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ARTICLE

Ectomycorrhizal fungal hyphae communities vary more along a pH and nitrogen gradient than between decayed wood and mineral soil microsites[1]

Jennifer K.M. Walker, Lori A. Phillips, and Melanie D. Jones

Abstract: Ectomycorrhizal (ECM) fungal community composition is structured by soil properties, but specialization for woody microsites by ECM fungi is equivocal. Because fungal mycelia explore the substrate and colonize nutrient patches, studies targeting ECM fungal hyphae may reveal niche preferences. Moreover, studying the distribution and composition of ECM fungal hyphal communities contributes to our understanding of nutrient cycling in forest soils. We used next-generation sequencing to determine whether the composition of forest floor fungal communities present as hyphae differed among three microsite types: decayed wood, mineral soil adjacent to intact logs, or control mineral soil of mature spruce forests in British Columbia. The microsites were located in three blocks that were separated by 1 km and varied in elevation. Across the site, the ECM fungal lineage /amphinema–tylospora was the most operational taxonomic unit (OTU)-rich group, while the saprotrophic order Mortierellales was also dominant. ECM fungal species differed among microsites. For example, ECM fungal OTUs identified as *Tylospora fibrillosa* and *Russula curtipes* were more frequent in decayed wood as compared with control mineral soil. However, ECM fungal communities were more strongly structured by block characteristics, and we conclude there is no distinct group of ECM fungi specializing in the soil microsites examined in this forest.

Key words: next-generation sequencing, forest soil, fungal hyphae, *Picea engelmannii*, ectomycorrhizal ecology.

Résumé : La composition d'une communauté fongique ectomycorhizienne (ECM) est structurée par les propriétés du sol, mais la spécialisation des ECM envers des microsites ligneux est équivoque. Parce que les mycéliums fongiques explorent le substrat et colonisent les parcelles comportant des nutriments, des études ciblant les hyphes fongiques ECM peuvent révéler leurs préférences sur le plan des niches. De plus, l'étude de la distribution et de la composition des communautés hyphales fongiques d'ECM contribue à notre compréhension du cycle des nutriments dans les sols forestiers. Nous avons utilisé le séquençage de dernière génération afin de déterminer si la composition des communautés fongiques du sol forestier présentes sous forme d'hyphe différait en fonction de trois types de microsites : le bois en décomposition, le sol minéral adjacent aux billes intactes ou le sol minéral contrôle de forêts matures d'épinettes en Colombie-Britannique. Les microsites étaient localisés dans trois blocs séparés d'un km et variaient sur le plan de l'élévation. À travers le site, le lignage fongique /amphinema–tylospora d'ECM était le groupe le plus riche en unités taxonomiques opérationnelles (UTO), alors que l'ordre saprotrophe des Mortierellales était aussi dominant. Les espèces d'ECM fongiques différaient en fonction du microsite. Par exemple les UTO fongiques d'ECM identifiées comme *Tylospora fibrillosa* et *Russula curtipes* étaient les plus fréquentes sur le bois en décomposition comparativement au sol minéral contrôle. Toutefois, les communautés fongiques d'ECM étaient plus fortement structurées par les caractéristiques du bloc, et nous concluons qu'il n'existe pas de groupe fongique distinct de d'ECM se spécialisant dans le sol des microsites examinés dans cette forêt. [Traduit par la Rédaction]

Mots-clés : séquençage de dernière génération, sol forestier, hyphes fongiques, *Picea engelmannii*, écologie des ectomycorhizes.

Introduction

The extensive mycelia of soil fungi play an important role in forest decomposition and nutrient cycling by releasing numerous degradative enzymes and subsequently absorbing soluble molecules (Carlile et al. 2001). Fungal saprotrophs break down complex forms of dead organic matter, including wood, and biotrophic mycorrhizal fungi provide their host plants with mineral nutrients such as nitrogen (N) and phosphorus (P) (Lindahl et al. 2007; Smith and Read 2008). Ectomycorrhizal (ECM) fungi may also play a role in decomposition (Talbot et al. 2008; Cullings and Courty 2009) and have been shown to translocate photosynthesis-derived carbon (C) into the soil (Jones et al. 1991; Clemmensen et al. 2013) and to other plants (Finlay and Read 1986). Although much work has been done on enzyme activities associated with ECM fungal root tips (Buée et al. 2007; Jones et al. 2012), enzyme production and nutrient absorption occur primarily in the extramatrical ECM fungal hyphae (Agerer 2001; Genney et al. 2006). Therefore, studying the distribution and composition of this important component of the fungal community contributes to our understanding of nutrient cycling in forest soils (Talbot et al. 2013).

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The distribution of ECM fungi is spatially patchy (Lilleskov et al. 2004; Pickles et al. 2010) and can be limited for some species by dispersal strategy (Peay et al. 2010, 2012), size of genet (Hortal et al. 2012), and exploration type (Agerer 2001; Tedersoo et al. 2010*a*). It is well established that ECM fungal communities can vary vertically (Rosling et al. 2003; Baier et al. 2006; Lindahl et al. 2007) and horizontally (Genney et al. 2006; Pickles et al. 2010) in soil on a variety of spatial scales. ECM fungal community composition can be strongly structured by deterministic processes (Bruns 1995; Koide et al. 2011), and some ECM fungal species have been detected in discrete soil niches (Tedersoo et al. 2003, 2008; Buée et al. 2007; Iwański and Rudawska 2007). Specialist wood decomposers are well characterized (Tanesaka et al. 1993; Fukami et al. 2010), but specialization for woody microsites by ECM fungi is equivocal (Talbot et al. 2013; Walker and Jones 2013), despite the potential wood-degrading enzymatic capabilities of ECM fungal root tips (Talbot et al. 2008; Cullings and Courty 2009). Because extramatrical fungal mycelia explore the substrate and subsequently colonize potential nutrient patches (Finlay and Read 1986), studies targeting ECM fungal hyphae may reveal stronger niche development as compared with root tips.

