, the OTU abundances for the two years for a particular site were combined in subsequent ordinations.

In addition to the MRPP analyses, which test for structuring of the entire fungal communities across all samples, we also examined the degree of variation in communities in the same site across years and among replicates of the same successional stage. These analyses are of particular relevance to interannual variability and resilience of fungal communities in soil. Again, Bray–Curtis dissimilarity indices from the species–site matrix from which species occurring in fewer than two samples and relativized to site totals were used. For ANOVA and regression of these dissimilarities, which range from 0 to 1, a logit transformation was applied to the Bray–Curtis values. We tested whether year to year dissimilarity within a site was related to soil horizon, successional stage (UP sites only), soil moisture class, and in situ pH, sodium, magnesium, and calcium contents (black spruce sites only). Nearly identical results were obtained with nonparametric Wilcoxon rank tests of untransformed Bray–Curtis dissimilarities (data not shown).

Results and discussion

Fungal diversity in 0.25 g of soil

Given the expansive goals of our project, we have conducted pilot studies aimed at quantifying sampling issues with regard to total diversity and its spatial structure in relation to sampling intensity. We find that, with current Sanger sequencing technologies, it is practically impossible to saturate fungal diversity in even miniscule samples of soil. For example, we obtained over 5000 high-quality sequences from a 0.25 g soil sample from a floodplain black spruce site and found 218 species, or OTUs (M.G. Booth, N. Lennon, C. Nusbaum, and D.L. Taylor, unpublished data) (see Molecular methods and bioinformatics section above).

Many of these OTUs occurred only once (singletons) and the rarefaction curve had not reached an asymptote, indicating that we failed to enumerate all of the species in this small sample (data not shown). Similar effort was applied to a second 0.25 g sample collected approximately 1 m from the first sample. The diversity characteristics were nearly identical to the first. Astonishingly, however, the two samples shared fewer than 14% of their combined OTUs, and even the dominant taxa differed starkly. Despite this study's demonstration of our inability to saturate fungal diversity using clone library sequencing due to extreme species richness and small-scale spatial patchiness of fungal communities, we have nevertheless been able to detect strong patterns in fungal community structure at larger spatial scales, i.e., at the site level.

Despite the high diversity and patchiness at the metre scale, we contend that only by constructing a detailed and accurate baseline picture of regional fungal community structure can we hope to assess impacts of climate change and other potential drivers. Therefore, we have allocated most of our sequencing capacity to spatially extensive characterization of fungal community composition among the core upland sites of Bonanza Creek LTER and among four black spruce vegetation types found widely in interior Alaska. In these two studies, our primary objective was to generate comprehensive site-level characterizations of the soil fungal communities in consecutive years and thereby quantify the degree of interannual variability as a first step in exploring community resilience. Toward this end, we collected relatively large numbers of soil cores at each site, which were then pooled within sites and subjected to high-throughput sequencing.

Successional and interannual studies

We have analyzed the black spruce (TKN sites) and uplands (UP sites) data sets separately, in detail, elsewhere. Here, we provide combined analyses for the first time. In total, this data set encompasses 52 875 clones after all of the quality filtering steps. These clones were partitioned into 3570 OTUs at 97% ITS sequence identity by Cap3. However, a great many of these OTUs are singletons, a high proportion of which may be of chimeric or other artifactual origin. Although of importance in diversity estimates, taxa that occur in only one sample have no impact on species-level community ordination and are not considered further here. There were 1578 OTUs following removal of singletons; 712 OTUs occurred in two or more of the black spruce samples and 763 OTUs occurred in two or more of the upland samples. This compares with a vascular plus nonvascular plant flora of 133 species in these black spruce sites and roughly 150 species in the upland sites (Hollingsworth et al. 2006) (www.lter.uaf.edu/). While some fungal taxa may have only been present as spores, and thus not been active members of the community, we feel that the contribution of spores to our results was minimal due to the extreme differences in community composition from site to site and between soil horizons discussed below.

A few taxa occurred in all three major stand types (black spruce, white spruce, and deciduous), such as *Cortinarius* cf. *sani*. However, the majority of taxa showed preferences for particular host trees or the associated habitats, such as

Hygrophorus olivaceoalbus, which was one of the most abundant taxa in black spruce sites but was essentially absent from other sites. Strikingly, the vast majority of clones belonged to mycorrhizal taxa. Of the top 30 OTUs shown in Fig. 1, only five are not mycorrhizal (*Clitocybe*, *Urnula*, two species of *Candida*, and *Botryosphaeria*). Most of the root-associated taxa form ectomycorrhizae, but there is also strong representation of ericoid taxa, although only a single arbuscular mycorrhizal species, *Paraglomus occultum*. This strong representation cannot be explained by any methodological bias favoring ectomycorrhizal Basidiomycota, as many of the most abundant taxa are Ascomycota (e.g., *Phialophora*, *Trichophaea*, *Candida*, and *Phialocephala*), and our primers have been extensively vetted (Taylor et al. 2008). Although we did not sample the most recent coarse litter and coarse woody debris, which are likely dominated by saprotrophic taxa (Lindahl et al. 2007), it is still surprising that our Oi horizon samples, containing recognizable plant material, were dominated by mycorrhizal taxa.

