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Influence of repeated prescribed burning on incorporation of ^{13}C from cellulose by forest soil fungi as determined by RNA stable isotope probing

Brigitte A. Bastias [a], Ian C. Anderson [a,b], J. Ignacio Rangel-Castro [c], Pamela I. Parkin [b], James I. Prosser [c], John W.G. Cairney [a,*]

[a] Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia
[b] The Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, United Kingdom
[c] Institute of Biological and Environmental Sciences, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3UU, United Kingdom

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ABSTRACT

Repeated prescribed burning is frequently used as a forest management tool and can influence soil microbial diversity and activity. Soil fungi play key roles in carbon and nutrient cycling processes and soil fungal community structure has been shown to alter with increasing burning frequency. Such changes are accompanied by changes to soil carbon and nitrogen pools, however, we know little regarding how repeated prescribed burning alters functional diversity in soil fungal communities. We amended soil with ^{13}C-cellulose and used RNA stable isotope probing to investigate the effect of biennial repeated prescribed burning over a 34-year period on cellulolytic soil fungi. Results indicated that repeated burning altered fungal community structure. Moreover, fungal community structure and diversity in ^{12}C and ^{13}C fractions from the unburned soil were not significantly different from each other, while those from the biennial burned soils differed from each other. The data indicate that fewer active fungi in the biennially burned soil incorporated ^{13}C from the labelled cellulose and that repeated prescribed burning had a significant impact on the diversity of an important functional group of soil fungi (cellulolytic fungi) that are key drivers of forest soil decomposition and carbon cycling processes.

1. Introduction

Soil fungal communities are central to decomposition processes in forest ecosystems and constitute essential components of carbon and nutrient cycles (Dighton, 2003). Forest soil fungal communities are taxonomically diverse and their structure is known to be altered by environmental disturbance (e.g. Cairney and Meharg, 1999). One such disturbance is fire which, as a result of natural wildfire or prescribed burning, can strongly influence the edaphic environment and below-ground biota (Neary et al., 1999). Although relatively few investigations of fire effects on soil fungal communities have been undertaken, it is evident that fungal community structure can be strongly influenced by single fire events, and that such effects, along with their duration, appear to be site- and/or fire-specific (reviewed by Cairney and Bastias, 2007).

Prescribed burning is used widely in forest management in order to prepare sites for planting, control pests and/or reduce the impact of wildfires on forests and neighbouring urban areas (Neary et al., 1999; Fernandes and Botelho, 2003). The benefits of fire-based forest management, however, may last for only a few years, necessitating the application of repeated prescribed burning for on-going management (Fernandes and Botelho, 2003). Such repeated burning can reduce surface and soil organic matter content, and may influence nutrient, especially nitrogen, availability (Wright and Hart, 1997; Neary et al., 1999; Guinto et al., 2001; Mao et al., 2002).

Aside from an investigation of ectomycorrhizal fungi in North America (Tuininga and Dighton, 2004), information on how repeated burning influences forest soil fungi is limited, but recent work at a long-term repeated prescribed burning experiment in a south-eastern Australian native sclerophyll forest indicates that it may have significant ramifications for soil fungal communities. Thus, direct DNA extraction from soil, coupled with denaturing gradient gel electrophoresis (DGGE) and terminal restriction length fragment polymorphism (T-RFLP) analyses, has been used to demonstrate that repeated burning over a >30-yr period significantly alters the structure of soil fungal and soil basidiomycete communities and that the effect is greater with burning at 2- than

* Corresponding author. Tel.: +61 (02) 4570 1404; fax: +61 (02) 4570 1103.
E-mail address: j.cairney@uws.edu.au (J.W.G. Cairney).

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4-yr intervals (Bastias et al., 2006a; Anderson et al., 2007). Analysis of mycelial communities of ectomycorrhizal fungi using similar methods revealed significantly altered community structure with burning at a frequency of 2-, but not 4-yr (Bastias et al., 2006b). Although clearly demonstrating that forest management, in the form of regular prescribed burning, over a prolonged period can alter the structure of soil fungal communities and significantly decrease soil C and N content (Bastias et al., 2006a), these observations provide no information on the likely functional significance of the changes to forest nutrient and carbon cycling processes.

