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Soil fungal communities respond compositionally to recurring frequent prescribed burning in a managed southeastern US forest ecosystem

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ABSTRACT

Prescribed fire is an important management tool to reduce fuel loads, to remove non-fire adapted species and to sustain fire-adapted taxa in many forested ecosystems of the southeastern USA. Yet, the long-term effects of recurring prescribed fires on soil fungi and their communities in these ecosystems remain unclear. We Illumina MiSeq sequenced and analyzed fungal Internal Transcribed Spacer (ITS2) amplicons from a long-term prescribed burn experiment that has implemented different regimes for nearly a quarter century to evaluate the effects of differing prescribed fire intervals and the season of their implementation on soil-inhabiting fungal communities. Unburned plots were used as a reference to represent the compositional state resulting from fire suppression. Our data show that while the recurring burning or the season of the prescribed burning do not affect richness and diversity of the fungal communities, the frequent (two and three year interval) fires maintain a fire-adapted community that is distinct from those in unburned reference plots. Subsequent indicator taxon analyses identified a total of 37 Operational Taxonomic Units (OTUs) that were more frequent in the frequently implemented burns and 26 OTUs that were more frequent in the fire suppressed treatment. We conclude that frequent prescribed burning maintains fire selected soil fungal communities that may support plant communities that are composed of desired fire adapted or fire tolerant species that dominate the frequently burned areas.

1. Introduction

Fire is a commonly implemented management tool used to reduce fuel loads in forested ecosystems of the southeastern USA (Waldrop et al., 1992; Callaham et al., 2012). Fire-regimes need to be continually refined to best fit the management goals (Stephens and Ruth, 2005). In the southeastern USA, silviculture is common and economically important (Talbert et al., 1985; Borders and Bailey, 2001). To properly maintain forest stands for silviculture, fire must be implemented to retain the fire-adapted species and remove competing fire sensitive species (Waldrop et al., 1992; Glitzenstein et al., 1995; Callaham et al., 2012). In many natural southern forest types, fire exclusion can lead to successional changes away from the diverse, fire-adapted ecosystems that developed there following Pleistocene glaciations (Abrahamson and Hartnett, 1990; Kirkman and Mitchell, 2006). Prescribed fire is viewed as a tool for restoration of these ecosystems, and frequent fires can result in overall improvement in terms of plant species richness, and animal food and habitat requirements (Ligon et al., 1986; Kowal et al., 2013; Steen et al., 2013). In determining optimal fire-regimes, a variety of ecosystem properties should be taken into account to ensure ecosystem stability and sustainability in the long-term. In non-fire adapted systems, or systems where fire has been long excluded, frequent fires can significantly reduce soil fungal biomass (Fritze et al., 1993; Pietikäinen and Fritze, 1993; Dooley and Treseder, 2012), reduce the quantity and quality of soil carbon (Neff et al., 2005) as well as change nutrient availability (Wan et al., 2001; Harden et al., 2003; Certini, 2005) indicating that both the biotic and abiotic soil properties are sensitive to fire, at least in the short-term.

Forest fires affect soil microbial communities both in short- and long-term (Holden and Treseder, 2013) and may preferentially select for fire adapted soil-inhabiting microbes. The fire effects and their duration depend on the intensity and frequency of burns (Hebel et al., 2009; Dooley and Treseder, 2012; Holden et al., 2013). Meta-analyses indicate that fires often reduce soil microbial biomass, at least in the short term (Dooley and Treseder, 2012; Holden and Treseder, 2013). Some microbial guilds are more sensitive to fire disturbance than others; Holden et al. (2013) concluded that ectomycorrhizal fungi exemplified such fire sensitive guilds. Others have pointed out specific taxa that are either resistant or adapted to fire disturbance. Examples of such genera include

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Wilcoxina (Baar et al., 1999; Fujimura et al., 2005; Kipfer et al., 2010), *Russula*, *Suillus* (Horton et al., 1998), *Coltricia*, *Thelephora* (Visser, 1994), *Rhizopogon*, (Baar et al., 1999; Kipfer et al., 2010), *Tomentella* (Baar et al., 1999), and *Cenococcum* (Kipfer et al., 2010). In conclusion, while the fire effects to fungal biomass are often negative, some fungi respond positively to fire treatments (Fritze et al., 1993; Visser, 1994; Baar et al., 1999; Kipfer et al., 2010) and fruit abundantly soon after fire (Wicklow, 1973, 1975).

Although prescribed burning has become an increasingly important management tool, much of our current understanding of the fire effects on soil properties comes from wildfires. It is still uncertain whether the impacts of wildfires are comparable to those of prescribed burning. Wildfires burn hotter, consume more of the aboveground biomass and organic matter, and result in greater nutrient volatilization (Hatten and Zabowski, 2010) than prescribed fires. This is partly attributable to one of the basic principles of fire management: prescribed fires are usually conducted under conditions that result in lower fire intensity and severity, and generally outside of the high risk conditions that are most conducive to the high intensity wildfires. Choromanska and DeLuca (2001) compared fire severity during a wildfire and prescribed burn in a temperate ponderosa pine forest. They observed that prescribed fire consumed 42% of the fine fuel and resulted in no overstory tree mortality. In contrast, the wildfire consumed all fine fuel and led to complete stand mortality highlighting the dramatic differences between wildfires and management through prescribed burning. Further, although both wildfires and prescribed fires tend to reduce soil fungal biomass (Dooley and Treseder, 2012), prescribed fires resulted in lesser loss of carbon in microbial biomass than wildfires (Choromanska and DeLuca, 2001). Evaluating the long-term effects of recurring prescribed fires on soils and fungal communities therein is important to properly manage forests to avoid compromising the composition and function of the soil fungal communities that are crucial to ecosystem services through nutrient cycling and facilitation of plant productivity (Wardle et al., 2004).