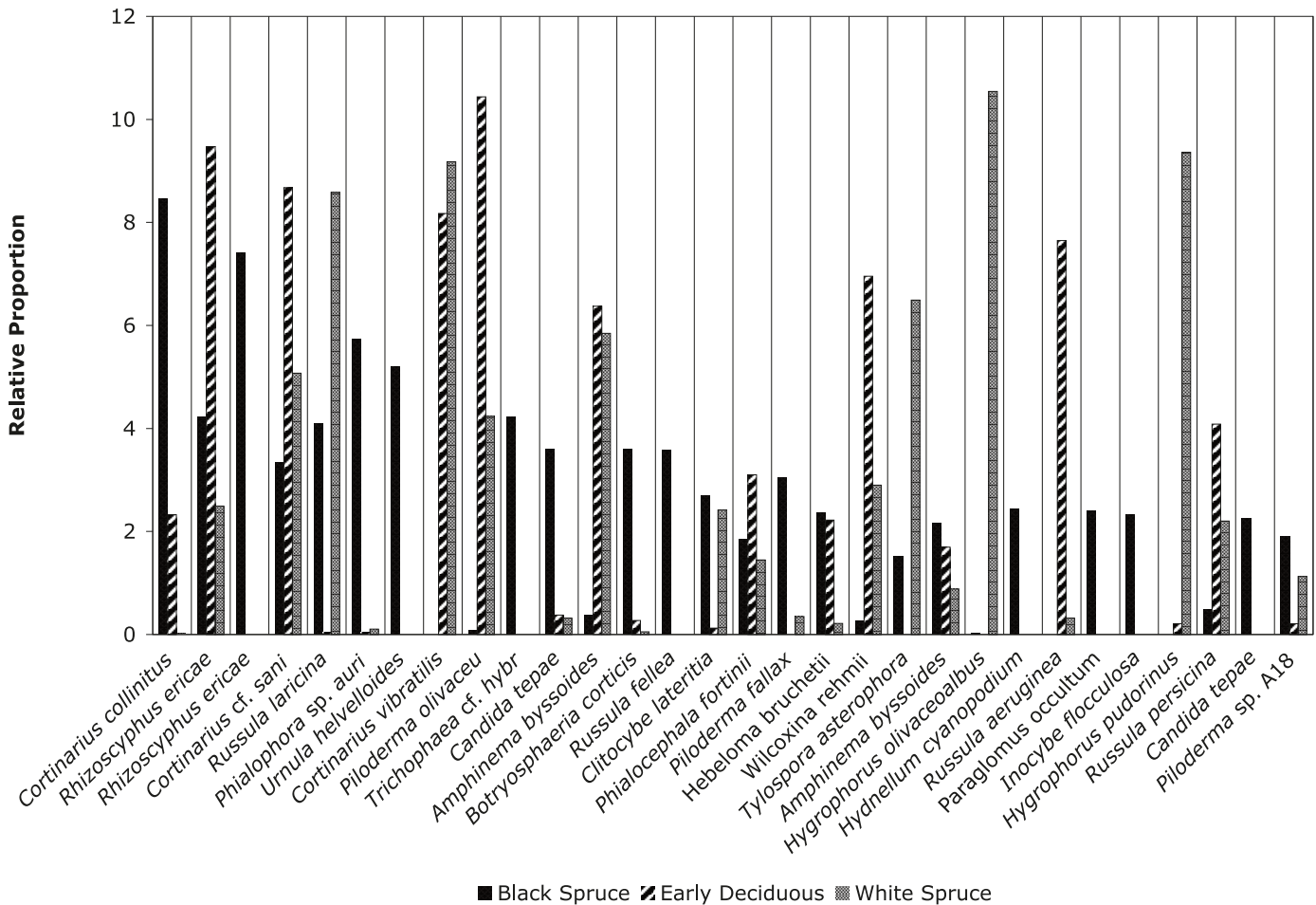
In general, NMS appeared to perform well in the ordination analyses. All ordinations had final instabilities below 0.0001 and the first two or three axes accounted for between 69.6% and 96.0% of the variation. In every case but one, a three-dimensional solution was chosen by PC-Ord based on maximal reduction in stress with the fewest axes.

MRPP tests showed that the fungal communities in 2004 were not statistically different from those in 2005 across all sites (effect size $A = -0.000678$, probability $p = 0.575$) (Fig. 2). We combined OTU counts across years in subsequent ordinations, since communities did not differ statistically between years. In contrast, the communities were significantly different when comparing organic with mineral horizons ($A = 0.0307$, $p < 0.00000001$) and when comparing upland with black spruce sites ($A = 0.0629$, $p < 0.00000001$). These patterns are also clearly evident in the NMS ordination plots, which show a strong distinction between the UP and black spruce plots and clustering of samples of the same horizon within these larger UP versus black spruce groupings (Fig. 3).