A functional understanding of the consequences of long-term repeated burning for decomposition processes requires knowledge of how relevant functional groups of soil fungi are affected. Cellulose is a major component of plant biomass, and a broad taxonomic range of fungi contribute to the degradation of this important polymer in soil (Lynd et al., 2002). Methods for identifying cellulolytic fungi have previously relied upon cultivation-based isolation but stable isotope probing (SIP) (Radajewski et al., 2000; Manefield et al., 2002) offers a more direct means of investigation. For example, Haichar et al. (2007) recently utilised SIP to identify cellulolytic bacteria by analysing ^{13}C-labelled DNA following incubation of soil with ^{13}C-cellulose. Since rates of RNA synthesis are higher than for DNA, RNA-SIP has been proposed as a more sensitive approach than DNA-SIP (Manefield et al., 2002). This is particularly true for fungi due to their relatively low growth rate compared with prokaryotes. RNA-SIP therefore provides a means of both determining which fungi are cellulolytic and which cellulolytic fungi are influenced by environmental change. This study therefore involved amendment of soil from the long-term prescribed burning experiment at Peachester State forest with ^{13}C-cellulose and the use RNA-SIP in combination with DGGE to determine the effect of repeated burning on incorporation of ^{13}C from cellulose by forest soil fungi.

2. Materials and methods

2.1. Site description and soil sampling

The field site used in this study comprises *Eucalyptus pilularis* Smith-dominated native wet sclerophyll forest at Peachester State Forest, situated in the Sunshine Coast hinterland of Queensland, Australia (26°50′S, 152°53′E). Since 1972, a long-term prescribed burning experiment has been maintained at the site and comprises replicated plots (30 × 27 m) subjected to biennial burning (2-yr burn plots), quadrennial burning (4-yr burn plots) or no burning (unburned plots). Further details of the site and soil characteristics are provided by Guinto et al. (2001). Previous investigations indicated that soil fungal communities in the upper 10 cm of the soil profile differed significantly between 2-yr burn plots and unburned plots (Bastias et al., 2006a,b; Anderson et al., 2007) and the current work focused on these two treatments. Ten 2.5 cm diameter soil cores (0–10 cm soil depth) were collected from two replicate plots of each treatment from two experimental blocks (a total of four plots for each treatment). To minimise the effects of within-plot heterogeneity, the ten soil samples from each plot were pooled, resulting in four pooled samples per treatment, which were sieved (2 mm mesh size) to facilitate relatively uniform mixing of ^{13}C-cellulose throughout the soil.

2.2. Amendment of soil with ^{13}C-labelled cellulose

Soil samples (four per treatment) were adjusted to 55% of field capacity by the addition of sterile milli-Q H_2O. Thirty grams of each soil sample was placed in sterile plastic specimen jars without lids, before being individually placed into 1 L glass preserving jars, containing sterile moist paper towel and sealed. Soil samples were conditioned in the preserving jars for one week at room temperature. After the conditioning period, 2 g of soil was removed from each sample for nucleic acid extraction prior to the addition of 10 mg of ^{13}C-cellulose (98 atom% ^{13}C) (*Solanum tuberosum*) (IsoLife, Wageningen, The Netherlands) which was mixed throughout the soil sample. Samples were incubated in sealed preservation jars in the dark for 35 d at 22 °C. Jars were opened on a weekly basis to remove CO_2 and replenish O_2.