Soil microbial communities may recover within a growing season or two after a fire disturbance, especially if undisturbed stands or undisturbed mineral soils serve as an inoculum source (Grogan et al., 2000; Barker et al., 2013). Yet, the recovery times of many ecosystem attributes tend to be long. Eddy covariance studies and vegetation surveys suggest that re-establishment of primary productivity may require more than a decade after fires in boreal forests (Mack et al., 2008; Amiro et al., 2010; Goulden et al., 2011). Following wildfires, fungal hyphal lengths in the organic horizon required more than two decades to return to pre-fire levels (Holden et al., 2013) and soil fungal succession can continue several decades after a wildfire disturbance (Visser, 1994). However, Fritze et al. (1993) estimated a 12-year recovery time to a pre-fire state after a prescribed fire event. Finally, post-fire effects on soil carbon and organic matter may similarly persist a decade after a fire in boreal systems (Johnson and Curtis, 2001; Treseder et al., 2004). These studies, as well as recent meta-analyses (Dooley and Treseder, 2012; Holden and Treseder, 2013), strongly suggest that post-fire community shifts back to an unburned state may require more than a decade. However, the community and abiotic soil property recovery after low-intensity prescribed fires remain largely unknown.

In this study, we took advantage of a long-term experiment that has implemented different burning regimes for nearly a quarter century, thus allowing a unique means to address questions about long-term effects of recurring fires on soils and communities therein. The objectives of the implemented fire regime management were to retain the native fire adapted plant community. Unburned plots were used as a reference to represent the undesirable compositional state resulting from no fire management or continuing fire suppression. Since the experiments have controlled both fire frequencies and the season of the fire treatments, we aimed to address (1) if recurring prescribed fires have a long-term effect on soil fungal communities; (2) if these responses depend on fire interval (2-yr, 3-yr, 6-yr); and (3) if these responses depend on timing (summer vs. winter) of the burn. Our data provide further evidence that fire regimes that apply prescribed burning at frequent intervals maintain fire adapted soil fungal communities that are distinct from those inhabiting areas under fire exclusion management. As the vegetation in the frequently burned plots is largely composed of desired species, it may be inferred that the soil fungal communities residing in these plots are adapted to the recurring fires and thus better support such plant communities under frequent prescribed burning regimes.

2. Materials and methods

2.1. Study site

The long-term prescribed burn experiment is located in the Hitchiti Experimental Forest (HEF) in Jones County, Georgia, U.S.A., part of the Oconee District of the Chattahoochee-Oconee National Forest. HEF is in the Ocmulgee branch of the Altamaha River Basin approximately 18 km east of Gray, Georgia. Overstory is composed primarily of ectomycorrhizal loblolly pine (*Pinus taeda* L.) stands that are typical and widely distributed throughout the Southern Piedmont region of the southeastern United States. Southern pine stands are important both economically and ecologically: they are managed for timber harvest as well as to provide wildlife habitat. Before conversion to forest, large proportions of HEF, including the sites for the current experiment, were used for cotton production. After the severely eroded and degraded lands were converted to forest, the stands remained a mix of pines and hardwoods and had not been burned for at least 50 years before a fire regime was established in 1989. Upon conversion to forest from cotton plantation, the area was planted with *P. taeda*, which has remained the dominant tree species at the site. The implemented fire treatments have resulted in compositional shifts in the stands and generally favor the more fire-adapted pines at the cost of the understory hardwoods.

2.2. Soils

Common to the Georgia Piedmont, the study site contains highly eroded Alifsols and Utilsols. According to the United States Department of Agriculture Soil Conservation Service mapping (http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_051233.pdf), the soils represent Wilkes and Davidson Series. Wilkes Series are shallow, well-drained soils with moderately slow to slow permeability. Davidson Series consists of very deep, well-drained, and moderately permeable soils. These soils formed on uplands in the Piedmont, and are classified as loamy, mixed, active, thermic, shallow Typic Hapludalfs and are found on gently sloping to moderately steep uplands in the Piedmont.

2.3. Experimental design

A long-term fire management experiment at HEF was been implemented from 1989 until 2008. The experiment contains 24 plots, each ~0.8 ha in size and assigned to burn treatments randomly. Plots are spread across an area of approximately 70 ha in the southwestern corner of HEF, and most are isolated on the landscape although some share a border with a neighboring plot. All plots were burned upon establishment, excluding unburned control plots, with low intensity backfires during the winter of 1988/

1989. The experiment includes five treatments: unburned control plots (4 replicates), 2-yr winter (4 replicates), 3-yr winter (8 replicates), 3-yr summer (4 replicates), and 6-yr summer burns (4 replicates). Thus, the experimental design permits assessing the effects of fire intervals as well as the effects of season of the prescribed fire (3 year fire intervals only). Since the establishment of the experiment, HEF has experienced damaging outbreaks of southern pine beetle (*Dendroctonus frontalis* Zimmerman) from 2004 to 2008, and some plots in the study were impacted. These plots had overstory trees salvaged in 2008, and were subsequently planted to longleaf pine (*Pinus palustris* Mill.). These plots were sampled regardless of damage and our previous analyses indicated that the insect damage had no or only minor effects on the fungal communities at the time of sampling in 2011 (Brown et al., 2013). The last prescribed fires occurred in 2008. The last burn treatments were implemented in this experiment as follows: 2-yr and 3-yr winter burns took place in 2008 or three years before sampling; 3-yr summer burn treatments were last applied in 2004 (or for one plot 2001) or seven or more years before sampling; and, 6-yr summer burns last occurred in 2001, 10 years before sampling for this study. The cessation of experimental fire management in 2008 was due to flood-related damage to a road (in 2009, repaired in 2014) that provided equipment access to the plots – without equipment access, the plots could not be burned safely. The cessation of experimental fires in the 3-yr and 6-yr summer burn plots in 2004 was due to a combination of extreme drought conditions and an ongoing outbreak of southern pine beetle, which resulted in unsafe conditions for application of prescribed fire.