Next-generation sequencing (NGS) is very effective at detecting fungal hyphae (Peay et al. 2008; Blackwell 2011) and is providing a much more intensive picture of soil fungal communities than was possible with culture-dependent methods. Pyrosequencing studies of forest soil fungal communities have ranged from Swedish spruce (Wallander et al. 2010) and European hardwood (Buée et al. 2009) plantations to tropical African (Tedersoo et al 2010*b*) forests. In all cases, tremendous diversity was revealed. In the current study, we used next-generation pyrosequencing to sample and identify forest floor hyphae from woody and mineral soil microsites. This first NGS study of soil fungal communities in the Engelmann spruce – subalpine fir (ESSF) biogeoclimatic zone complements only one other comprehensive study of British Columbia forest soils (Hartmann et al. 2012). The specific objective of this study was to determine whether the composition of forest floor fungal communities present as hyphae differed among three microsite types: decayed wood, mineral soil adjacent to intact logs, and control mineral soil (i.e., greater than 1 m from any woody substrate) of mature ESSF forests at Sicamous Creek, British Columbia. We focused on the role of downed and decayed wood in this spruce forest system because it provides critical habitat for ECM fungal root tips (Harvey et al. 1979; Goodman and Trofymow 1998), and may also be important for structuring the ECM fungal community present as hyphae, which directly impacts plant nutrition (Smith and Read 2008). The microsites were located in 1 ha plots in three blocks, which were separated by over 1 km and varied in elevation by hundreds of metres. We hypothesized that while the fungal community would vary across the blocks, there would be specific ECM fungal species that characterized communities in decayed wood, hard downed wood, and mineral soil microsites within blocks.

Material and methods

Field site description and experimental design

The field site comprised three replicate 1 ha mature forest blocks within the 150 ha Sicamous Creek Silvicultural Systems Trial experimental area (http://www.for.gov.bc.ca/rsi/research/sicamous/index.htm). This high elevation forest is located in the Engelmann spruce – subalpine fir wet-cold4 biogeoclimatic zone (ESSFwc4), which is characterized by long snowy winters. A detailed description of this subzone can be found at http://www.for.gov.bc.ca/hre/becweb/resources/classificationreports/subzones/index.html. We established these blocks in 2007, 30 m inside the margin of cutblocks originally created in 1996. The forest blocks range in elevation and aspect from 1580 m a.s.l., northwest-facing (Block A), through 1675 m a.s.l., north-facing (Block B), to 1770 m a.s.l., west-facing (Block C) (Hollstedt and Vyse 1997), and they are located approximately 1 km apart. These unlogged areas are dominated by subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.), with a smaller proportion of Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.), a white rhododendron (*Rhododendron albiflorum* Hook.), and a huckleberry/blueberry (*Vaccinium* spp. L.) understory (Craig et al. 2006). Soils are Humo-Ferric Podzols, and the current forest began to re-establish after a fire event more than 350 years ago (Hollstedt and Vyse 1997).

Hybrid *Picea engelmannii* × *Picea glauca* (Moench) Voss (native interior hybrid spruce) were grown from surface-sterilized seed (seedlot number 26212; B.C. Ministry of Forests Seed Center, ID DWD 20070064A) in sterile 1:1 peat–vermiculite potting mix in a greenhouse. In early July 2007, 8-week-old seedlings were gently shaken free of the potting mix and planted in each of three different microsite types at randomly selected locations within each of the 1 ha forest blocks. Control microsites comprised primarily mineral soil located at least 50 cm from any visible downed wood. Downed wood microsites were mineral soil within 5 cm, and on the northern and downhill side, of a piece of downed wood at least 10 cm in diameter and of the BC Ministry of Forests, Lands and Natural Resources Vegetation Resource Inventory decay class 1–3 (http://www.for.gov.bc.ca/hts/vri/standards/gs_vri.html). Decay class 1–3 ranges from hard, intact logs with bark and twigs attached, to sagging, partly decayed logs with roots invading the sapwood. The decayed wood microsites were within wood of Vegetation Resource Inventory decay class 4, 5, or beyond: sunken logs that are no longer round and that have roots invading the heartwood, as well as small, soft portions of wood on the ground.

Seedling harvesting and sample processing

Five replicate seedlings were harvested from each of the microsite types in all three forest blocks (5 × 3 × 3 = 45 seedlings with their surrounding substrate) in late August through mid-September 2009, two years after planting. For every seedling harvested, a 10 cm wide × 10 cm wide × 20 cm deep block of soil and/or decayed wood, including all seedling roots, was cut with a pruning saw and transported to the lab on ice.

Samples were stored in the lab at 4 °C and processed within two days of collection. Seedlings were gently teased apart from the surrounding soil or decayed wood, and then the bulk substrate fraction (i.e., the substrate that fell away from the roots with gentle shaking) was collected. The bulk substrate sample was passed through a 2 mm sieve, and any visible sclerotia or roots of other plants were removed, leaving behind primarily fungal hyphae, but also spores. Approximately 20 g of this root-free substrate was ground with a sterilized mortar and pestle until it was of uniform size and colour. Of this, 10 g was used for dry mass calculations (65 °C for 24 h) and subsequent chemical analyses, and 1.0 g was frozen at –80 °C in a 1.5 mL microcentrifuge tube for subsequent fungal hyphae DNA extraction.

Microsite abiotic properties

Air-dried and ground subsamples (45 × 10 g) of the bulk substrate fraction were individually tested for mineralizable N (ammonium, 1 mol·L^{-1} KCl anaerobic incubation), available nitrate-N and ammonium-N (2 mol·L^{-1} KCl extraction), available phosphate-P (Bray P-1), and total C and N (combustion elemental analysis) at the Analytical Chemistry Services Lab of the British Columbia Ministry of Forests and Range, Victoria, B.C., and for pH (H_20) in-house. There was sufficient material to allow further testing of a subset of these (i.e., substrate was available from 3 replicate seedlings × 3 microsite types × 3 blocks = 27 samples). Loss-on-ignition (Schulte and Hopkins 1996) and the chemistry of the organic C fraction were also analysed in-house via proximate analysis into polar extracts (PE) comprising simple sugars, proteins, and polyphenols, and non-polar extracts (NPE) comprising fats and waxes (Trofymow et al. 1995). The acid-soluble fraction

(AS), representing cellulose and hemicellulose, and acid-insoluble fraction (AIS), representing lignin, cutin, and humics, were also included in the proximate analysis (Trofymow et al. 1995).