2.3. Nucleic acid extraction, RNA purification and ^{13}C analysis

Following incubation, 12 × 1.0 g sub-samples were collected from each soil sample for nucleic acid extraction using the method of Griffiths et al. (2000) and bead beating for 30 s at a speed of 5 m s^{-1} in a FastPrep™ (FP120) Thermos Savant bead beating system (Bio-101, Vista, California, USA). RNA was purified from each nucleic acid extract using the RNase-free DNase set (Qiagen, Doncaster, Australia) and the RNeasy MinElute Cleanup Kit (Qiagen) following the manufacturer's instructions. The twelve replicate extracts for each soil sample were then pooled and stored at −80 °C. One microgram of RNA per sample, cut with 61.6 μg of glucose, was freeze-dried in 6 × 4 mm tin cups (Elemental Microanalysis, Okehampton, UK) and submitted for ^{13}C analyses by Isotope Ratio Mass Spectrometry (IRMS). Isotope analysis was carried out using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyser with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific, Crewe, UK). When operated in the dual isotope mode, δ^{13}C is measured along with %N and %C (Scrimgeour and Robinson, 2003). ^{13}C enrichment values were compared to the international standard Pee Dee Belemnite as indicated in Rangel-Castro et al. (2005).

2.4. Density gradient separation and fractionation of RNA

RNA concentration in each purified RNA extract was determined using a Nano Drop (ND-1000) Spectrophotometer (Labtech International, Ringmer, UK). ^{13}C-labelled and unlabelled RNA were separated by isopycnic ultracentrifugation using density gradient solutions of CsTFA (Amersham Biosciences, Little Chalfont, UK) and *ca* 500 ng of RNA per sample as described by Rangel-Castro et al. (2005). Fractionation using a Fraction Recovery system (Beckman Coulter, Palo Alto, CA, USA) yielded 10 fractions (each *ca* 85 μl), of which fractions 1 and 2 were discarded as these are likely to contain labelled molecules other than nucleic acids (Manefield et al., 2002; Rangel-Castro et al., 2005). RNA from the remaining eight fractions for each sample was precipitated with ice-cold isopropanol and then re-suspended in 20 μl of RNase-free water, before being stored at −80 °C.

2.5. cDNA synthesis and PCR amplification

RNA from each fraction (for each sample) was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Paisley, UK) and the reverse primer ITS4 (White et al., 1990) following the manufacturer's instructions, except that incubation was performed at 52 °C (Rangel-Castro et al., 2005). Reverse transcription reactions included 10 μl RNA template, 1 μl 10 pmol reverse primer and 1 μl 2.5 mM dNTP. Two negative controls [no template (water only) and template without RT enzyme] were conducted for all reactions. PCR amplification was conducted using the resulting cDNA and the primers ITS1-F (Gardes and Bruns, 1993) and ITS4. Reactions were performed using a Dyad DNA Engine thermal cycler (MJ Research, Waltham, MA) in 50 μl volumes as described by Bastias et al. (2006a). A nested PCR was conducted

using PCR products as template and the primers ITS2 (White et al., 1990) and ITS1-F with a 40 base GC-clamp attached to the 5′ end using the conditions and parameters described by Anderson et al. (2003). Negative controls (containing no DNA template) were included in each reaction run.

2.6. DGGE analysis

DGGE analysis was performed on PCR products from each fraction using a DCode universal mutation detection system (Bio-Rad Laboratories, Hertfordshire, UK) as described by Bastias et al. (2006a) using a 20–60% denaturing gradient and a standard marker constructed from nested PCR products from DNA from fungal cultures. DGGE gels were silver stained and banding patterns of digitalised gel images for each fraction were analysed using Phoretix 1D Advanced software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). A binary matrix based on the presence/absence of bands was produced for each DGGE gel. The binary data were then subjected to principal coordinate analysis using unconstrained metric multi-dimensional scaling of Bray–Curtis distances. Canonical analysis of the principal coordinates (CAP) was subsequently conducted using the discriminant analysis option (Anderson and Willis, 2003). All analyses were conducted using the CAP program with 999 permutations (Anderson, 2002).

