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Title: The potential for mycobiont sharing between shrubs and seedlings to facilitate tree establishment after wildfire at Alaska arctic treeline

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Running title: Post-fire shrub-seedling interactions

Abstract

Root-associated fungi, particularly ectomycorrhizal fungi (EMF), are critical symbionts of all boreal tree species. Although climatically-driven increases in wildfire frequency and extent have been hypothesized to increase vegetation transitions from tundra to boreal forest, fire reduces mycorrhizal inoculum. Therefore, changes in mycobiont inoculum may potentially limit tree-seedling establishment beyond current treeline. We investigated whether ectomycorrhizal shrubs that resprout after fire support similar fungal taxa to those that associate with tree seedlings that establish naturally after fire. We then assessed whether mycobiont identity correlates with the biomass or nutrient status of these tree seedlings. The majority of fungal taxa observed on shrub and seedling root systems were EMF, with some dark septate endophytes and ericoid mycorrhizal taxa. Seedlings and adjacent shrubs associated with similar arrays of fungal taxa, and there were strong correlations between the structure of seedling and shrub fungal communities. These results show that resprouting post-fire shrubs support fungal taxa compatible with tree seedlings that establish after wildfire. Shrub taxon, distance to the nearest shrub, and fire severity influenced the similarity between seedling and shrub fungal communities. Fungal composition was correlated with both foliar C:N ratio and seedling biomass and was one of the strongest explanatory variables predicting seedling biomass. While correlative, these results suggest that mycobionts are important to nutrient acquisition and biomass accrual of naturally establishing tree seedlings at treeline and that mycobiont taxa shared by resprouting post-fire vegetation may be a significant source of inoculum for tree seedling establishment beyond current treeline.

Introduction

Both paleoecological and present-day demographic studies emphasize that the controls over seedling establishment are key to predicting latitudinal and alpine treeline advance (Germino *et al.* 2002; Lloyd 2005). Yet, the key drivers of successful seedling establishment at and beyond treeline are not clear. Tree seedling establishment is influenced by a myriad of factors including propagule

dispersal, substrate availability, physiological tolerance, life history traits, and facilitative and/or competitive species interactions (Pickett *et al.* 1987). One often-overlooked yet physiologically important factor in seedling establishment is symbiosis with root-associated fungi. In fact, ectomycorrhizal fungi (EMF) are obligate mycobionts of all boreal tree species (Smith & Read 2008). Given the considerable ecological repercussions of treeline advance into tundra, including increased aboveground carbon storage, decreased albedo, and increased latent and sensible heat fluxes (Chapin *et al.* 2005), it is important to understand the relative strength of abiotic and biotic controls over tree-seedling establishment at the latitudinal margins of their current range.

In the boreal forest, and at treeline in Alaska, tree seedling recruitment is strongly linked with fire regime (Johnstone *et al.* 2004; Lloyd *et al.* 2007). In the last half-century wildfire frequency and extent have increased across the boreal forest and in regions of tundra (Macias Fauria & Johnson 2008; Rocha *et al.* 2012). Increased prevalence of wildfire in areas with historically rare fires, i.e. both tundra and treeline, is expected to facilitate vegetation transitions from tundra to boreal forest (Landhausser & Wein 1993) by opening up new, high-quality microsites for seedling establishment and reducing competition from extant vegetation (White 1979). However, fires at treeline and in tundra may have detrimental effects on critical belowground mycobionts, through combustion, heating of soil and mortality of host plants (Dahlberg 2002). In boreal and temperate regions, severe wildfire activity can alter EMF community structure (Baar *et al.* 1999; Grogan *et al.* 2000) and reduce EMF biomass (Stendell *et al.* 1999) and root colonization (Treseder *et al.* 2004). Changes in mycobiont composition due to wildfire are likely to influence seedling success because of taxon-specific functional traits, such as successional status (early-, multi-, or late-stage) or the efficacy of nutrient mobilization and translocation (Hobbie & Agerer 2010; Last *et al.* 1987), that may benefit host plants. The roots of ectomycorrhizal plants are also colonized by dark septate endophytes (DSE) and ericoid mycorrhizal fungi (ERM), yet the effects of fire on these fungal groups and the influence of these fungi on plant performance is less well studied (Grelet *et al.* 2010; Hewitt *et al.* 2016; Hewitt *et al.* 2013; Mandyam & Jumpponen 2005; Mandyam & Jumpponen 2015; Newsham 2011).