Molecular identification of fungi from soil samples

DNA was extracted from previously frozen subsamples (45 × 1.0 g) of the root-free bulk substrate with the MoBio Ultra Clean Soil DNA Extraction Kit using the alternative protocol for maximum yields (MoBio Laboratories Inc., Carlsbad, California, USA). Polymerase chain reaction (PCR) amplification of the fungal internal transcribed spacer (ITS)1 region was performed in triplicate for each sample and subsequently pooled. To distinguish samples during downstream data analysis, a unique multiplex identifier (MID) tag was used for each of the 15 samples within each block. Every 50 μL PCR reaction mixture included 5.0 μL 10X buffer, 1.0 μL 10 mmol·L^{-1} dNTPs, 2.0 μL 50 mmol·L^{-1} $MgCl_2$, 1.0 μL 10 μmol·L^{-1} of each forward and reverse fusion primer, 1 U Platinum *Taq* polymerase (Invitrogen Corporation, Carlsbad, California, USA), and 1.0 to 2.0 μL of template DNA (for a final concentration of 0.2 ng·μL^{-1}). The sequence of the forward fusion primer was 5′-CCATCTCATCCCTGCGTGTCTCTCCGACTCAG (Titanium A Primer) XXXXXXXXXX CTTGGTCATTTAGAGGAAGTAA (ITS1F)-3′ (Gardes and Bruns 1993), where "XXX…" represents one of 15 unique MID tags. The reverse primer for all reactions was 5′-CCTATCCCCTG TGTGCCTTGGAGTCTCAG (Titanium B Primer) GCTGCGTTCTTCA TCGATGC (ITS2)-3′ (White et al. 1990). Thermocycler conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min, and a final extension step of 68 °C for 10 min. Samples yielding good quality single bands of expected size (i.e., as visualized on 1% agarose gels) were cleaned with the Agencourt Ampure XP magnetic bead PCR purification system (Beckman Coulter Inc., Danvers, Massachusetts, USA), gel-checked again for band quality, primer dimers, and other low molecular mass product, and quantified against a low mass DNA ladder and with a NanoDrop micro-volume spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). The final 20 ng·μL^{-1} sample from each block, containing an equimolar mixture of 15 pooled amplicon samples, each representing the bulk substrate fraction from one seedling, was amplified in a ¼ plate pyrosequencing reaction on a Roche GS-FLX at the McGill University and Génome Québec Innovation Center.

Sequence processing and phylogenetic-based naming

Sequence and quality files from the NGS were imported into MOTHUR version 1.16.0 (Schloss et al. 2009), where primers were removed, and sequences were filtered and trimmed (min 100 bp, max 400 bp, pdiffs = 1, maxambig = 0, maxhomop = 4). The Fungal ITS Chimera Checker (Nilsson et al. 2010*a*) and Fungal ITS Extractor (Nilsson et al. 2010*b*) were used to isolate the ITS1 region, and these sequences were submitted to the ITS Pipeline (Nilsson et al. 2009) for matches to the GenBank database (BLASTn, Altschul et al. 1997) with and without uncultured fungi. The ITS Pipeline matches fungal ITS sequences to the GenBank database, and then groups those that share 50% of their top 15 closest BLAST database hits, based on their taxonomic names. Groups of closely related ITS1 sequences were aligned using MAFFT version 5 (Katoh et al. 2002), then assembled into distance matrices and clustered in MOTHUR (countends = F, cutoff = 0.10). The appropriate similarity cutoff for grouping sequences into operational taxonomic units (OTUs) was determined by plotting a taxon accumulation curve for a range of sequenced similarities: 91% through 99% in MOTHUR (Buée et al. 2009; Jumpponen and Jones 2009; Amend et al. 2010; Tedersoo et al. 2010*b*). We selected a 95% sequence similarity cutoff curve, which did not reach an asymptote (highly unlikely for these data), yet did not increase exponentially either, and used this to define the OTUs prior to naming them. It is more common to employ 97% (Buée et al. 2009; Tedersoo et al. 2010*b*), but 95% has also been used (Jumpponen and Jones 2009), and the correct choice is often ambiguous (Amend et al. 2010). One randomly chosen representative sequence from each OTU was imported into MEGAN version 4.40.1 (Huson et al. 2007) for taxonomic placement (based on lowest common ancestor (LCA) parameters: min support = 1, minimum score = 200, top percent = 10, disable = environmental samples). Once the OTUs were grouped taxonomically by MEGAN, we used a conservative approach for subsequent analysis to acknowledge short NGS reads (e.g., median >250 bp; 97th percentile <340 bp): most non-ECM OTUs were identified at the level of order, and OTUs identified as ECM taxa were named according to fungal lineage (comparable with family or order, Tedersoo et al. 2010*a*).

Statistical analyses

The experimental design was a balanced mixed model with two factors: block (random) and microsite (fixed). Abiotic properties were tested using a multivariate ANOVA for non-independent variables, followed by univariate ANOVAs and post-hoc Tukey's honestly significant difference (HSD) tests when microsite or block effects were significant at $P \leq 0.05$ (R version 2.15.0; R Core Team 2012). Fungal community data (presence–absence) were analysed using permutational multivariate ANOVA (adonis, in the vegan package of R; Oksanen et al. 2012) followed by univariate analyses of individual fungal OTUs (anova, R; R Core Team 2012). Read abundance was transformed to presence–absence for analyses of community composition, because read abundance from pyrosequencing is not directly related to the abundance of DNA of a taxon (Amend et al. 2010). Soil chemical data (with the exception of pH and % organic matter) were log-transformed to improve normality and minimize variance, and a Bonferroni correction was applied when considering results of univariate tests of non-independent soil properties. The six soil chemical properties related to C, N, and P were considered to be non-independent of each other, as were the four C fraction variables. Nonmetric multidimensional scaling (NMDS) in the vegan package of R version 2.15.0 (Oksanen et al. 2012; R Core Team 2012) was used to visually explore the relative relationships among fungal communities.

Results

Soil abiotic properties

The three types of microsites varied in their chemistry, with decayed wood generally differing from mineral soil (i.e., control mineral soil and (or) mineral soil beside hard downed wood; Tables 1*a*, 2*a*, and Supplementary Table S1[2]). Not surprisingly, total C and % organic matter were highest, and pH was lowest, in decayed wood. Of the components of organic matter, polar extractables (representing simple sugars, proteins, and polyphenols) were highest, and the acid-insoluble fraction (representing lignin, cutin, and humics) was lowest, in decayed wood (Table 2*a* and Supplementary Table S1*b*[2]). None of the mineral nutrients differed among microsites after Bonferroni correction for multiple variables (Table 1*a* and Supplementary Table S1*a*[2]). Significant block effects were detected only for extractable NO_3–N and pH, which were lowest at block A (Tables 1*b*, 2*b*, and Supplementary Table S1[2]).

ITS reads and fungal OTU richness

Rarefaction curves based on 95% molecular sequence similarity over the entire site (not shown) and per microsite per block (Supplementary Fig. S1[2]) did not reach an asymptote, suggesting that many fungal taxa remain undetected in spite of the deep sequenc-

[2]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2013-0239.

Table 1. Soil chemical properties* compared by (*a*) microsite and (*b*) block in a mature spruce-fir forest.