Separate ordinations of the black spruce and upland data sets again highlighted the distinctiveness of fungal communities between organic and mineral horizons. The ordination of the upland sites indicated that successional stage was strongly correlated with turnover in fungal community composition (Fig. 4), while the intercorrelated abundances of tree species were also significant (Pearson correlations: successional stage axis 1 $r^2 = 0.486$, axis 2 $r^2 = 0.450$; Alaska paper birch axis 2 $r^2 = 0.207$; trembling aspen axis 1 $r^2 = 0.286$; white spruce axis 1 $r^2 = 0.343$, axis 2 $r^2 = 0.502$). Similar results have been reported (Palfner et al. 2005; Twieg et al. 2007).

Because we resampled the identical sites in 2004 and 2005, we were able to test whether the community of each site shifted from year to year. Year 2004 was extremely dry, while 2005 was moderately dry. Inspection of the NMS ordination with samples from the two years kept separate clearly shows that some sites occupied nearly identical positions in ordination space, while some shifted considerably (Fig. 2). For the black spruce sites, there were no clear relationships between the degree to which a site shifted from 2004 to 2005 and soil moisture class, in situ pH, and ammo-

Fig. 1. Proportional rank abundances of the 30 most abundant OTUs by stand type. Early-successional deciduous sites are the upland UP1 and UP2 sites, white spruce (*Picea glauca*) are the upland UP3 sites, and the black spruce (*Picea mariana*) sites are mostly lowland. Numbers of clones for OTUs were normalized by the total numbers of clones recovered from each stand type. The OTUs are named by the top identified BLAST hit in GenBank; note that some of the OTU sequences are rather divergent from their best match in GenBank and so likely belong to different species or genera than those listed.



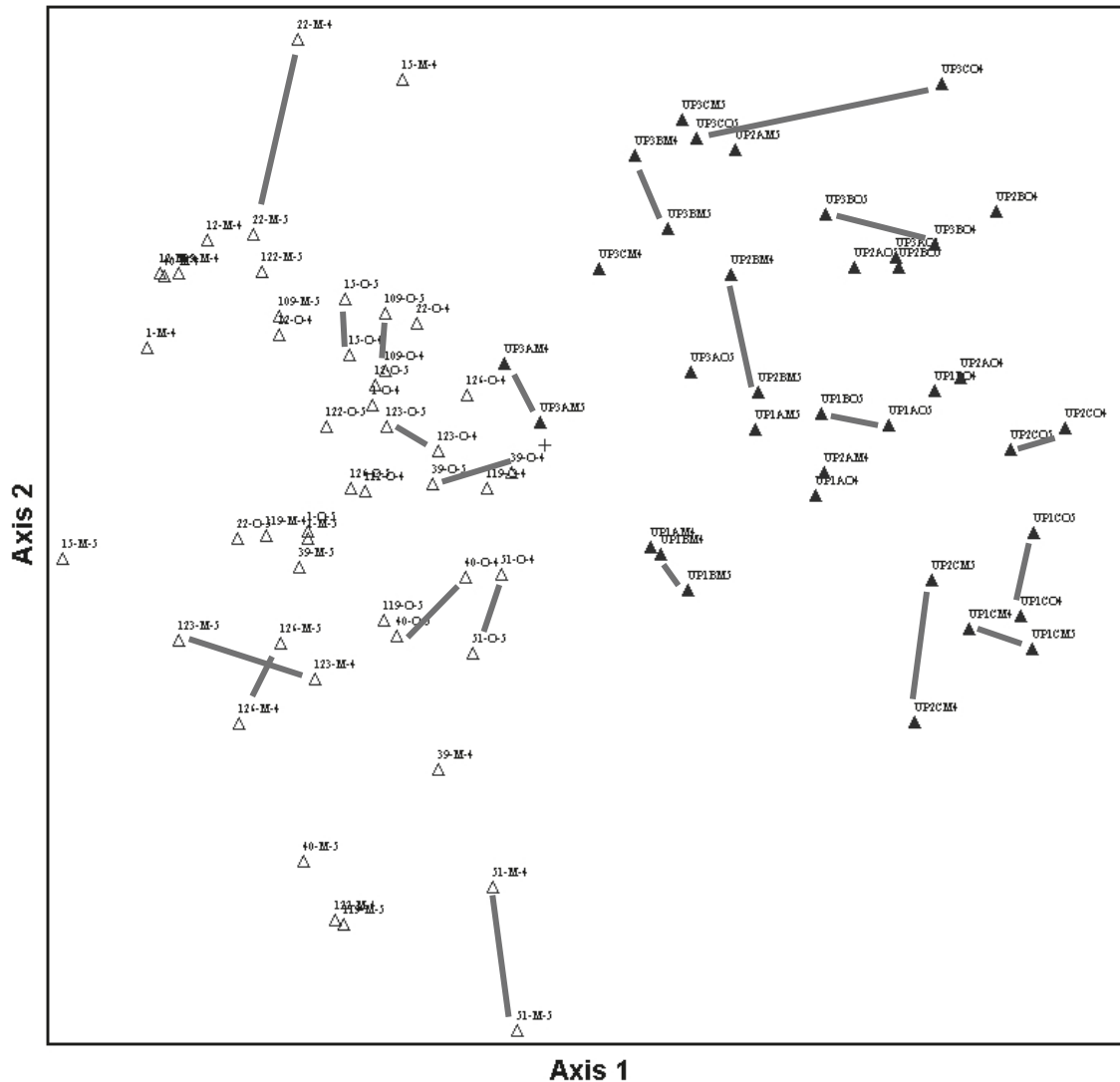
nium, calcium, magnesium, or sodium contents (for details on these measurements, see Hollingsworth et al. 2006). However, the mineral horizon varied between the two years more than did the organic horizon (Student's $t = 2.07$, $\alpha = 0.050$). For the upland sites, horizons did not differ in their year to year variation. We also investigated the similarities of replicate sites within a successional stage. The early-stage sites (UP1s) were most variable, while the late-stage sites (UP3s) were least variable, and the differences were significant (ANOVA: $F = 4.17$, $p = 0.036$) (Figs. 2 and 5).