2.7. Reamplification and sequencing of excised bands

Selected ITS DGGE bands from the 2-yr burn fractions were excised from SYBR gold stained DGGE gels (McCaig et al., 1999). Excised bands were placed into 50 μl of milli-Q water and used as the template for PCR amplification. Re-amplified PCR products were re-run on DGGE gels (as described above) along with the corresponding fraction (i.e. either fraction 4 or 9) to ensure the amplified product corresponded to the target DGGE band from the original profile. PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega, Southampton, UK) according to the manufacturer's instructions and sequenced using either an Applied Biosystems 3730xl or 3700 sequencer (Applied Biosystems, Warrington, UK). Sequences were analysed using the FastA algorithm to determine closest sequence identities in the EMBL nucleotide database.

3. Results

IRMS analysis of RNA extracted from soil (from both unburned and 2-yr burn plots) that was incubated with ^{13}C-cellulose had higher δ^{13}C values than RNA extracted from the same soil prior to incubation (Table 1), demonstrating that RNA from the ^{13}C-cellulose treated soils was highly enriched with ^{13}C.

Following nested PCR of ITS1-F/ITS4 RT-PCR products, DGGE profiles of fractions 3–10 from unburned and 2-yr burn samples comprised *ca* 30–61 and 6–44 distinct bands, respectively (Figs. 1 and 2). Repeat SIP density gradient centrifugation, PCR amplification and DGGE analysis yielded reproducible profiles (data not shown). Previous work indicates that the density gradient centrifugation and fractionation protocols used here yield a gradient rather than a clear sharp separation between ^{12}C and ^{13}C fractions (Rangel-Castro et al., 2005) which is consistent with the observations of others (Manefield et al., 2002). The majority of the total accountable ^{13}C in such a gradient is, however, present in fractions 3–6 while fractions 7–10 are predominantly ^{12}C (Rangel-Castro et al., 2005). Hereafter, we therefore refer to fractions 3–6 as ^{13}C and 7–10 as ^{12}C. All fractions across the ^{12}C/^{13}C gradient from the unburned samples had complex DGGE profiles. In contrast,

Table 1
Atom% ^{13}C values of RNA extracted from unamended soil and soil incubated with ^{13}C-cellulose.

RNA sample	Atom% ^{13}C (mean ± s.d.)
2-year burn soil	1.097 ± 0.001
Unburned soil	1.097 ± 0.002
2-year burn soil (+^{13}C-cellulose)	1.353 ± 0.039
Unburned soil (+^{13}C-cellulose)	1.237 ± 0.105

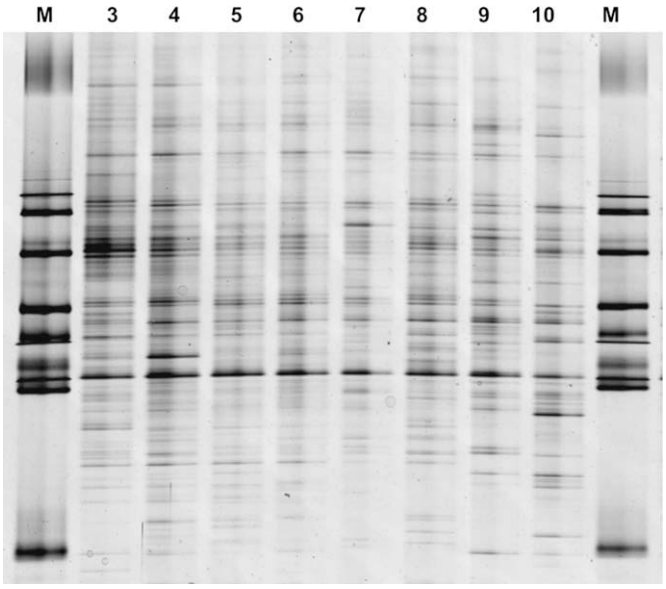

Fig. 1. DGGE patterns of partial fungal ITS regions amplified from soil RNA extracts (unburned plot) following incubation of the soil with ^{13}C-cellulose. Fractions 3–6 (^{13}C) and 7–10 (^{12}C) were obtained by ultracentrifugation of CsTFA density gradients and subsequent fractionation. M represents a marker constructed using DNA from fungal cultures.