	%		Soil extracts (mg·kg^{-1})			
	Total C	Total N	Extractable P	Extractable NH_4–N	Extractable NO_3–N	Mineralizable N
(*a*)						
Microsite						
Control soil	17.8 (11.6)a	0.78 (0.44)	11.8 (9.1)	24.5 (20.0)	29.1 (29.1)	352.9 (173.2)
Downed wood	26.0 (16.0)a	1.02 (0.54)	16.3 (10.0)	41.1 (33.0)	20.6 (29.0)	462.2 (209.8)
Decayed wood	54.3 (7.8)b	0.71 (0.17)	18.3 (7.4)	21.2 (20.9)	17.2 (9.5)	272.0 (92.6)
P	**<0.001**	0.19	0.03	0.06	0.20	0.03
(*b*)						
Block						
A	34.1 (19.7)	0.9 (0.5)	13.8 (9.7)	38.3 (30.1)	16.4 (25.0)a	408.9 (191.6)
B	33.2 (22.0)	0.8 (0.4)	17.2 (7.6)	17.9 (9.6)	16.6 (6.4)b	297.7 (134.1)
C	30.8 (18.9)	0.9 (0.4)	15.4 (10.1)	30.7 (30.7)	33.9 (31.3)b	380.5 (200.6)
P	0.91	0.78	0.20	0.07	**0.007**	0.26

*Values represent untransformed means (SD), but *P* values are based on log-transformed data analysed with a univariate ANOVA after significant MANOVA (Block and Microsite $P \leq 0.006$). Significance after Bonferroni correction for these six non-independent variables is <0.01. Lowercase letters denote significant differences ($P \leq 0.05$) based on post-hoc Tukey's honestly significant difference tests; $n = 5$.

Table 2. Soil pH, % organic matter, and carbon fraction* for (*a*) microsites and (*b*) blocks in mature forest blocks.

			Soil extracts (mg·g^{-1})			
	pH (H_2O)	% Organic matter	Non-polar extractables	Polar extractables	Acid-soluble	Acid-insoluble
(*a*)						
Microsite						
Control soil	4.9 (0.57)a	28.0 (10.9)a	11.6 (9.8)	25.9 (13.0)a	215.1 (91.4)	791.4 (67.5)a
Downed wood	4.7 (0.51)a	42.3 (24.1)a	18.0 (14.0)	36.9 (25.1)a	171.1 (58.0)	730.0 (121.5)ab
Decayed wood	4.4 (0.41)b	96.0 (2.6)b	20.3 (8.2)	73.8 (15.7)b	261.3 (42.8)	644.6 (57.9)b
P	**0.003**	**<0.001**	0.19	**<0.001**	0.05	**0.01**
(*b*)						
Block						
A	4.2 (0.3)a	60.7 (32.0)	24.2 (12.5)	58.9 (31.9)	214.3 (96.0)	702.6 (137.1)
B	4.8 (0.4)b	51.6 (34.3)	16.8 (9.5)	38.5 (20.5)	216.8 (80.0)	727.9 (104.6)
C	4.8 (0.6)b	54.0 (36.8)	8.9 (5.1)	39.2 (26.9)	216.3 (50.0)	735.6 (67.0)
P	**<0.001**	0.48	0.02	0.04	0.98	0.75

*Values represent untransformed means (SD), but *P* values are based on log-transformed data analysed with a univariate ANOVA after significant MANOVA (Block and Microsite $P \leq 0.002$). Significance after Bonferroni correction for the four non-independent variables (i.e., the carbon fractions only) = 0.01. Lowercase letters denote significant differences ($P \leq 0.05$) based on post-hoc Tukey's honestly significant difference tests; $n = 3$.

ing effort. Following all sequence editing, 406 467 ITS reads, comprising 13 869 OTUs summed across three blocks, remained in the data set (Supplementary Table S2[2]). Over 50% of the OTUs were singletons and doubletons. Collectively, OTUs with greater than 100 reads represented 73% of total reads (Supplementary Table S2[2]). There were 267 OTUs with at least 100 reads used for subsequent analysis. These represented 17 ECM fungal lineages (Tedersoo et al. 2010*a*) and 20 other identified fungal orders, classes, or phyla. Among these, there were 99 ECM fungal species, 83 other (saprotrophic, endophtyic, or pathogenic) fungi, and 85 OTUs that remained unidentified. Across all samples, the most OTU-rich fungal group (i.e., based on number of OTUs) was the ECM fungal lineage /amphinema–tylospora (Table 3 and Supplementary Table S3[2]). The saprotrophic order Mortierellales was also dominant in these soils (Table 3 and Supplementary Table S3[2]). In addition to those shown in Table 3, the ECM lineages /meliniomyces (2.0%), /wilcoxina (1.1%), and /tomentella–thelephora (0.9%), were among the groups containing OTUs with greater than 100 reads, but that made up only a small percentage of the community overall based on OTU richness (Supplementary Table S3[2]).

Table 3. Dominant ectomycorrhizal (ECM) fungal lineages (Tedersoo et al. 2010*a*) overall and per block based on OTU richness (i.e. the number of operational taxonomic units (OTUs) represented by each fungal group divided by the total number of OTUs per sample), and compared with the OTU richness of the saprotrophic order Mortierellales.

ECM lineage or fungal order[a]	Overall	Block A	Block B	Block C
/amphinema–tylospora	16.2%	20.3%	19.4%	11.0%
/russula–lactarius	7.0%	5.3%	4.3%	10.6%
/hygrophorus	6.6%	14.3%	5.1%	3.9%
/piloderma	4.7%	10.5%	5.5%	0.8%
/pseudotomentella	3.7%	3.8%	5.5%	2.0%
/inocybe	2.3%	1.5%	1.6%	3.5%
/cortinarius	2.2%	3.0%	2.0%	2.0%
Mortierellales	10.8%	6.8%	8.7%	14.9%
Percentage of fungal community[b]	**53.5%**	**65.4%**	**52.2%**	**48.6%**