The potentially negative effects of wildfire on plant-fungal interactions and seedling establishment may be amplified at treeline and in tundra due to low densities or the absence of ectomycorrhizal host plants (Read 1991). Although seedlings can be colonized by spores or sclerotia of the resistant propagule community that survives fire (Baar *et al.* 1999; Taylor & Bruns 1999), colonization by vegetative growth of mycelium hosted by established vegetation is often more rapid and vigorous because the fungus has already established a thallus (Horton & van der Heijden 2008). Established plants have a greater ability to compete for soil resources, so seedlings that integrate into a common mycorrhizal network (CMN) with established plants often have reduced carbon costs per unit of nitrogen gained (He *et al.* 2003). However, the effect of CMNs on seedlings depends upon differences in the magnitude of benefits or costs conferred to seedlings by different fungal taxa (Booth 2004; Booth & Hoeksema 2010; Koide *et al.* 2008; Nara 2006a; Simard & Durall 2004). Potential sources of EMF and other root-associated fungal inoculum for tree seedlings after fire at treeline and in tundra are ectomycorrhizal tundra shrubs, some of which have the capacity to resprout after fire (Hollingsworth *et al.* 2013) and putatively maintain their pre-fire, late-stage mycorrhizal communities (Hewitt *et al.* 2013).

Although the most common shrubs at treeline and in tundra, *Betula nana* and *Salix* sp., support generalist EMF communities (Timling *et al.* 2012), it is unknown whether these EMF assemblages facilitate natural tree seedling establishment via provision of inoculum or mycorrhizal linkages at the edge of the boreal forest and beyond treeline. The provision of inoculum by resprouting shrubs could play an important role in tree-seedling establishment success at treeline and promote treeline migration into tundra after fire. To probe this issue, we carried out a study with three main goals: 1) to investigate whether seedlings that establish after fire share mycobiont taxa with nearby resprouting shrubs, 2) to determine if fungal composition or specific fungal taxa that associate with seedlings are related to measures of seedling performance (biomass and foliar nitrogen), and 3) to examine whether root-associated fungi that are compatible with seedlings and shrubs have geographic ranges that extend into areas of predicted forest expansion. Together, these investigations contribute to our understanding of whether shrubs have the potential to provide compatible and

beneficial mycobionts for establishing seedlings and whether these fungi are currently found beyond

the range limit of boreal tree seedlings.

Materials and Methods

Study Site

Our study area at treeline in the upland boreal forest of interior Alaska is bounded by the

Brooks Range and latitudinal treeline to the north (67˚N), and the Alaska Range to the south (63˚N).

Interior Alaska has a continental climate and is underlain by discontinuous permafrost. Sampling was

focused in two treeline sites: 1) the uplands of the southern foothills of the Brooks Range at Finger

Mountain (66°21'35.20"N, 150°28'35.22"W, elevation 560 m) north of the Yukon River and 2) the

White Mountains at Nome Creek (65°19'26.88"N, 146°41'5.86"W, elevation 609 m) located between

the Yukon and Tanana Rivers (Supporting Information Figure 1). Upland forest cover at both study

sites is dominated by black spruce (*Picea mariana* (Mill.) Britton, Sterns & Poggenb.), with patches

of deciduous broadleaf trees, mainly trembling aspen (*Populus tremuloides* Michx.) and Alaska paper

birch (*Betula neo-alaskana* Sarg.). Treeline at these sites is dominated by a mix of white spruce

(*Picea glauca* Moench) and black spruce. Alpine tundra at both sites is dominated by ericaceous

shrubs, dwarf birch (*Betula nana* L.), tall shrubs including willow (*Salix* L. sp.) and alder (*Alnus*

viridis (Chaix) DC.), graminoids, and feather mosses. Both sites burned in 2004 when over 2.7

million ha of forest burned across the interior of Alaska. Site data are available at the Bonanza Creek