We expect that environmental factors such as soil moisture may well influence the degree of interannual variability in fungal communities but we were unable to detect such patterns due to the limited number of sites and years examined. Even though we sampled the same sites in 2004 and 2005, 50 soil cores is far from sufficient to fully characterize the sites, and we did not attempt to collect each 2005 core within a few metres of a corresponding 2004 core, as would be required to have a reasonable likelihood of resampling the same fungal genet (e.g., Redecker et al. 2001). Detection of environmental drivers that are more subtle than soil horizon or dominant tree species will require enormous sam-

pling and sequencing efforts. Our intensive clone sequencing has failed to saturate diversity within any of the sites studied despite our concentration on very few sites (data not shown). Again, the massive diversity and spatial patchiness of fungal communities in soil are likely responsible for the limitations on the inferences that can be drawn.

The upland and black spruce studies illustrate considerable interannual stability in fungal community composition at the plot level in the sense that year was not a significant factor in MRPP analyses and that the same site often occupied similar positions in ordination space in consecutive years. This stability is somewhat unexpected given the unusually dry conditions in both sampling years and the likely contribution of spatial patchiness to variation when attempting to resample the same fungal community. On the other hand, the actual Sorenson dissimilarity values for the same site in consecutive years were quite high, ranging from 0.47 to 0.94, indicating that there were considerable differences in the identities and abundances of fungi across years (data not shown). The close positions in ordination space result from the fact that other sites were even more dissimilar in species composition. Because we pooled soils from 50 cores

Fig. 2. NMS ordination of upland and black spruce (*Picea mariana*) sites. Lines connect samples from the same site and soil horizon in successive years (2004–2005), demonstrating the general consistency of communities at a site across years. For clarity, only a subset of year to year connections are shown. Upland sites are shown with solid triangles and black spruce sites are depicted with open triangles.



per plot, we cannot evaluate the degree to which patchiness and (or) migration of species across the site were at play. In their study of ectomycorrhizal root tips in the Sierra Nevada mountains, Izzo et al. (2005) found that plots were relatively stable from year to year, with similar patterns in ordination space as ours, but that some of the dominant species occupied different locations within the plot in different years. The issues of sampling intensity and spatial scale deserve even greater scrutiny in future studies.

In our upland study, we found that replicate successional stands were most distinct from each other in early succession and increasingly similar as the communities reached the mixed white spruce climax stage. This observation fits the predictions of resilience theory wherein a major disturbance resets species organization and permits new and variable combinations (Holling and Gunderson 2002). However, there are many potential state factors that may vary more among these particular early-successional sites than among the late-successional sites, resulting in the observed patterns

in fungal communities. It will be exciting to learn in additional studies whether convergence of fungal communities through succession is a general phenomenon.

The foremost aspect of fungal community structure that is so clear as to be unassailable in all three studies described here is the preference of particular fungal species for different soil horizons. Such preferences have received increasing support recently (Dickie et al. 2002; Lindahl et al. 2007). Of interest with respect to fungal function and resilience are the observations in our studies that the horizon preferences are neither guild specific nor lineage specific. For example, several ectomycorrhizal *Cortinari* species are among the most abundant taxa in our studies, and some have strong preferences for the organic horizon, while others have strong preferences for the mineral horizon. The Russulaceae is also ectomycorrhizal, and several taxa in this family were also dominants in our studies, yet most of these were found in similar abundances in both horizons (Geml et al. 2009). These results illustrate the key point that soil-dwelling fungi

Fig. 3. NMS ordination of upland and black spruce (*Picea mariana*) sites with abundances across years combined. Note the strong split between upland and black spruce sites and further subdivision between organic and mineral communities. Upland sites are shown with solid triangles and black spruce sites are depicted with open triangles. Overlays have been manually added.