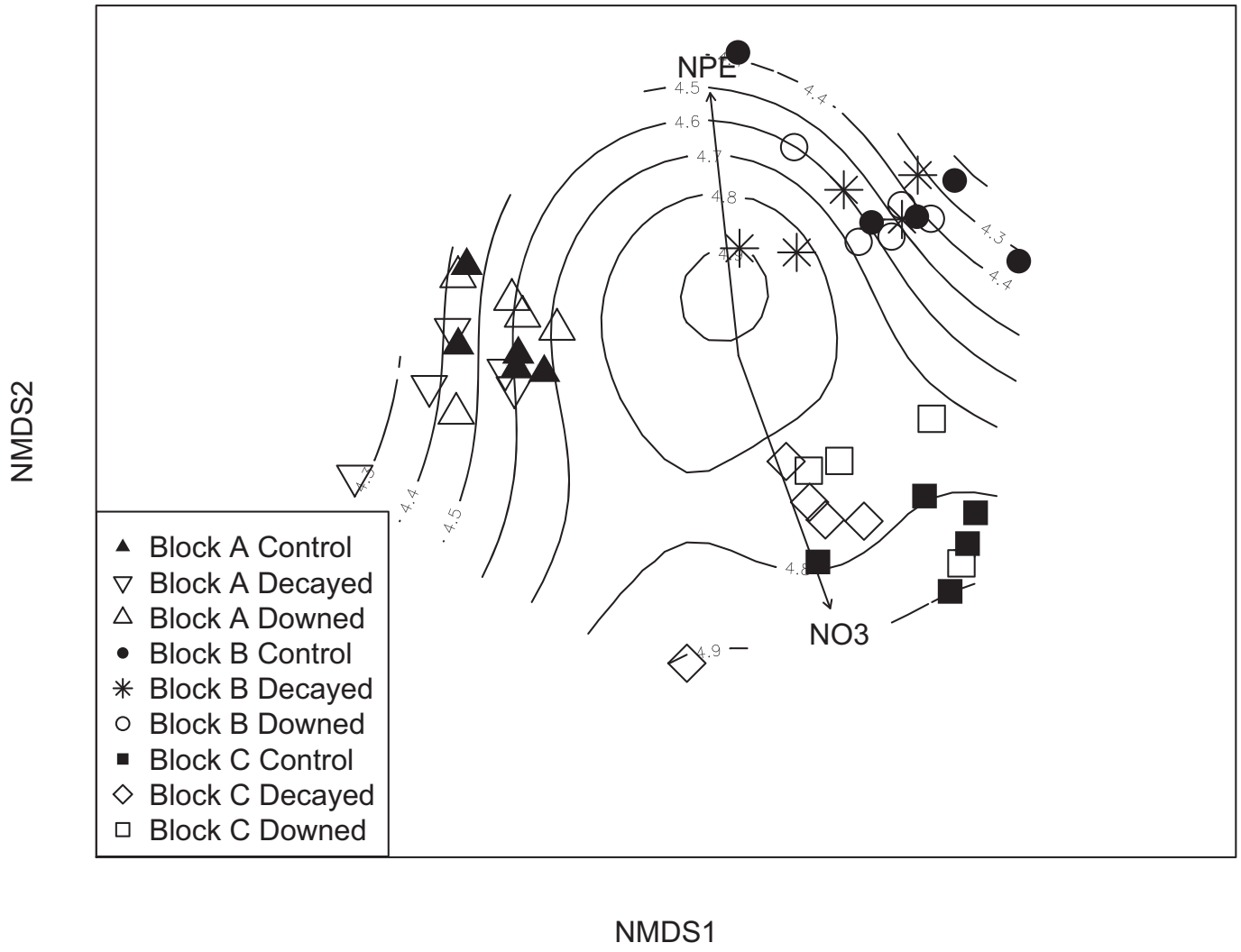
[a]These eight fungal taxa represent the top five most OTU(species)-rich fungal groups from each block when all OTUs with greater than 100 reads were identified.

[b]The total contribution of these eight taxa to the fungal community detected in forest soils as compared to all other fungal taxa with OTUs containing at least 100 reads.

Analysis of the distribution and composition of fungal communities

Initial qualitative observations of ITS read abundance and OTU richness (Supplementary Fig. S1, Supplementary Table S2[2]) indicated that fungal community composition varied more across blocks than among microsites. For example, the number of edited NGS reads and the number of fungal OTUs were much lower at Block A, at approximately 65% and 55%, respectively, of the num-

Fig. 1. Nonmetric multidimensional scaling (NMDS) ordination of the presence–absence of 267 dominant fungal species (i.e., all fungal Operational Taxonomic Units with at least 100 internal transcribed spacer reads) among blocks and among microsites (n = 5). Stress is excellent for this 2-dimensional Bray–Curtis solution (0.11). Non-polar extractables (NPE) and available nitrate (NO_3) are shown as vectors; the arrows point to an increasing gradient, while the length of the line represents the strength of the correlation between the variable and the ordination. The pH vector is shown as a surface gradient (isocline) with values as labeled. Only statistically significant and (or) strongly correlated environmental variables shown.



ber in the other two blocks (Supplementary Table S2[2]). In addition, there was a shift in OTU richness among blocks for many of the dominant ECM fungal groups and for the saprotrophic order Mortierellales (Table 3, Supplementary Table S3[2]). Fungal communities were again more similar within blocks (P = 0.001; Supplementary Table S4[2]) than within microsite types, when OTUs with at least 100 reads were considered (Fig. 1). For example, representative members of the ECM fungal lineage /amphinema–tylospora were detected at all blocks (Supplementary Table S3[2]), however, the identity of individual /amphinema–tylospora OTUs (i.e., species composition) differed significantly among blocks (univariate ANOVA $P \leq 0.05$). In contrast, OTUs of the ECM fungal lineages /cenococcum, and /wilcoxina were not detected at all blocks (the former was absent at Block C and the latter absent at Block A; Supplementary Table S3[2]), while the /hygrophorus lineage was significantly more species-rich at Block A (Tukey's HSD tests, $P \leq 0.05$).

Nevertheless, type of microsite influenced composition of fungal communities (P = 0.018; Supplementary Table S4[2]) across the site. Univariate ANOVA analyses of individual fungal OTUs (species) revealed that several ECM, saprotrophic, and unknown fungi differed among microsites across all blocks (Table 4). Specifically, the ECM fungal OTUs /amphinema–tylospora6 (*Tylospora fibrillosa*) and /russula–lactarius5 (*Russula curtipes*), the saprotrophic and potentially pathogenic ascomycetes Helotiales20 (*Helicodendron websteri*), and Leotiomycetes1 (*Leptodontidium elatius*), and Unknown2 (uncultured mycorrhizal fungus) were more frequently detected in decayed wood as compared with control mineral soil (Tukey's HSD tests, $P < 0.05$), with intermediate frequencies in downed wood microsites. By contrast, the ECM fungal OTU /pseudotomentella1 (*Pseudotomentella mucidula*), Helotiales4 (Helotiaceae sp.), Ascomycota8 (uncultured *Schizosaccharomyces*), and Unknown6 (unknown ECM fungus) were significantly more frequent in mineral soil microsites, either those under downed wood or away from any visible wood (i.e., control soil).

With block acting as a strong driver of fungal community composition, there was a weak interaction between microsite and block effects (Supplementary Table S4[2]; P = 0.07). A number of fungal OTUs varied significantly among microsites at one block, especially Block C (Table 4). For example, the ECM fungal OTUs /laccaria3

Table 4. (*a*) Known ectomycorrhizal, (*b*) potential saprotrophic, endophytic, or pathogenic, and (*c*) unknown fungal operational taxonomic units (OTUs) (species) whose occurrence (presence–absence) varied among microsites overall and (or) among microsites within a single block when tested independently with univariate ANOVAs (anova, R).