Long Term Ecological Research (LTER) site (http://www.lter.uaf.edu/data/data-detail/id/342). The

Finger Mountain site (listed as site DC 31 on the LTER site) and the Nome Creek site (site BF 91 on

the LTER site) were categorized as moderate fire severity in 2005. Both sites have patches that burned

at high severity and patches that burned at low severity. To account for this variability we measured

the residual organic soil depth at each focal plant (described below).

Field sampling

Due to the low seedling densities at both treeline sites, an uneven sampling design was employed to maximize the number of seedlings sampled at each site. We therefore sampled 40 pairs of tree seedlings and adjacent shrubs at our Finger Mountain site and 29 pairs of tree seedlings and shrubs at our Nome Creek site in July and August of 2009. Both our sites were approximately 35 m x 30 m. We then subdivided our sites into five parallel 1 X 30 m transects located five meters apart. We excavated the root systems and aboveground parts of aspen, birch, or spruce seedlings and the closest resprouting dwarf birch, Alaska paper birch, willow, or bearberry (*Arctostaphylos uva-ursi*) shrub. Each pair of seedling-shrub samples was at least 2 meters from another sampled pair. Due to low seedling density along the transects (approximately two seedlings per 25 m-2 at Finger Mountain and one seedling per 60 m-2 at Nome Creek), we continued to sample seedlings and shrubs by haphazardly searching for tree seedlings. We harvested the root system as an intact root ball (i.e. intact mass of soil containing the root system of the focal plant). The root ball of each focal plant was approximately 30 cm deep and varied in diameter based on the distance between focal plants. Seedlings located close (<50 cm) to the resprouting shrub were harvested together with the shrub in one root ball. On average, root balls were 40 cm in diameter with the shrub and/or seedling in the center. The distance between seedlings and shrubs varied from 1-200 cm with the median distance 40 cm. We recorded the substrate and presence of the dominant vegetation within one meter of the focal seedling, the depth of the organic layer where the seedling and shrub were collected, and the distance between each seedling and the nearest shrub. Excavated rootballs were transported back to the University of Alaska Fairbanks (UAF).

Seedling age and biomass

For all harvested seedlings, aboveground biomass was separated into stems and leaves, dried at 60°C for 48 hours in a drying oven, and weighed. We verified that each seedling established after the wildfire by sanding the base of each seedling stem with 400-600 grit sandpaper and counting

growth rings on a sliding bench micrometer. Carbon and nitrogen concentrations for leaf material were determined on a Thermo Scientific Delta V interfaced with a Carlo Erba elemental analyzer at the Alaska Stable Isotope Facility, UAF Water & Environmental Research Center.

Fungal sampling and characterization

Root systems were washed gently with DI water, and then we harvested roots that were clearly connected to the seedling or shrub. Intact roots were preserved in RNA later for up to ~20 weeks before root tip sampling (Bent & Taylor 2010). Root systems were cut into 4 cm segments and floated in ultrapure DI water. Healthy ectomycorrhizal root tips were picked from each segment. In preliminary trials we assessed whether a pooled sample of single and multiple tips accurately captured fungal richness using an ARISA approach (see below). Fungal gene richness per seedling plateaued at a low tip sampling density. To determine the proportion of the root system needed to capture fungal richness, we ran a trial on four root systems with sampling of 9, 18, 27, and 36 root tips each. The fungal taxon richness obtained via ARISA was not related to the number of root tips sampled (F=0.3032, p=0.5895) suggesting that the low diversity of fungi detected on a single root system was captured when sampling a smaller proportion of tips from the root system (nine or eighteen tips). This result was supported by in-house trials from other sites and EMF host plants in burned areas within interior Alaska. We chose to sample 18 root tips because this sample size would adequately capture the low fungal diversity on seedlings and shrubs and is a sample size that represents equal sampling effort across roots systems of both larger host plants and small seedlings where eighteen tips was in some cases the maximum number of tips recovered. Eighteen root tips were randomly selected and pooled from the root system and stored in ultrapure water at -80˚ C and lyophilized.