2.4. Soil sampling and sample processing

To avoid any confounding temporal effects resulting from time of sampling, at least one representative of each treatment was sampled each day of sampling. All plots were sampled in a total of four days between November 1 and 16, 2011. Fifteen dominant canopy *P. taeda* were selected a minimum of 10 m from plot edge to avoid edge effects. At each target tree, one 10 cm deep 5 cm diameter soil core was collected 3 m directly south of the tree bole for a total of 15 soil cores per plot. The cores were composited into one sample per plot. Pine beetle outbreak caused extensive damage in one plot leaving few remaining overstory trees. From this plot, soil cores were sampled systematically using a grid with fifteen points spaced ~5 m apart.

The pooled samples were frozen at −20 °C immediately on each sample date upon return to the laboratory, and then shipped frozen on dry ice to Kansas State University where stored at −80 °C until processing. The samples were thawed at room temperature and sieved through 1 mm mesh (No. 18 American Society for Testing and Materials (ASTM) E-11 Specification, Fisher Scientific Inc. Waltham, Massachusetts) to remove leaf litter, rocks, large particles, and roots. Samples were sieved into a large sealable plastic bag, in which they were manually mixed for ~5 min. A total of four 50 ml aliquots were collected from each sample and stored for DNA extraction at −80 °C. The remaining soil was dried at 50 °C for 48 h and analyzed for pH, total nitrogen, and total carbon at the Kansas State University Soil Testing Laboratory. Total genomic DNA was extracted from ~10 g (9.07–10.7 g fresh weight) subsample of one aliquot using UltraClean Mega Soil DNA Kit (MoBio, Carlsbad, California) following the manufacturer's instructions and stored at −80 °C until PCR amplification.

2.5. PCR amplification and sequencing

The DNA was quantitated with ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) and standardized to 5.0 ng/μl for PCR amplification of the Internal Transcribed Spacer 2 (ITS2) region of the ribosomal RNA gene cluster. ITS2 was chosen as it has been proposed as the universal barcode for fungi (Schoch et al., 2012) as well as to target shorter reads available with the paired-end Illumina MiSeq and amplified with the forward primer fITS7 (5′-GTGARTCATCGAATCTTTG-3′, Ihrmark et al., 2012) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′, White et al., 1990). All PCR reactions were performed in 25 μl reaction volumes in a two-step PCR process following protocol recommended by Berry et al. (2011) with three technical replicates of each of the 24 experimental units and a negative control (molecular biology grade water). The primary PCRs contained the following amounts/concentrations: 25 ng DNA template (5 μl), 200 μM dNTPs, 1 μM of both forward (fITS7) and reverse (ITS4) primers, 5 μl 5× Phusion Green HF Buffer containing 1.5 mM $MgCl_2$, 7.3 μl molecular biology grade water, and 0.5 unit (0.25 μl) of the proof-reading Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, Pittsburgh, USA). PCR cycling parameters included an initial denaturing at 98 °C for 30 s, followed by 25 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 8 min. A unique, sample-specific 12-base-pair sequence barcode was incorporated in a secondary PCR using an ITS4 fusion primer synthesized with sample specific DNA-tags (Supplemental Table S1). Secondary PCR conditions were identical to those for the primary PCR except that they included 5 μl of the primary PCR product as template, tagged reverse primers (ITS4), and the number of PCR cycles was reduced to five.

The secondary PCR amplicons were cleaned using Agencourt AmPure XP magnetic 96-well SPRIplate system (Beckman Coulter, Indianapolis, Indiana) following the manufacturer's protocol with 1:1 AmPure XP solution to amplicon ratio. The three technical replicates per experimental unit (24 in total) were combined and the experimental units equimolarly pooled into one amplicon library. The libraries were AmPure cleaned again to remove any residual short DNA contaminants and submitted to the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA), where Illumina specific adapters and indices were ligated using a NEBNext® DNA MasterMix (Protocol E6040, New England Biolabs Inc., Ipswich, MA, USA) and sequenced using a MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA) with 500 cycles. Paired fastq files (SRR1508275) are available in the Sequence Read Archive at NCBI (www.ncbi.nlm.nih.gov).

2.6. Sequence analysis

The sequence data were analyzed using the program mothur (v. 1.32.2; Schloss et al., 2009). After initial contig construction the paired-end read library contained 5,425,946 sequences. The data were screened to remove contigs with less than 100 bp overlap, any ambiguous bases, any disagreements with primer or DNA-tag sequences, sequences shorter than 250 bp, or homopolymers longer than 8 bp (1,113,584 remaining sequences). The sequences were truncated to 250 bp, near identical sequences preclustered to reduce potential sequencing bias (Huse et al., 2008) and screened for chimeras (uchime; Edgar et al., 2011). After quality control and removal of chimeras, the experimental units were subsampled to an equal 15,000 sequences. A pairwise distance matrix was calculated and sequences clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity. Low frequency OTUs (representing $\leqslant 10$ sequences) that may be PCR and/or sequencing artifacts were removed (Tedersoo et al., 2010; Brown et al., 2015) resulting in a total of 1412 OTUs and 348,994 total sequences in the dataset. The 1412 OTUs were assigned to taxa using the Naïve Bayesian Classifier at 97% sequence similarity (Wang et al., 2007) and the UNITE taxonomy reference (http://unite.ut.ee/repository.php) as implemented through mothur.