Taxon name[a]	Best identity[b]	Overall microsite *P* value	Block × microsite *P* value	Block[c]	Microsite[d]
(*a*)					
/amphinema-tylospora6	*Tylospora fibrillosa*	0.017		all	+D
/pseudotomentella1	*Pseudotomentella mucidula*	0.018		all	+M, H
/russula-lactarius5	*Russula curtipes*	0.043		B, C	+D
/cortinarius2	*Cortinarius caperatus*		0.042	A	+H
/piloderma10	*Piloderma croceum*		<0.001	B	–D
/amphinema-tylospora13	*Amphinema byssoides*		<0.001	C	–H
/amphinema-tylospora20	*Tylospora asterophora*		0.002	C	+M
/laccaria3	*Laccaria nobilis*		0.003	C	+H
/meliniomyces5	*Cadophora finlandica*		<0.001	C	+D
/pseudotomentella7	*Pseudotomentella tristis*		0.006	C	+H
(*b*)					
Leotiomycetes1	*Leptodontidium elatius*	0.023		all	+D
Leotiomycetes3	*Cylindrosympodium lauri*	0.043		B, C	+H
Helotiales4	Helotiaceae sp.	0.003		A, B	+M, H
Helotiales13	uncultured Helotiales	0.028		B, C	+H
Helotiales20	*Helicodendron websteri*	0.022		B, C	+D
Ascomycota8	uncultured Schizosaccharomyces	0.026		B, C	+M, H
Mortierellales7	*Mortierella* sp.	0.037		B, C	–D
Mortierellales8	*Mortierella alpina*	0.035		B, C	–D
Onygenales1	*Oidiodendron pilicola*		0.002	A	–D
Basidiomycota3	*Botryobasidium subcoronatum*		<0.001	A	+H
Helotiales9	*Hyaloscypha aureliella*		0.019	B	–M
Helotiales14	*Chalara microchona*		0.041	B	+H
Helotiales15	*Hyalodendriella betulae*		<0.001	B	+M
Helotiales17	uncultured Helotiales		0.025	B	–D
Ascomycota5	*Cladophialophora minutissima*		<0.001	B	–D
Mortierellales10	*Mortierella humilis*		<0.001	B	+D
Pezizomycotina1	uncultured Pezizomycotina		0.005	B	–D
Helotiales21	*Phialocephala virens*		0.044	C	+D
Mortierellales6	*Mortierella humilis*		0.001	C	–D
Mortierellales11	*Mortierella alpina*		0.025	C	–D
Mortierellales13	*Mortierella horticola*		<0.001	C	+D
Mortierellales15	*Mortierella elongata*		0.043	C	–D
Mortierellales17	*Mortierella elongata*		0.046	C	+M
Mitosporic Ascomycota3	*Tetracladium setigerum*		0.002	C	+M
Agaricales8	*Clavaria acuta*		0.044	C	–D
Basidiomycota9	uncultured Basidiomycota		0.001	C	+D
Tremellales3	*Cryptococcus terricola*		<0.001	C	+M
(*c*)					
Unknown2	uncultured mycorrhizal fungus	0.026		all	+D
Unknown6	uncultured ectomycorrhizal fungus	0.039		A, C	+M, H
Unknown11	uncultured fungus		<0.001	A	+H
Unknown17	uncultured ectomycorrhizal fungus		0.015	B	–M
Unknown26	*Rickenella pseudogrisella*		0.024	B	–H
Unknown35	Tremellales sp.		0.008	B	+H
Unknown39	uncultured fungus from ectomycorrhizal root		0.006	C	+H
Unknown40	uncultured fungus		0.011	C	–M
Unknown41	uncultured fungus		0.001	C	+G
Unknown42	uncultured fungus		0.044	C	–D
Unknown45	uncultured fungus		<0.001	C	+H
Unknown51	uncultured Thelephoraceae		0.001	C	+D
Unknown52	*Peltigera britannica*		0.044	C	–D

[a]Taxon names are derived from the lineage within which the OTU (species) belongs and the number of different OTUs within the same group.
[b]Identities are based on the best BLAST and UNITE database hits for each OTU; these can be found in Supplementary Table S3(*a*–*c*).
[c]Block in which the OTU was detected and for which the reported *P* value is relevant.
[d]Microsite (control mineral soil (M), hard downed wood (H), or decayed wood (D)), in which the OTU was significantly more (+) or less (–) frequent as compared with the other microsite types at $P < 0.05$ based on post-hoc Tukey's honestly significant difference tests.

(*Laccaria nobilis*) and /pseudotomentella7 (*Pseudotomentella tristis*) were significantly more frequent in downed wood microsites, while /amphinema–tylospora20 (*Tylospora asterophora*) was most frequently detected in control mineral soils and /meliniomyces5 (*Cadophora finlandica*) in decayed wood at Block C (Table 4). Fungal communities in Block C were correlated with high soil pH, high extractable NO_3–N, and low NPE as visualized by vectors and isoclines on the NMDS (Fig. 1, but also see Tables 1*b* and 2*b*).

Discussion

Specialist soil fungi have been described in mineral soils (Hoffland et al. 2004) and in wood (Siitonen 2001). These fungi play

an important role in the nutrient cycles of forest soils by decomposing the most recalcitrant molecules (van der Heijden et al. 2008). We were interested in the potential niche preferences of ECM fungi, which are directly responsible for nutrient acquisition by their woody plant hosts (Read and Perez-Moreno 2003). Coarse woody debris in varying stages of decay has been shown to be effective ECM fungal habitat (Christy et al. 1982, Elliott et al. 2007). Several studies have documented a clear difference in the frequency and abundance of ECM fungal root tips found in logs versus forest floors (Goodman and Trofymow 1998; Tedersoo et al. 2003; Iwański and Rudawska 2007; Tedersoo et al. 2008). In addition, Tedersoo et al. (2003) were able to determine microsite preferences for some ECM fungal lineages and other fungal orders in a spruce forest: /amphinema–tylospora (Order Atheliales) and /tomentella–thelephora (Order Thelephorales) mycorrhizae were strongly associated with decayed wood, while members of the Helotiales and Agaricales were prominent in mineral soils. Buée et al. (2007) found that the woody debris in a hardwood forest was inhabited by the mycelia of saprotrophs, but also by the root tips of the ECM fungal genera *Tomentella* and *Lactarius*. Although we previously detected no significant differences in ECM fungi colonizing roots of spruce seedlings in decayed wood versus mineral soils at Sicamous Creek (Walker and Jones 2013), we remained unconvinced that specialist ECM fungal inoculum did not occur in the microsites, and therefore expected to see differences in ECM fungal hyphal communities among microsites, as well as differences in the distribution of individual ECM fungal species. Dahlberg (2001) noted that a link between ECM fungal species richness or diversity and ecological function had yet to be determined. However, the contribution by ECM fungi to soil nutrient cycling — apart from host provision — is well supported (Read and Perez-Moreno 2003). Moreover, the role played by ECM fungal hyphae in mineral and organic material breakdown is substantial (reviewed by Finlay 2008), and suggests that soil fractions rich in ECM fungal hyphae, regardless of species identity, are ecologically important (Phillips et al. 2014).