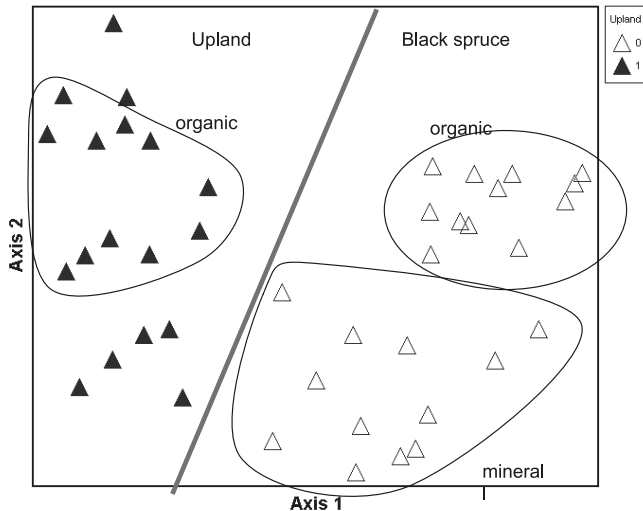
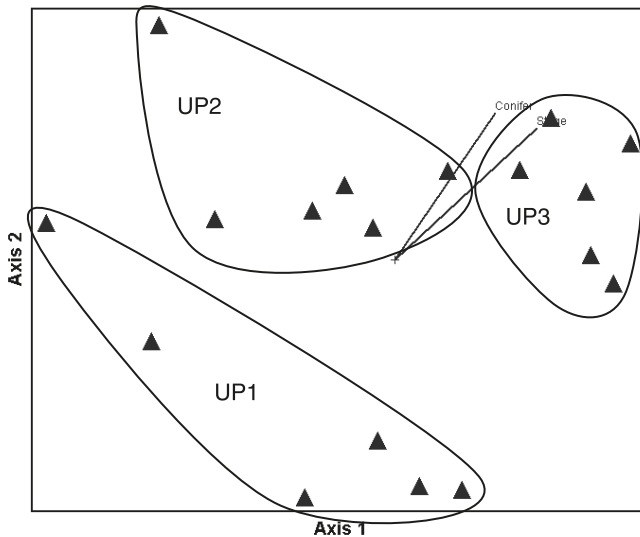


Fig. 4. NMS ordination of upland sites with years combined. Note strong clustering of replicate sites within each successional stage and greater intersite variation in the earliest UP1 sites. Vectors are shown for environmental variables correlated with ordination axes at $r^2 > 0.300$.

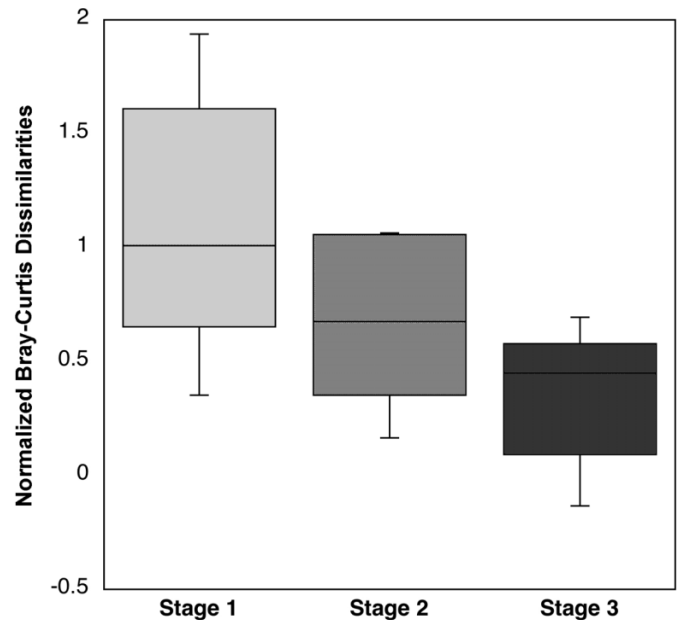


display considerable niche partitioning within functional guilds. Thus, depending on the functional question being asked, analyses at the level of guild (e.g., ectomycorrhizal, ericoid mycorrhizal, and saprotrophic) may not provide satisfactory answers.

Seasonal studies

DNA was isolated from all sample pools collected from our intraannual seasonal study site. Of the 9216 clones of fungal ITS gene sequenced, 7622 passed stringent quality control filters and were grouped into 211 nonsingleton

Fig. 5. Box plot of intersite Bray–Curtis dissimilarities by successional stage. Fungal abundances were normalized by site totals and the resulting pairwise Bray–Curtis dissimilarity values between replicate sites of a given successional stage were logit transformed because the raw index ranges from 0 to 1.