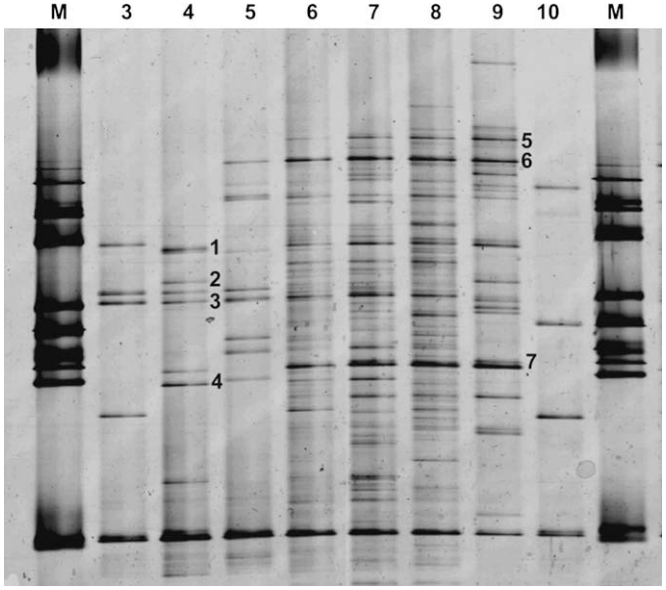

Fig. 2. DGGE patterns of partial fungal ITS regions amplified from soil RNA extracts (2-yr burn plot) following incubation of the soil with ^{13}C-cellulose. Fractions 3–6 (^{13}C) and 7–10 (^{12}C) were obtained by ultracentrifugation of CsTFA density gradients and subsequent fractionation. M represents a marker constructed using DNA from fungal cultures. Numbers indicate bands that were excised and sequenced (Table 2).

while DGGE profiles for the ^{12}C fractions were complex, these became simpler in the ^{13}C fractions with fraction 3 containing only 5 discrete bands (Figs. 1 and 2). CAP analysis indicated a significant separation of DGGE profiles along both axis 1 and 2 ($p = 0.04$). Unburned samples were separated from 2-yr burn samples along the first canonical axis which explained 35% of the total variation. Profiles from fractions 3–6 (^{13}C-labelled) and fractions 7–10 (^{12}C-labelled) from 2-yr burn samples were further separated from each other along canonical axis 2 which explained 14.3% of the total variation (Fig. 3). In contrast, there was no separation between ^{12}C- and ^{13}C-labelled fractions from the unburned samples (Fig. 3).

Sequences corresponding to seven ITS DGGE bands from the 2-yr burn SIP fractions (Fig. 2) were obtained (Table 2). Of the few bands that were present in the ^{13}C fractions, sequences were obtained from four bands and, although sequence matches for three of these were relatively low (≤93%), these were most similar to sequences for *Cryptococcus podzolicus*, *Monacrosporium sichuanense* and *Metarhizium anisopliae* (Table 2). Bands at positions similar to these were also present in some of the ^{12}C fractions (Fig. 2). Three bands which were intense in the ^{12}C fractions but decreased in relative intensity in the ^{13}C fractions (bands 5–7) were most similar to ITS sequences of *Cryptococcus* spp. and *Monacrosporium ellipsosporum* (Table 2). With the exception of band 6 (99% sequence identity to *C. podzolicus*), sequence matches were relatively low (≤93%).

Table 2
Closest FastA matches of sequenced ITS DGGE bands from 2-yr burn SIP fractions to reference sequences in the EMBL database. Band numbers correspond to those in Fig. 2.