Coverage {Good's coverage ($G = 1 - (n_1)/(N)$), where n_1 = the number of OTUs that have been sampled once and N = the total number of individuals in the sample}, observed (S_{Obs}: number of OTUs) and extrapolated [Chao1: $S_{\mathrm{Chao1}} = S_{\mathrm{Obs}} + \{n_1(n_1 - 1)/2(n_2 + 1)\}$, where n_1 = the number of OTUs with only 1 sequence and n_2 = the number of OTUs with only two sequences] richness, diversity ($1-D$ or complement of Simpson's diversity index: $1-D = 1-\Sigma p_i^2$, where D = Simpson's diversity index and p_i = proportional abundance of the ith species), and evenness {Simpson's equitability or E_D: $E_D = (1/\Sigma p_i^2)/S$), where p_i = proportional abundance of the ith species and S = richness} were calculated using mothur (v. 1.31.2; Schloss et al., 2009).

2.7. Statistical analyses

We first tested for differences in soil properties including pH, total nitrogen, and total carbon across all treatments using one-way ANOVA (ANOVA: $P > 0.05$) in JMP® (version 7.0.2). We tested for differences in diversity and richness estimators across treatments to detect any effects of season or time-interval using both one way ANOVA and mixed models with season as a random variable in JMP® (version 7.0.2). For these analyses data were transformed to meet the assumptions of variance homogeneity and normality: response variables ranging from 0 to 1 (percentages) were arcsine square root transformed – others were $\log_{10}$-transformed. Both mixed model and one-way ANOVA analyses gave congruent results. As a result, only one-way ANOVA results will be presented. We first analyzed the effects of spring and winter burns relative to the control using only the unburned control treatments and prescribed fires that occurred every three years, because this was the only fire interval that included fires scheduled for both burn seasons. These analyses indicated that season of the prescribed fire had no effect on communities; therefore, we resolved to compare all treatments to conclude on any effects of the fire interval.

To detect compositional differences in the fungal communities across treatments, we first derived a Bray–Curtis distance matrix and compared treatments by Multi-Response Permutation Procedure (MRPP) in PC-ORD (version 5; McCune and Mefford, 2006). To visualize these community data clouds, we estimated Non-metric Multidimensional Scaling (NMS) axis scores for first two ordination axes that represented 84.3% of the variation to obtain a stress of 0.15. In addition to evaluating the responses on the whole community level, we used Indicator Species Analysis in PC-ORD to identify OTUs that were present in one treatment more commonly than in others. To ensure true significance of indicator OTUs the results were run using false discovery rate (FDR) with the false detection rate (q-value) set to 0.20.

3. Results

3.1. Soil parameters

Soil parameters did not differ between the five prescribed fire interval treatments (ANOVA: $P > 0.05$; Table 1). However, soil pH tended to be higher in the most frequent fire treatments (Table 1).

3.2. General fungal community data description

The dataset initially contained 5,425,956 sequences. After quality control, subsampling to equal number of sequences per experimental unit, and removal of low frequency OTUs ($\leqslant$10 sequences) we included a total of 1412 OTUs and 348,994 sequences in the final analyses. A total of 15.4% (53,971 sequences; 244 OTUs, 17.2%) of sequences were only assigned to Kingdom fungi but remained unclassified at the phylum level. Communities overall were dominated by Basidiomycota (203,990 sequences, 58.5%; 801 OTUs, 56.7%), followed by basal lineages – mainly taxa formerly assigned to Zygomycota (53,729 sequences, 15.4%; 42 OTUs, 2.9%), and a smaller proportion to Ascomycota (36,806 sequences, 10.5%; 308 OTUs, 21.8%). On the order level, our data were dominated by sequences that remained unclassified (55,759 sequences, 15.9%; 396 OTUs, 28.0%), but included Russulales (52,729 sequences, 15.1%; 100 OTUs, 7.1%) with many taxa that form ectomycorrhizae (ECM), Agaricales (46,043 sequences, 13.2%; 260 OTUs, 18.4%) with diverse ecologies, and Mucorales (10,246 sequences, 11.5%; 22 OTUs, 1.6%) that likely represent soil-inhabiting, free-living fungi. On the family level, Russulaceae composed of mainly ECM members contributed 15.1% (52,729 sequences) with 100 OTUs (7.1%), followed by the largely soil-inhabiting family Umbelopsidaceae (11.4%, 39,873 sequences) with 17 OTUs (1.2%), and basidiomycetous Geminibasidiaceae (5.5%, 19,414) with 19 OTUs (1.3%). The most common OTUs represented mainly soil-inhabiting fungi (OTU0001 with affinity to *Umbelopsis dimorpha* – 28,163 sequences, 8.1% and OTU0002 *Geminibasidium* 16,897 sequences, 4.8%), but included also unclassified, unknown taxa (OTU0003 unclassified, 8635 sequences, 2.4%). In addition, the ten most common OTUs included common ECM taxa (OTU0005 *Russula* sp. – 7297 sequences, 2.1%; OTU0007 *Suillus* sp. – 5223, 1.5%; and OTU0010 *Russula* sp. – 4239, 1.2%). A complete list of OTUs with their taxon assignments, sequence counts, and frequencies among treatments is included in the Supplemental Table S2.