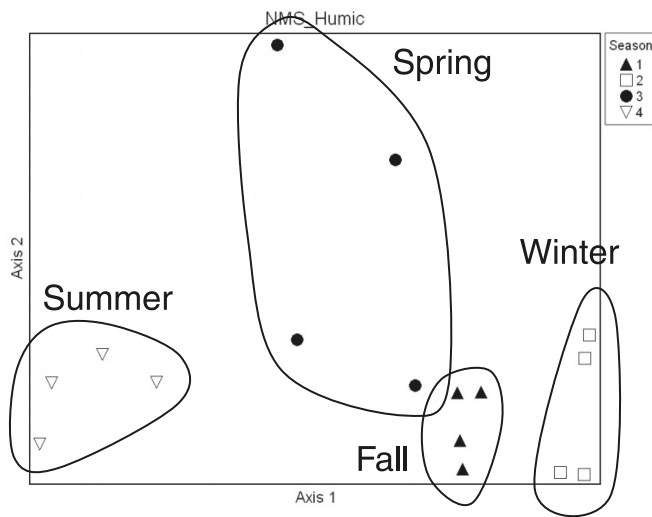
OTUs at 97% sequence similarity, as described above. The effect of our snow exclusion treatment resulted in significantly altered soil thermal regime and significantly retarded soil thaw in the months following snowmelt (data not shown).

The NMS ordination conducted on the entire data set of 211 OTUs showed two major gradients containing much of the variation inherent in the data set and almost completely distinct groupings of datapoints that correspond to horizon of origin (ordination not shown). Thus, as with the upland and black spruce sites described above, which were sampled in summer only, soil horizon appears to be the overall strongest factor influencing community structure in the white spruce, seasonal site. However, NMS conducted on individual data sets separated by horizon shows structuring of samples according to season within the humic (Oi) horizon (Fig. 6) and in the mineral (Oe) horizon (not shown).

In agreement with the NMS ordinations, MRPP analyses of the seasonal data set suggest that overall, the communities at this site were most strongly structured by soil horizon, moderately structured by season, and weakly but significantly affected by the exclusion of winter snowpack (not shown). On data sets divided by horizons, additional MRPP runs suggested that the organic horizon community did not change significantly across seasons, while the humic and mineral horizons did change significantly, with the former displaying the strongest seasonality. Winter and spring communities were most similar, while the summer community differed most from the others.

We also highlighted through indicator species analysis the OTUs whose variable abundance across samples contributes significantly to differences in community composition across

Fig. 6. NMS ordination plot from the seasonal study. The first two dimensions are plotted. Datapoints represent the communities of OTUs derived from the humic horizon across seasons and snow conditions.



soil horizon and season (Table 2) and snow condition (not shown). A few of the highly abundant OTUs belonging to, e.g., *Tricholoma* sp., *Piloderma* sp., and *Cortinarius* sp., were implicated in multiple tests. Interestingly, some of the same OTUs implicated in one horizon as significantly indicative of a particular season were implicated in another horizon in a different season. Overall, the abundant OTUs with significantly variable abundances could be viewed as potentially influential species that are adapted to certain spatio-temporal conditions. On the other hand, those species of relatively high overall abundance across space, time, and treatment that did not exhibit significant seasonality, horizon preference, or snow condition preference could be considered candidates for characterization as resilient members of the community, able to withstand extremes of conditions imposed by seasonal and climatic conditions. Whether the less frequent species exhibit strong seasonality is not apparent from this study, as statistical power suffers by virtue of their proportional scarcity.

The single humic soil sample collected in spring 2007 harbored 30 nonsingleton fungal OTUs across both DNA and RNA communities. The DNA library contained 29 OTUs. The 16 OTUs in the RNA library were a subset of those in the DNA library, except for one OTU at very low abundance (0.6%), which was not found in the DNA library. The estimated richness and diversity of the DNA library were also significantly greater than those of the RNA library (data not shown). In the DNA library, *Tricholoma* sp. was by far the most dominant OTU, in agreement with its status as the overall most abundant OTU in the DNA-only seasonal study conducted 2 years prior at the site. This OTU was also a significant indicator species in all significant MRPP groups in that study. In contrast, although the *Tricholoma* sp. did occur in the RNA library, *Cenococcum geophilum* was the most abundant OTU in the RNA library. *Cenococcum geophilum* was rare (0.1%) in the corresponding DNA library and of relatively low abundance overall in

the first study (rank abundance = 66), yet was a significant indicator species for season among the 95 humic soil OTUs.