DNA extraction and ARISA of mycorrhizal fungi

Lyophilized pooled samples were ground in lysis buffer using a sterile plastic pestle (Kontes - Rockwood, Tennessee, USA). DNA was extracted from the pooled root-tip samples from each host plant using the DNEasy Plant Mini Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturer's instructions, slightly modified as suggested in Bent and Taylor (2010). Genomic DNA was amplified for Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher & Triplett 1999) of fungal community structure following the protocol of Bent & Taylor (2010) using the forward primer ITS1-F (CTTGGTCATTTAGAGGAAGTAA; (Gardes & Bruns 1993), labeled on the 5' end with FAM, a fluorescein amidite (Applied Biosystems, Carlsbad CA, USA)), the reverse primer ITS4 (TCCTCCGCTTATTGATATGC; (White *et al.* 1990)), and then run through an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad CA, USA; Pop6, 50cm array, T-RFLP_1500 protocol) (see Supporting Information Appendix A for full detail of all molecular and bioinformatics methods). In our experience, ARISA is a sensitive and cost-effective method to obtain richness and abundance data for the low diversity EMF communities in our study region (Bent & Taylor 2010). By verifying ARISA ribotype identities with sequence identities from clone libraries, we have reduced the uncertainties associated with fragment-length based ARISA ribotypes that might influence the conclusions drawn from the community profiles.

Amplicon cloning and sequencing

To assign taxonomic identities to ARISA ribotypes we matched ARISA ribotypes with sequence identities from both direct and clone sequences. For pooled tip samples that yielded a single ARISA ribotype, we obtained fungal ITS sequences by direct sequencing. For direct sequencing we followed the PCR protocol described earlier except that we used primers ITS1-F and ITS4 without fluorescent labels. For all other samples we constructed pooled clone libraries using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA, S.I. Appendix A, Taylor *et al.* 2007). Cloned cells were selected from colonies from each library and used in direct PCR using primers

M13F and M13R. PCR products were then subject to RFLP analysis using Hae III (New England

BioLabs, Ipswich, MA, USA) in order to pick unique clones for sequencing. PCR products for

samples with unique RFLP patterns were sequenced at Functional Biosciences Inc., Madison, WI.

Sequence lengths did not always exactly match the ARISA fragment length, apparently due to

migrational differences caused by secondary structure or the fluorescent moiety. Therefore, to directly

match DNA sequences with our ARISA profiles, we performed ARISA on the clone PCR product that

was sequenced. ARISA ribotype identities were obtained for two representative PCR samples from

each Operational Taxonomic Unit (OTU) in our sequence dataset. The representative sequence for

each OTU has been archived with GenBank under accessions KU176235-KU176321. For additional

details see S.I. Appendices A and B. We sequenced 188 clones and direct sequenced 56 PCR products

with one dominant ribotype. All root-associated fungi were included in the community analysis

described below.

Bioinformatics

We used GeneMapper 3.7 to read ARISA electropherograms. Fragment length

(ribotype) and peak height represent identity and abundance of fungal taxa, respectively. The

peak heights for each fragment were relativized by dividing the fluorescence height for each

peak by the total fluorescence for a sample profile (Fisher & Triplett 1999). As recommended

elsewhere, we used 1.0 base pair (bp) ARISA ribotype bins (Dunbar *et al.* 2001) as surrogates

for "species" in fungal community analyses.

Clone and direct sequences were assembled using CodonCode Aligner 3.7 (CodonCode

Corporation, Centerville, MA, USA) and processed using the pipeline described in Taylor and Huston

(2011, see S.I. Appendix A for full description). We grouped all direct and clone sequences into

Operational Taxonomic Units (OTUs) using CAP3 (Huang & Madan 1999) at 97% sequence identity.