3.3. Effect of prescribed fire season

We used 3-yr summer and 3-yr winter burn treatments to evaluate the effects of burn season. Overall, the season of the prescribed fire implementation had only minimal effects on the soil-inhabiting fungal communities, particularly when compared to the responses to fires recurring at two and three year intervals. The richness and diversity estimators were unaffected by the choice of burning season: Good's coverage, observed (S_{Obs}) and extrapolative (Chao1) richness, diversity ($1-D$), or evenness (E_D) did not differ across treatments (Table 2).

Similarly, the season of prescribed burn implementation had no effect on the fungal community composition: whereas the communities in both winter and summer burns at three year intervals differed clearly from those in the unburned control (MRPP: $T_{2,7} = -4.25$, $A_{2,7} = 0.25$, $P = 0.001$; $T_{2,11} = -2.8$, $A_{2,11} = 0.18$, $P = 0.006$, respectively), the communities in the winter and summer fire regimes at three year intervals did not differ from each other (MRPP: $T_{2,11} = 1.05$, $A_{2,11} = -0.04$, $P = 0.862$). The observed

Table 1

Means and standard deviations of soil parameters across different prescribed fire treatments. Comparison of treatments across all measured soil parameters indicates no significant differences between the treatments. F-statistics refer to one-way ANOVA that compares means of all five treatments. Note that while no pairwise differences were observed (ANOVA: $P > 0.05$), the ANOVA suggests a nearly significant difference among the fire treatments in the soil pH.

Soil parameter	Control	2-yr winter	3-yr winter	3-yr summer	6-yr summer	$F_{4,23}$; Prob > F
pH	4.68 ± 0.096	5.15 ± 0.238	4.95 ± 0.256	4.95 ± 0.125	4.88 ± 0.171	2.57; 0.07
Total N (%)	0.08 ± 0.0001	0.09 ± 0.0009	0.09 ± 0.0002	0.09 ± 0.0009	0.13 ± 0.0007	1.22; 0.33
Total C (%)	2.00 ± 0.000	2.75 ± 0.027	2.25 ± 0.005	2.62 ± 0.005	2.75 ± 0.009	1.12; 0.38

Table 2
Means and standard deviations of community diversity and richness estimators across different prescribed fire treatments. *F*-statistics refer to one-way ANOVA that compares means of all five treatments. One-way ANOVAs ($F_{4,23}$, Prob > *F*) comparing all treatments indicate no significant differences across treatments. None of the richness estimators differ among the burn treatments implemented at different intervals or during different seasons.

Parameter	Control	2-yr winter	3-yr winter	3-yr summer	6-yr summer	$F_{4,23}$; Prob > *F*
Coverage	0.99 ± 0.2%	0.99 ± 0.1%	0.99 ± 0.1%	0.99 ± 0.2%	0.99 ± 0.04%	0.44; 0.78
Richness (S_{Obs})	668.00 ± 77.42	656.5 ± 59.80	680.5 ± 41.92	659.75 ± 7.9	687.5 ± 43.02	0.34; 0.85
Richness (Chao1)	902.71 ± 142.05	899.16 ± 71.57	934.87 ± 51.22	883.90 ± 72.21	915.31 ± 35.17	0.41; 0.81
Diversity (1−*D*)	0.98 ± 0.001	0.97 ± 0.007	0.98 ± 0.003	0.97 ± 0.009	0.98 ± 0.009	0.68; 0.61
Evenness (E_D)	0.07 ± 0.003	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	1.23; 0.33

community responses were thus mainly affected by the short, three year fire interval treatments, consistently with the broader analyses of fire interval effects (see below).

3.4. Effect of prescribed fire intervals

None of the diversity and richness estimators (Good's coverage, S_{Obs}, E_D, Chao1, and 1−*D*) differed among the fire interval treatments (Table 2). In contrast, burn treatments resulted in compositional shifts in soil fungal communities (Fig. 1; MRPP: $T_{5,23} = -2.19$, $A_{5,23} = 0.09$, $P = 0.021$). In pairwise comparisons, the short fire interval (2-yr winter, 3-yr winter, 3-yr summer) treatments differed strongly and significantly from the unburned control (Fig. 1; MRPP: $T_{2,7} = -3.12$, $A_{2,7} = 0.18$, $P = 0.007$; $T_{2,7} = -4.26$, $A_{2,7} = 0.26$, $P = 0.001$; $T_{2,11} = -2.8$, $A_{2,11} = 0.17$, $P = 0.006$, respectively). It is of note that the last burn events occurred during the same year for both the 2- and 3-yr winter treatments, whereas the last burns in the 3-yr summer treatment occurred seven or more years before our sampling. In contrast to the frequent fire treatments, the 6-yr treatments that were burned last a decade before our sampling were only marginally significantly different from the control ($T_{2,7} = -1.62$, $A_{2,7} = 0.10$, $P = 0.05$). The treatments with different fire intervals did not differ from each other. The observed community distinctions were most obvious along the first NMS axis that represented 48.0% of the variability (Fig. 1). The 6-yr fire treatments, which only marginally differed from the unburned control, were mainly located between control and 2- or 3-yr treatments (Fig. 1).