Importantly, our small spatial scale RNA study shows that abundance via a DNA assessment does not necessarily correlate with an RNA assessment in terms of proportional representation of species. This is in concordance with previous similar approaches on various microbial communities (Moeseneder et al. 2005). Some species appear in large abundance in the DNA community but in small abundance in the RNA community. Notably, *Tricholoma* sp. and *C. geophilum* dominated DNA and RNA communities, respectively. Both of these are ectomycorrhizal species, and the recovery and demonstration of this proxy for metabolic activity at near freezing conditions for mycorrhizal species are noteworthy.

Stable isotope probing of cellulose degradation

The SIP study revealed a wealth of information about the identities of bacteria and fungi present in the organic horizon at a single midsuccessional upland site (UP2A) involved in either the direct or indirect utilization of cellulose. Our q-PCR results of density gradient fractions suggest that fungi dominated carbon acquisition from cellulose, with a high proportion of fungal DNA becoming highly ^{13}C labeled. Meanwhile, bacterial DNA was less heavily labeled, suggesting that these bacteria (known cellulose degrader *Cellvibrio*, as well as unclassified Rhizobiales, Sphingobacteriales, and Actinobacteria) may be generalists deriving carbon from a variety of sources in addition to cellulose. The fungi *Sebacina*, *Geopyxis*, and *Geomyces* were frequently detected in [^{13}C]DNA clone libraries T14 and T28 but were not found colonizing birch tongue depressors. Interestingly, the *Sebacina* species belongs to the putatively ectomycorrhizal clade A of Weiss et al. (2004). However, this family also includes known saprophytes such as *Sebacina incrustans* and taxa with uncertain, complicated trophic niches (Deshmukh et al. 2006), illustrating the complicated task of assigning functional significance to certain fungi. The most abundant fungal taxa found in the birch tongue depressor clone library were *Gloeopeniophorella*, *Xylaria*, and *Phaeococcomyces*. The first two of these are well-known wood decomposers. The most frequent fungal clones from the total community clone library were *Hygrophorus*, *Phaeococcomyces*, and *Geomyces vinaceus*. *Hygrophorus* species are known to be ectomycorrhizal (Visser 1995), which may explain why they did not appear in our SIP microcosms. *Phaeococcomyces* have been found in soil and associated with plant material (de Hoog et al. 1995). *Geomyces vinaceus* was also detected using SIP and is widely recovered from soil, air, and other diverse environments (Gianni et al. 2003). None of the taxa recovered from birch tongue depressors or as labeled cellulose degraders were abundant in the broad-scale soil clone libraries from UP2A described above (Fig. 2). This contrast suggests that rare members of the total fungal community are nevertheless important, as they may be able to respond rapidly to new resources or novel conditions to which they are well adapted. In future studies, it would be valuable to identify cellulose utilizers in the surface litter layer, which is likely to be a highly active zone of cellulose degradation.

Table 2. Indicator species status among the 10 most frequent OTUs in the seasonal study.

Indicator species BLAST ID	Rank abundance	Indicator of:	Ecology	Subphylum	Family
Seasonal indicator species in the humic horizon					
<i>Tricholoma orirubens</i>	1	Winter	Ectomycorrhizal	Agaricomycotina	Tricholomataceae
<i>Piloderma lanatum</i>	4	Spring	Ectomycorrhizal	Agaricomycotina	Atheliaceae
<i>Orbilbia auricolor</i>	6	Summer	Saprotrophic	Pezizomycotina	Orbiliaceae
<i>Piloderma lanatum</i>	7	Summer	Ectomycorrhizal	Agaricomycotina	Atheliaceae
<i>Cortinarius erythrinus</i>	8	Spring	Ectomycorrhizal	Agaricomycotina	Cortinariaceae
Seasonal indicator species in the mineral horizon					
<i>Tricholoma orirubens</i>	1	Summer	Ectomycorrhizal	Agaricomycotina	Tricholomataceae
<i>Piloderma lanatum</i>	4	Summer	Ectomycorrhizal	Agaricomycotina	Atheliaceae
<i>Cortinarius erythrinus</i>	8	Summer	Ectomycorrhizal	Agaricomycotina	Cortinariaceae
<i>Orbilbia auricolor</i>	10	Spring	Saprotrophic	Pezizomycotina	Orbiliaceae

Note: OTUs are listed whose variable abundance across samples contributes significantly to differences in community composition across seasons. Identity, abundance, taxonomy, ecology, and environment of significant indicator value are shown.

Conclusions

Fungal diversity in Alaskan boreal forest soil is much greater than might be expected based on simple extrapolations from plant diversity. We found over 1500 fungal OTUs in a handful of sites occupied by only four tree species: white spruce, black spruce, Alaska paper birch, and trembling aspen. The understory vegetation is also simple, with fewer than 200 vascular species across these sites. Rarefaction curves (not shown) indicate that we would find many more fungal species were we to sequence more clones, to collect more soil cores, or to visit more sites. Although not discussed in detail here, we have amassed evidence at some sites for extreme patchiness of fungal abundance and species composition at the metre scale, as has been noted in many other studies (Lilleskov et al. 2004; Koide et al. 2005; Genney et al. 2006; Lindahl et al. 2007). Undersampling, patchiness, and extreme diversity each poses a challenge to detecting correlations between fungal community composition and environmental factors that may be the drivers. Nevertheless, our large sampling and sequencing efforts have yielded data sets that make certain trends conspicuous and suggest other weaker patterns.