ECM fungal communities differed less than expected among microsites

We approached this experiment from the perspective that ECM fungal communities are strongly structured by niche partitioning (Bruns 1995; Koide et al. 2011), but recognize that neutral (i.e., stochastic) processes also play a role. These processes may include ongoing spore dispersal into the microsites, and regular fine root turnover and recolonization, both of which might serve to moderate a "niche" signal. Nevertheless, we detected a difference in the occurrence of some ECM fungal species among microsites. For example, in this study, *T. fibrillosa* and *Amphinema byssoides* (both members of the ECM fungal order Atheliales) were consistently present in decayed wood microsites. By contrast, *T. asterophora* was more frequent in mineral soils. Tedersoo et al. (2008) found *T. fibrillosa* and *A. byssoides* to be the dominant ECM taxa on rotted logs in an undisturbed spruce forest in Estonia; these taxa were also relatively frequent in most forest microsites, including the undisturbed forest floor. Little competitive exclusion has previously been observed between *A. byssoides* and *T. fibrillosa*, because they have different growth forms (Agerer 2001; Tedersoo et al. 2008). It is not surprising that these species co-occur in this study. Landeweert et al. (2003) found that the mycelia of *T. asterophora* were only detected in the strongly weathered East horizon of a spruce and pine forest in northern Sweden, a soil layer where total C was low. This is consistent with our detection of *T. asterophora* in nutrient poor control soil. Ecological and physiological differences are common among fungal genera and among fungal species (Smith and Read 2008; Kranabetter et al. 2009). Therefore, while these three species occur in similar habitats in the Northern Hemisphere, it is not surprising that the two species within the genus *Tylospora* are adapted to two different substrates at Sicamous Creek.

Piloderma spp. are often considered as taxa that favour decayed wood microsites because their mycorrhizae are found there, and some species are only abundant in older forests (Twieg et al. 2007; Smith et al. 2000). For example, *Piloderma fallax* mycorrhizae appear to consistently prefer, and in fact require, decayed wood substrates (Goodman and Trofymow 1998; Smith et al. 2000). While the root tips (Rosling et al. 2003) and hyphae (Landeweert et al. 2003) of *Piloderma* OTUs have been found in all soil horizons, the mycelia of *Piloderma* were more abundant in C-enriched (Landeweert et al. 2003) and mineral N-poor (Lilleskov et al. 2002) spruce forest soils. Therefore, the absence of *Piloderma croceum* (/piloderma10) from decayed wood in our study was unexpected given that *Piloderma* spp. are known to colonize the soils surrounding both spruce (Arocena et al. 2001) and fir (Arocena et al. 1999), which are the dominant host trees at Sicamous Creek.

In this study, *Cadophora finlandica* was more frequent in decayed wood microsites. *C. finlandica* is an ascomycetous fungal endophyte that forms ectomycorrhizae very similar to those of *Laccaria bicolor* (Peterson et al. 2008). It is one of only three ECM-forming Helotialian species currently known to make up the /meliniomyces lineage, the others being *Meliniomyces bicolor* and *Rhizoscyphus ericae* (Tedersoo et al. 2010*a*). These three species are very closely related, and can also form ericoid mycorrhizae (Grelet et al. 2010). It is possible that *C. finlandica* was associated with *Rhododendron* spp. and *Vaccinium* spp., which form a large part of the understory at this site (Craig et al. 2006). Related taxa also include endophytes and saprobes (Tedersoo et al. 2010*a*) that can live on organic debris in the absence of a host plant (Day and Currah 2011). Genney et al. (2006) found *C. finlandica* to be twice as frequent as hyphae than as root tips, and while the root tips were limited to the organic horizon, the mycelia were found at all depths in that study. Our findings of *C. finlandica* in decayed wood are consistent with the success of this species in organic substrates.

Substrates from the three types of microsites at Sicamous Creek had distinct abiotic properties. For example, decayed wood had lower pH and a higher proportion of C and organic matter than either of the other microsites, while control soils were shown to remain significantly drier throughout the growing season as compared with the woody substrates (Walker and Jones 2013). It is possible that we would have detected greater differences in the ECM fungal community among microsites if the substrates had differed from each other more strongly in their N properties, since the capacity for N-acquisition varies among ECM fungi (Jones et al. 2009; Kranabetter et al. 2009), potentially driving niche development.

A major difference in approach between this study and most of those cited above is that we focused on the occurrence of ECM fungal hyphae, not ECM fungal roots. Other studies that have compared the occurrence of ectomycorrhizae and the extramatrical hyphae of the same fungal species, found a correlation in distribution for some ECM fungi, but not others (Izzo et al. 2005, 2006; Kjøller 2006; Genney et al. 2006). Living ECM fungal hyphae are the predominant form of inoculum in undisturbed forests (Jones et al. 2003), and given the substantial biomass of ECM fungal hyphae in spruce forest soils (Wallander et al. 2004), we are confident that we have primarily targeted ECM fungal hyphae in this study. In a recent experiment using mesh bags to isolate the active ECM fungal community in a Norway spruce forest, Wallander et al. (2010) found that *T. fibrillosa* amplicons constituted 80% of the community; this strongly mirrors and supports our results. In our past work at this site, we were surprised that ECM fungal species colonizing spruce seedling roots were not unique to a microsite type (Walker and Jones 2013). We hypothesized that the colonization ability of ECM fungi preferring a particular microsite type, and present as living inoculum, may have been limited by the C-provision of the seedling (i.e., the successful

colonization of seedling roots by ubiquitous fungi with low C demands masked the presence of substrate specialists). However, while several ECM fungal species appeared to be associated with a particular microsite in some blocks, we conclude that, overall, there is no distinct community of ECM fungi that specialize in each of the soil microsites we examined in this spruce forest.

Fungal communities differed among geographically distinct experimental blocks

In addition to differences among microsite types, we hypothesized that taxonomic differences would be found among the experimental blocks across the site. Indeed, fungal community structure differed among blocks based on number of NGS reads, OTU richness, and OTU (species) composition. The ECM fungal lineages that differed statistically among blocks (e.g., /cenococcum and /wilcoxina) may be structured by spatial organization and (or) abiotic soil characteristics.