We used indicator taxon analyses as a conservative tool to identify OTUs that may be adapted to recurring prescribed fires. Across the five fire treatments, we identified a total of 77 indicator OTUs that occurred more frequently in one treatment compared to others (Supplemental Table S3). More than a third of the indicator taxa (26 OTUs) were enriched in the unburned control plots relative to other treatments and thus likely represent fire sensitive taxa. The unburned control indicators included a large proportion of fungi that remained unclassified beyond a phylum (13 OTUs), but also a variety of basidiomycete OTUs (1 in family Atheliaceae,

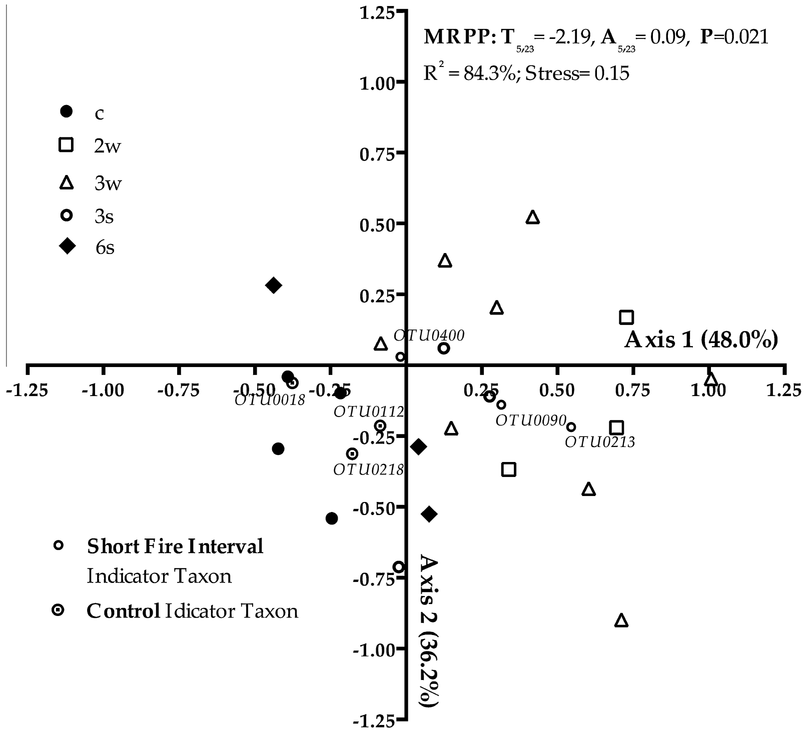

Fig. 1. Non-metric Multidimensional Scaling (NMS) ordination of long-term prescribed fire treatments (c = unburned control, 2w = winter burn at 2 year intervals, 3s = spring burn at 3 year intervals, 3w = winter burn at 3 year intervals, 6s = summer burn at 6 year intervals). The NMS ordination was optimally resolved on two axes (stress = 0.15) that represented 48.0% and 36.2% of the variability for a total of 84.2%. Multiple Response Permutation Procedure (MRPP) indicated significantly different communities ($T_{5,23} = -2.19$, $A_{5,23} = 0.09$, $P = 0.021$) across the five fire treatments. Subsequent pairwise comparisons among the treatments indicated that communities in the short fire interval treatments (2-yr and 3-yr) differed the from the unburned control and are mainly located right of NMS axis 2 (MRPP: $P < 0.05$), whereas the long fire interval treatment (6-yr) marginally differed from control (MRPP: $P = 0.05$) but did not differ from other fire treatments (MRPP: $P \geqslant 0.05$). Small open circles exemplify dominant indicator taxa for short fire intervals (OTU0090 (*Amanita* sp.), OTU0213 (*Clavulina* sp.), and OTU0400 (*Suillus* sp.)) and small dotted circles for the unburned control treatments (OTU 0018 (*Trechispora* sp.), OTU 0218 (*Russula* sp.), and OTU0112 (*Sistorema* sp.)).

1 Thelephoraceae; 1 in genus *Callistosporium*, 1 *Geminibasidium* 3 *Russula*, 1 *Sistotrema*). There were also 4 ascomycete OTUs (3 OTUs in Eurotiomycetes, 1 in genus Xylaria). Short fire intervals (2- and 3-yr burns, for a total of three treatment combinations and sixteen experimental units) included a total 37 indicator OTUs consisting of thirteen unclassified OTUs and one OTU assigned to a genus *Umbelopsis* representative of basal, early diverging lineages. The remaining OTUs included five ascomycetes (3 OTUs in order Verrucariales, 1 in family Herpotrichiellaceae, 1 in genus *Bionectria*). There were an additional 19 basidiomycete OTUs identified as indicators for the short fire intervals (4 unclassified Agaricomycetes; 2 in family Clavariaceae, 1 Russulaceae, 4 Thelephoraceae; 1 in genus *Amanita*, 1 *Clavulina*, 1 *Geastrum*, 1 *Ramaria*, 3 *Suillus*, 1 *Tylospora*). The indicator taxon analyses identified no OTUs that were completely lost in the frequent fire treatments. As a result, we conclude that the overall compositional community responses to recurring prescribed fires are largely driven by community member reordering rather than taxon replacement.

4. Discussion

Effects of recurring prescribed burning on soil microorganisms and their community composition in the long-term have remained largely unknown, despite their essential roles in nutrient cycling as well as in soil and plant health. Intense wildfires tend to have greater impacts and longer lasting effects than low intensity fires, often implemented with prescribed fire management (Carter and Foster, 2004; Certini, 2005). Thus, it is important to evaluate the effects of prescribed fire as the results from studies on wildfires may not permit extrapolation into prescribed fires. Similarly, more frequent fires often have effects on soil fungi (Cairney and Bastias, 2007) that may differ from those observed in single occurrence fire events. Here we evaluated the effects of different fire regimes on soil fungal communities over time using an experimental design that has implemented prescribed burning in the long term.