Band no.	Accession code	Size (bp)	FastA closest match	Sequence similarity (%)	Overlap (bp)
1[a]	EU247515	173	*Metarhizium anisopliae* (EF051711)	93	162
2[a]	EU247516	150	*Cryptococcus podzolicus* (AB035576)	99	138
3[a]	EU247517	177	*Monacrosporium sichuanense* (AY902795)	83	179
4[a]	EU247518	220	*Monacrosporium sichuanense* (AY902795)	92	220
5	EU247519	152	*Cryptococcus* sp. (AM262437)	93	145
6	EU247520	145	*Cryptococcus humicolus* (AB035575)	99	134
7	EU247521	219	*Monacrosporium ellipsosporum* (DQ999832)	75	167

[a] Bands present in ^{13}C fractions.

4. Discussion

Fungi are important cellulolytic microorganisms in forest soils and, in this investigation, we utilised RNA-SIP to demonstrate the influence of a forest management strategy (repeated prescribed burning) on soil fungi that obtain carbon from cellulose. While there are numerous reports of the use of nucleic acid SIP to investigate soil bacteria (e.g. Buckley et al., 2007; Cébron et al., 2007), to date there are only two records of the application of the methods for analysis of soil fungal communities (Lueders et al., 2004; Rangel-Castro et al., 2005) although there has been a recent report of the use of this approach to demonstrate movement of plant-derived C to root inhabiting symbiotic mycorrhizal fungi (Vandenkoornhuyse et al., 2007). DNA- and RNA-SIP were recently used to investigate bacterial utilisation of ^{13}C-labelled wheat residue (Bernard et al., 2007) and DNA-SIP to identify cellulolytic bacteria in an agricultural soil (Haichar et al., 2007) but, as far as we are aware, this investigation is the first to utilise an insoluble high molecular weight substrate such as cellulose to target a functional group of fungi in soil using RNA-SIP.

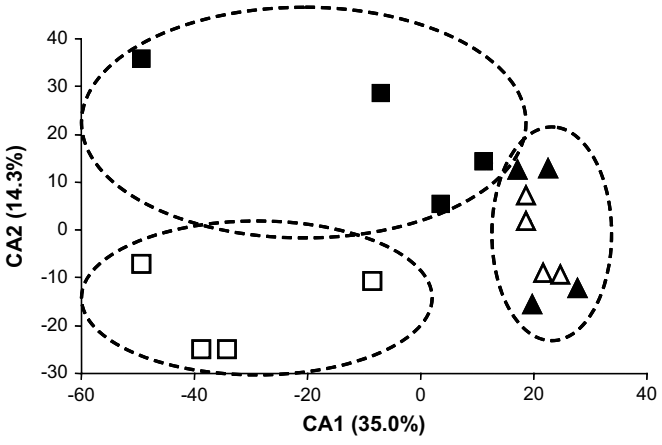

Fig. 3. Plot of the first two canonical axes produced by canonical analysis of principal coordinates (CAP) of DGGE profiles for partial ITS sequences from RNA extracted from unburned (triangles) and 2-yr burn (squares) soil incubated with ^{13}C-cellulose ($p = 0.04$). Open symbols represent fractions 3–6 (^{13}C) and closed symbols represent fractions 7–10 (^{12}C).

IRMS clearly revealed that RNA extracted from soil that had been incubated with ^{13}C-cellulose was considerably enriched with ^{13}C compared to RNA from unamended soil from both the 2-yr burn and unburned plots at the Peachester site. This indicates that the fractionation of RNA that occurred during density gradient centrifugation was due to differences in ^{13}C–^{12}C incorporation into RNA rather than differences in GC content (Holben et al., 2004) but further individual IRMS analyses on individual fractions would be required to confirm this. This notwithstanding, previous research using similar ultracentrifugation conditions to those used here has shown that fractions 3–6 are predominantly ^{13}C, while fractions 7–10 are predominantly ^{12}C although there is a gradient rather than a sharp separation between ^{12}C and ^{13}C fractions (Manefield et al., 2002; Rangel-Castro et al., 2005). The similarity of the DGGE profiles in the ^{13}C and ^{12}C fractions from the unburned plot provides further evidence that GC content did not significantly bias the fractionation.