Most investigations of ECM fungal community structure have sought to detect the spatial organization among ECM fungi over centimetres to metres within plots (reviewed by Lilleskov et al. 2004; Genney et al. 2006; Pickles et al. 2010). Izzo et al. (2005, 2006) compared plots in a temperate fir forest that were separated by 200 m to over 1.5 km. They found that while some members of the ECM fungal root tip community, including *Cenococcum geophilum* and *Wilcoxina* spp., were widespread and detectable at virtually all plots, most ECM fungal taxa were detectable as root tips in only one plot (Izzo et al. 2005). Interestingly, while it has been documented that the distribution of ECM fungal root tips and their extramatrical hyphae are rarely the same (Genney et al. 2006; Kjøller 2006), when spores and hyphae were sampled in a subsequent study, *C. geophilum* and *Wilcoxina* spp. remained dominant (Izzo et al. 2006). Moreover, in that later study, the overall differences among plots were diminished as compared to when ECM fungal root tips were assessed. Because we sampled fungal hyphae instead of root tips in the present study, we would expect to have found a more uniform distribution of these taxa, even though blocks were approximately 1 km apart.

ECM fungi exhibit variable patchiness (Lilleskov et al. 2004; Pickles et al. 2010), but we do not believe our findings, at least for /cenococcum, which was not detected at all blocks, to be related to patch size. *Cenococcum geophilum* forms small (i.e., less than 300 cm^3) patches (Genney et al. 2006) of low biomass (Lilleskov et al. 2004), but these are evenly dispersed throughout the soil and root systems (Genney et al. 2006; Lilleskov et al. 2004). Moreover, our detection of *Cenococcum* was successful overall, in spite of pyrosequencing technology limitations for this genus (Kauserud et al. 2012). *Cenococcum geophilum* also appears to be well-suited to numerous site conditions (Dickie 2007 and references therein), and its ecology is not related to N-supply (Avis and Charvat 2005). In contrast, *Wilcoxina* mobilizes N more efficiently than *Cenococcum* does (Jones et al. 2009), therefore its absence from a block with high available ammonium is unexpected. We conclude that the uneven distribution of *Cenococcum* and *Wilcoxina* hyphae at this site does not appear to be explained by spatial organization or niche preference.

The ECM fungal community, as both root tips (Toljander et al. 2006) and hyphae (Nilsson et al. 2005), changes along natural biotic and abiotic gradients in temperate forests. We speculate that the natural elevation gradient, increasing from Block A to Block C, and a related shift in understory plant composition (Lloyd and Inselberg 1997), overstory tree distribution (J. Walker, personal observation), and seasonal conditions (especially precipitation, see Walker and Jones 2013), contributed to the structure of the ECM fungal community. We also detected changes among blocks in chemical properties such as pH and the availability of inorganic N, which are known to be strong drivers of mycorrhizal community structure in forest soils (Rousk et al. 2009; Cox et al. 2010). However, we could not distinguish relationships between changes in the ECM fungal community and changes in soil abiotic properties.

A greater number of ECM fungal taxa were identified using NGS in this study than in our previous experiments at this site

Innovative molecular techniques have made it possible to look at ECM fungal hyphae (Horton and Bruns 2001; Anderson and Cairney 2007), and next-generation pyrosequencing has revealed the overwhelming diversity of ECM fungi (Buée et al. 2009; Hibbett et al. 2009). NGS is not without limitations, however, including fundamental PCR bias (Amend et al. 2010), short sequence lengths that may be artefactual (Lindahl et al. 2013), and database identification issues (Nilsson et al. 2006; Ovaskainen et al. 2010). NGS can also result in a different view of the community based on the primers chosen (Tedersoo et al. 2010*b*; Mello et al. 2011) and may overestimate diversity due the misinterpretation of rare species (Reeder and Knight 2009; Dickie 2010; Kunin et al. 2010).

To ensure that our samples reflected dominant fungal species, we restricted our analyses to OTUs containing more than 100 NGS reads, and while only a small percentage of OTUs fell into this category, we were still able to identify the majority of fungal taxa that we detected in soil and wood samples. Nevertheless, accumulation curves including all OTUs did not reach an asymptote, demonstrating that many fungal taxa remain undetected despite an enormous sequencing effort. This is common even in other deep sequencing efforts on fungi (Buée et al. 2009; Jumpponen and Jones 2009, Taylor et al. 2010). The number of reads detected in this study per block and per 1 g soil or substrate sample (including singletons and doubletons) were similar to other pyrosequencing studies of ECM fungi in forest soils (Buée et al. 2009), but the number of OTUs, even with a conservative cutoff at 95% similarity, was high (Buée et al. 2009; Jumpponen and Jones 2009; Tedersoo et al. 2010*b*). The identities of dominant soil fungal species were also similar to those detected in other studies of temperate forest soils, and the increased diversity in this study is likely due to our sampling over a much larger area. For example, the areas in the studies cited above range from the fungal ecology of single leaves (Jumpponen and Jones 2009), and the ECM community in 0.1 ha closely-spaced plots (Buée et al. 2009), to one 12 ha forest area (Tedersoo et al. 2010*b*). Our 1 ha blocks were within 30 ha forest units spaced 1 km apart; we sampled at both the metre and centimetre scale within these blocks. In addition, we encountered a far larger proportion of ECM fungal species among our identified OTUs than were encountered in oak and beech plantations (Buée et al. 2009), but similar to samples from boreal forests subjected to conventional cloning and sequencing (Taylor et al. 2010). The similarity with the boreal forest samples may be because both were from natural stands with similar host species. Most importantly, we identified twice the number of ECM fungal taxa than in our past studies at this site.

The most abundant fungal taxon overall was the ECM fungal lineage /amphinema–tylospora, which was uniformly frequent at all blocks. This is consistent with our earlier findings that *Tylospora* spp. had colonized root tips in all forest blocks (Walker and Jones 2013), and that DNA of *Amphinema* and *Tylospora* spp. together contributed almost 50% to the overall fungal community in mesh bags from adjacent clearcuts (Walker et al. 2012). Closer inspection of Supplementary Table S3[2] shows that *Tylospora* spp. contribute much more than *Amphinema* spp. to the abundance of the /amphinema–tylospora lineage detected as hyphae. This corresponds with the identification of *Tylospora* spp. as an indicator species of the forest community at this site (Walker and Jones 2013). Hence, at Sicamous Creek these closely related fungal taxa dominated both the hyphal and root tip communities in the forest. While these taxa are commonly found separately and together in temperate spruce forests as root tips or hyphae (Baier

et al. 2006; Landeweert et al. 2003; Rosling et al. 2003), they are rarely among the most abundant taxa (Tedersoo et al. 2008). Finally, this sequencing effort resulted in a broad view of the entire fungal community at Sicamous Creek, including the identity of dominant saprotrophs (e.g., members of the Mortierellales) and potential pathogens or endophytes. It therefore presents a comprehensive view of the soil fungal community at many different scales, and one that is unique among these investigations thus far.

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