As a result of the complexity of the experimental design, we separately addressed the effects of timing of the fire (summer vs. winter burn) and the interval (2-yr, 3-yr, 6-yr and control unburned) between the fire treatments on fungal richness and diversity as well as overall community composition. Our data show that while season of the burn has minimal effects, frequent burns will shift overall community structure and may select for fire adapted and/or fire tolerant fungi. Further, as indicated by our 3-yr summer burn treatments that were discontinued in 2004, the prescribed burn effects remain visible in the fungal communities for more than half a dozen years. In contrast to composition, neither the fire season nor the interval had any detectable effects on species richness or diversity. Thus, our data suggest that frequent recurring fires likely lead to taxon re-ordering rather than loss of the common community constituents and indicate that the fungal communities in shorter (two and three year) fire intervals were most distinct from the unburned control treatments. In contrast, we detected no significant differences between communities in any of the treatments that had been burned during our experiment. Interestingly, the fungal communities residing in the 6-yr interval treatments were positioned intermediately between the unburned control and the frequently burned treatments in our ordination – possibly indicating that the six-year prescribed fire intervals may be inadequate to maintain the fire-adapted communities. However, it is important to bear in mind that these 6-yr interval treatments were last burned a decade prior to our sampling and may reflect the proposed decadal community shift towards communities that resemble those in the unburned reference treatments. Our studies were not designed to decouple mechanisms that result in the distinct fire adapted communities. Thus, observed differences between frequently burned treatments and unburned treatments may reflect the difference in fire adapted and fire sensitive aboveground plant communities: unburned plots include hardwood competitors and shrubs whereas the frequently burned plots are mainly composed of fire-adapted pines and grasses. These data suggest that short fire intervals that prevent the expansion of unwanted vegetation have similar effects in the associated fungal communities below ground and may inhibit fungi with a preference for the hardwoods and/or their litter. Further, since frequent fires do not negatively affect diversity and richness of fungal communities, they can be perceived as stable and likely composed of fire-tolerant members.

Consistently with other reports and meta-analyses that broadly cover biomes ranging from boreal and temperate forests to woodlands and shrublands (Dooley and Treseder, 2012; Holden et al., 2013; Holden and Treseder, 2013), our data suggest that fungal communities respond to fire disturbance. Despite the low intensity stripped head-fires used in our experimental implementation, our results are consistent with findings from other studies (Johnson and Curtis, 2001; Treseder et al., 2004; Mack et al., 2008; Amiro et al., 2010; Goulden et al., 2011; Dooley and Treseder, 2012; Holden et al., 2013) and indicate that shifts back to an unburned state following fire disturbance require several years. However, these responses may vary among ecosystems or biomes: while the meta-analyses (Dooley and Treseder, 2012; Holden and Treseder, 2013) suggest that fungi – in general – respond negatively to disturbance, boreal systems may be particularly sensitive to fire. Fritze et al. (1993) estimated a 12 year recovery time after prescribed fire in a boreal forest system. Similarly, meta-analyses (Dooley and Treseder, 2012) and fire chronosequence studies in Alaskan boreal forests (Holden et al., 2013) arrive at similar conclusions and suggest that at least a decade, or even up to two and a half decades may be necessary for complete shift to the unburned state after a fire disturbance. Our results are in line with these: while the short interval fire treatments clearly differed in their community composition from the unburned plots, the 6-yr interval burns that had not been burned in a decade differed neither from the other fire treatments nor from the unburned controls. Based on this, we conclude that community shifts from even low-intensity prescribed fires take more than a decade to reach a community state clearly distinguishable from the frequent fire implementation. With the management goals in mind, conservative interpretation of our findings suggests that fire intervals exceeding six years may allow soil fungal communities to shift towards states associated with unwanted vegetation.

Analyses of fungal community responses to fire are few (Holden and Treseder, 2013); our current studies provide a detailed analysis of soil fungal community responses to low intensity prescribed fire. Similarly to our study, previous research on Australian sclerophyll forests using Restriction Fragment Length Polymorphism (RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) analyses suggested that long-term short-interval fire regimes alter fungal communities (Bastias et al., 2006a; Anderson et al., 2007). Holden et al. (2013) utilized a chronosequence approach in boreal coniferous forests in Alaska and concluded that recently burned sites were distinct from those with more distant fire history. ECM fungal communities may be particularly responsive to repeated fire disturbance: short fire intervals often have greater effects on ECM fungal communities than long-interval fire regimes (Bastias et al., 2006b; Buscardo et al., 2010). Our indicator taxon analyses identified seventeen OTUs representing likely ECM fungi that were favored by either the unburned (1 OTU in family Thelephoraceae; 3 OTUs in genus *Russula*, 1 *Sistotrema*) or frequently burned (1 OTU in family Russulaceae, 4 Thelephoraceae, 1 in genus *Amanita*, 1 *Clavulina*, 1 *Tylospora*, 1 *Ramaria*, and 3 *Suillus*) treatments indicating that, across the ECM fungi, taxa can have opposite responses to fires. These taxa potentially respond to fire or – alternatively – to the concomitant shifts in the plant communities (i.e., from broadleaf hardwoods and shrubs to pines and grasses). While some ECM associations are host specific (Horton et al., 1998; Newton and Haigh, 1998) and ECM communities of different hosts distinct (Morris et al., 2009; Tedersoo et al., 2009), our data do not permit decoupling mechanisms underlying the observed community responses. It is perhaps tempting to speculate that ECM fungi respond most strongly to fire disturbance, particularly when considering stand replacing wildfires. However, this may not be the case for low intensity prescribed burning where soil physicochemical and biotic responses below ground may be minimal (Callaham et al., 2012).