Ordination by CAP indicated that DGGE profiles for both ^{13}C and ^{12}C fractions from the 2-yr burn plot differed significantly from those of the unburned plot, which is consistent with previous DNA-DGGE- and DNA-T-RFLP-based investigations of soil fungal communities at the Peachester site. Thus repeated prescribed burning over a period of >30 yr has been shown to significantly alter communities of total soil fungi, soil basidiomycetes and ectomycorrhizal fungi (Bastias et al., 2006a,b; Anderson et al., 2007). Since analysis of fungal RNA extracted directly from soil targets active fungi in soil (e.g. Pennanen et al., 2004; Anderson and Parkin, 2007), the RNA-based data from the present study further indicate that the 2-yr burning treatment significantly altered the active fungal community in the upper 10 cm of soil at Peachester forest. We are mindful, however, that our experimental approach of sieving soil prior to incorporation of ^{13}C-cellulose is likely to have disrupted established mycelial networks, thus the fungi that were most active during soil incubation may not necessarily reflect those that were most active in the field situation.

The lack of significant separation of the DGGE profiles for ^{13}C and ^{12}C fractions from the unburned plot in the CAP ordination suggests that the fungal communities identified in both were broadly similar. Since ^{12}C fractions are considered to represent the total fungal community (Rangel-Castro et al., 2005), this implies

that most of the active fungi in soil from the unburned plot incorporated ^{13}C from the labelled cellulose. In contrast, the ordination revealed a significant separation of DGGE profiles for the ^{13}C and ^{12}C fractions from the 2-yr burn plot. Together with the fact that there were far fewer bands in DGGE profiles for the ^{13}C fractions than for the ^{12}C fractions, this suggests that fewer active fungi in the 2-yr burn plots incorporated ^{13}C from the labelled cellulose.

Incorporation of ^{13}C from ^{13}C-cellulose into fungal RNA does not necessarily indicate that the fungi were involved in primary assimilation of the substrate. Given the 35-d duration of the incubation, it is perhaps likely that some of the fungi may have incorporated ^{13}C from degradation products following degradation of cellulose by those involved directly in primary assimilation. Three of the bands that were excised from the 2-yr burn ^{13}C fractions and sequenced had similarity to *Cryptococcus* spp. and *Monacrosporium* spp., although for the latter the sequence similarity was relatively low. There is evidence that some members of each genus either utilise cellulose or produce cellulolytic enzyme activities (e.g. Khalid et al., 2006; Nakase et al., 1996; Zacchi et al., 2003), suggesting that these fungi may have contributed to primary degradation of the ^{13}C-labelled cellulose. In contrast, while sequence similarity was relatively low, one of the sequenced bands from the 2-yr burn ^{13}C fractions was most closely related to the entomopathogenic genus *Metarhizium*, which does not appear to have cellulolytic abilities (St Leger et al., 1997). It is therefore possible that this fungus derived ^{13}C via an indirect pathway.

The fact that fewer active fungi in the 2-yr burn plots incorporated ^{13}C compared to the unburned plots strongly suggests that diversity of cellulolytic fungi was reduced by repeated prescribed burning every two years over a period of >30 years. Since previous chemical analyses of soils from the Peachester site revealed significant reductions in total soil carbon in 2-yr burn plots compared to unburned plots (Guinto et al., 2001; Bastias et al., 2006a), this may reflect reduced availability of cellulose substrate in the 2-yr burn plots.

In conclusion, our data, obtained using stable isotope probing, indicated that repeated prescribed burning affects the diversity of an important functional group of soil fungi (cellulolytic fungi) that are key drivers of forest soil decomposition and carbon cycling processes. These observations, along with future investigations of decomposition of complex organic matter, will be critical in understanding the responses of decomposer organisms and carbon cycling processes to forest fires. This is particularly important given that forest fire frequency in many locations is expected to increase in line with current climate change predictions (e.g. Pitman et al., 2007).

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