In every site that we have studied, the fungal communities differ strongly and predictably between soil horizons. Some species appear to be horizon generalists, while others appear to be horizon specialists. The distinction between generalists and specialists does not fall out along groupings by ecological guild (e.g., the ectomycorrhizal guild). For example, several *Cortinarius* species are found nearly exclusively in the organic horizon and others primarily in the mineral horizon, although from the seasonal study, it appears that this genus has niches on temporal axes as well. Several *Russula* species are equally represented in these horizons. Both genera are ectomycorrhizal, and a number of species in these genera are among the 30 most abundant OTUs in this study overall.

We find similar trends with respect to season. Overall, there are significant changes in fungal community structure through the seasons, but these trends differ among soil horizons. Furthermore, some species respond strongly to season, while others appear to be much less variable throughout the year. Again, we find ectomycorrhizal taxa that represent both of these extremes. Despite these seasonal shifts, it appears that the fungal communities of the upland and black

spruce sites are remarkably stable from year to year. In every comparison, year did not have a significant effect on community composition. However, it should be noted that both years in which the upland and black spruce sites were sampled were drier than usual, which may have driven the communities toward a drought-tolerant subset. Furthermore, several consecutive years of sampling, with careful consideration of spatial patchiness, are required to adequately assess the interannual stability of fungal communities.

Our results add to a growing body of evidence suggesting strong niche partitioning among soil fungi along a variety of environmental axes. Niche diversification can occur at very fine phylogenetic scales, e.g., different species of *Cortinarius* that are specialized to different soil horizons and (or) seasons, and is not restricted to a particular ecological guild such as saprotrophs. Relating all of this diversity to function will be a gargantuan but exciting and worthwhile undertaking.

The blessing and curse of microbial ecology has been the large degree to which advances have been technologically driven. The high diversity and spatial patchiness of fungal communities demonstrated in our studies and many others suggest that this technological throne will continue its ascendancy for some time to come. While our efforts to characterize fungal diversity in Bonanza Creek with respect to space and time are of an unprecedented scale, we cannot yet call our survey exhaustive. Enumerating every species may not be attainable, or particularly enlightening from an ecological perspective. However, bringing environmental parameters that shape fungal communities, such as seasonal dynamics, horizon preferences, and successional changes, into the light is ecologically revealing. The fact that we have conducted more intensive sampling and sequencing than any studies prior to next-generation sequencing (Bueé et al. 2009) and are beginning to see strong correlations between environmental parameters and fungal community structure suggests that these efforts are worthwhile. However, some of the patterns, such as decreasing community variation through succession and lower interannual variation in black spruce mineral horizons, are only weakly supported, perhaps due to limited statistical power. Additional data will allow us to test the validity of these patterns and likely detect additional drivers of community structure. Next-genera-

tion sequencing offers huge potential to overcome some of the problems posed by extreme diversity, namely failure to saturate species diversity within a particular sample. However, the spatial and temporal issues require intensive and strategic sampling beyond simply generating large numbers of sequences. Less reliance on sample pooling would be a significant step forward, as we could begin to estimate the spatial scales of phenomena below the plot level.

With respect to the resilience of fungal communities, our current perspective remains limited, given the diversity-sampling issues emphasized throughout this paper. However, several of the strong patterns that we have detected do allow preliminary inferences concerning resilience in the *composition* of soil fungal communities. For example, the seasonal changes in fungal communities that we documented suggest that these fungi are sensitive to soil temperatures and thus are not stable with respect to this environmental parameter. Hence, ongoing climate change, particularly warmer soils in winter, is likely to cause shifts in fungal community composition. On the other hand, these communities appear to be resilient in the sense that they return to a similar composition in successive summers. We therefore tentatively suggest that these communities are resilient to transient temperature shifts but may be vulnerable to longer-term, persistent changes in temperature. Whether their capacity to respond is gradually eroded with a prolonged change in conditions remains to be determined. In addition, the high species turnover from site to site suggests that, at broad spatial scales, disturbances such as large, high-intensity fires will alter community composition at the landscape scale and that these communities may not recover to the same composition if narrowly distributed species are eliminated. Hence, the increasing areal extent and severity of fires are likely to impact fungal community composition. With respect to functional resilience, the cellulose SIP study indicates that soil fungi in this system are highly responsive to the introduction of new resources. Hence, process rates for cellulose consumption are likely to be highly dynamic. From a strict definition, such dynamic rates indicate low resilience. But from the broader perspective of the capability of the community to respond to novel conditions, these data suggest considerable functional plasticity due to a reservoir of biochemical capacities.

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