To further elaborate on the community level responses, our indicator taxon analysis identified a total of 77 indicator taxa across our treatments. Previous studies have identified early-successional fungi that increase in abundance following a fire and include several ECM genera (*Coltricia*, *Thelephora* – Visser, 1994; *Russula*, *Suillus* – Horton et al., 1998; *Rhizopogon*, *Wilcoxina* – Horton et al., 1998; Baar et al., 1999; Kipfer et al., 2010; *Tomentella* – Baar et al., 1999; and *Cenococcum* – Kipfer et al., 2010). Although our analyses included many of these taxa, many were not identified as indicators – only one OTU assigned to *Suillus* and four to Thelephoraceae may be among taxa that seem consistently abundant after fire in our study and those of others. However, it is important to bear in mind that many of the earlier studies have focused on recent fires (e.g., Baar et al., 1999; Chen and Cairney, 2002) or followed the effects of a single fire application over time (e.g., Fritze et al., 1993; Visser, 1994; Longo et al., 2011; Holden et al., 2013). In contrast, our study focused explicitly on the long-term legacy effects of recurring fire regimes that had been implemented in the long term. As a result, our study operates on a different time scale in terms of community responses and is therefore perhaps difficult to compare to earlier studies.

Inferences about changes in functional attributes of soil inhabiting communities are rare and our analyses permit them only cursorily by taxon assignment. In contrast to previous suggestions that ECM taxa may be most sensitive to fire disturbance (e.g., Holden et al., 2013), our data suggest that many functional guilds respond but mainly through a few disturbance sensitive and adapted taxa. More detailed functional assays targeting either soil enzyme activities directly or genes that code for those enzymes permit further functional inference. Artz et al. (2009) analyzed laccase genes of soil basidiomycetes in a clone library study of fire effects in a *Eucalyptis pilularis* dominated wet sclerophyll forest in Australia. They observed a greater diversity and evenness of these carbon polymer-modifying genes following the fires than in the unburned control. Holden et al. (2013) assayed directly a number of enzyme systems that contribute to the cycling of macro-nutrients in soil (carbon, nitrogen, phosphorus). These studies suggest that metabolic activities as well as the metagenomic content permitting those activities shift in response to recent fires. This is likely a result of a combination of changes in substrate quality and quantity (Wan et al., 2001; Harden et al., 2003; Certini, 2005; Neff et al., 2005), reductions in soil inhabiting microbial biomass (Fritze et al., 1993; Pietikäinen and Fritze, 1993; Dooley and Treseder, 2012; Holden et al., 2013; Holden and Treseder, 2013), and shifts in the soil-inhabiting communities (Brown et al., 2013; Holden et al., 2013) in response to fires.

Burning litter layer and ground vegetation often leads to a short-term decrease in soil organic matter as well as loss of nitrogen and carbon as a result of combustion and volatilization (Hernández et al., 1997; Certini, 2005). Different forms of nitrogen may also respond differently to fire: while inorganic nitrogen may increase in the short term, organic nitrogen in soil may greatly decline (Prieto-Fernández et al., 2004). Most commonly in forest ecosystems, fires tend to reduce soil organic matter and nutrient availability in the short term, although the addition of burnt plant material can increase these parameters in the long-term (Prieto-Fernández et al., 2004; Certini, 2005). Williams et al. (2011) and Toberman et al. (2014) observed that a greater frequency in prescribed fires lead to greater loss in available soil nutrients in the long-term. We observed no, or only minimal, differences in the soil parameters (pH, total *N*, and total *C*) across the different burn treatments, even though our experimental manipulations have been in place for two decades. Comparison across experiments is difficult because fires can have a wide range of effects on soil parameters depending on fire intensity, duration, or the soil properties (e.g., volumetric soil moisture) before the fire (Certini, 2005; Glass et al., 2008). While we observed no shifts in the soil chemistry resulting from frequent burning, our results are congruent with Glass et al. (2008), who also found no effects of low-intensity fires in the short- or long-term. This demonstrates the range of variation in fire effects across fire intensities and soil parameters.

Our study identifies clear community shifts resulting from frequently implemented prescribed fires. In contrast to fire frequency, we found no effects of season of fire implementation. However, Smith et al. (2004) suggested that seasonal timing of burn regimes can have short-term (<two years) effects on fungal communities finding a greater richness in spring burns compared to fall burns. In our study, the longer (six year) fire intervals are particularly interesting, as they provide clues to how soil fungal communities return to states preceding disturbance over time. While the long fire interval treatments did not clearly differ from any of the other treatments ten years after the cessation of the burn treatments, they still only marginally differed from the unburned treatments. Based on this, we speculate that return to community state preceding the fires takes a decade or more. These estimates are congruent with those of others. While our data suggested clear compositional shifts as a result of the frequent fires, the richness or diversity of the communities remained unchanged suggesting likely taxon reordering. With the management goals in mind, we conclude that frequent burns maintain stable diversity and richness of fungal communities over time. We speculate that these communities are associated with the desirable vegetation and suggest that fire intervals should not surpass a decade to avoid communities returning to the unfavored unburned state.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2015.02.020.

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