To assign species identities we ran a BLAST search using a representative sequence for each OTU

against both the UNITE version 5.0 reference sequence database and the entire nucleotide database in

GenBank utilizing the filter to exclude uncultured and environmental samples. We assessed the top matches with the highest bit score from the UNITE and GenBank databases. We followed the conventions for assigning the level of taxonomic hierarchy from Timling *et al.* (2012) based on sequence similarity. When the best matches were identified to the same taxonomic level we reported the UNITE Species Hypothesis, which provides a stable name and accession number (Kõljalg *et al.* 2013). We used a tiered approach to assign OTU identities derived from Sanger sequencing to ARISA ribotypes based on direct sequencing and matched fragment lengths of ARISA ribotypes and DNA sequences (S.I. Appendix A, S.I. Appendix B, S.I. Table 1). We used the ARISA dataset matched with OTU identities in all further analyses. For additional details see S.I. Appendices A and B.

Statistical analysis

Ordination analysis of vegetation and fungal composition for naturally established seedlings

To examine fungal composition associated with each naturally established focal seedling we used Bray-Curtis ordinations (Bray & Curtis 1957) with the Sørensen distance and the variance-regression method of endpoint selection (Beals 1984). As part of our evaluation of seedling root-associated fungi we tested for correlations between fungal composition and factors pertaining to environment, fire regime, and vegetation composition. The fire regime was characterized in terms of fire severity and measured as the depth of the residual organic horizon (Johnstone *et al.* 2008). We also characterized the substrate upon which the seedling was growing: charred organic soil, mineral soil, moss, or graminoid cover. We specifically tested whether each shrub and seedling species differed in their mycobiont community composition using the Multiple-Response Permutation Procedure (MRPP) (Berry *et al.* 1983) (shrubs: *Betula nana*= 23, *Arctostaphylos uva-ursi* = 9, *Salix* sp. =15; seedlings: *Populus tremuloides*= 34, *Picea mariana* = 5, *Betula neo-alaskana*. =8). For MRPP, we used the Euclidean distance measure (McCune & Grace 2001). Because fungal composition was strongly correlated with organic soil depth, a proxy for fire severity, we performed an Indicator Species Analysis (Dufrene & Legendre 1997) to detect ribotypes that were over-

represented for a particular fire-severity class (high severity <3 cm residual organic soil depth or low >3 cm residual organic soil depth) (Johnstone & Chapin 2006). For the Indicator Species Analysis we used 4999 permutations in the Monte Carlo test of indicator values. To represent vegetation composition in our environmental matrix, we performed an additional Bray-Curtis ordination on vegetation composition within 1 meter of the focal seedling. We exported axis 1 from the vegetation ordination (explaining approximately 70% of the variation) and included it in the environmental matrix as part of the fungal ordination. Axis 1 from both the vegetation and fungal ordinations (fungal ordination axis 1 explained approximately 43% of the variation) were exported and used in Random Forest analysis. All ordination analyses of root-associated fungal communities were performed in PC-ORD 6.0 (MJM Software Design, Gleneden Beach, Oregon (USA)).

Analysis of fungal community similarity between seedlings and shrubs

Our central question concerned the degree to which seedlings and shrubs shared mycobionts. Therefore, we tested for a relationship between the structures of the fungal communities associated with pairs of seedlings and adjacent shrubs. We used the Mantel test (Mantel 1967) with 999 Monte Carlo permutations to evaluate the null hypothesis that there was no significant relationship between the fungal community structure of seedlings and shrubs. The matrices representing seedling and shrub fungal communities from the Nome Creek and Finger Mountain treeline sites had 69 seedlings or shrubs (rows) and 94 ribotypes (columns). We also computed Sørensen dissimilarity distances for all seedlings and shrubs. We used matched pairs T-tests to evaluate whether seedlings paired with the adjacent shrub had fungal communities that were more similar than seedlings that were unpaired, i.e., randomly paired with shrubs that were not adjacent to the sampled seedling. We evaluated Spearman correlations to detect collinearity between variables (Supporting Information Table 2). Data visualization and univariate statistical procedures were performed in JMP 10.0 (SAS Institute Inc., Cary, NC, USA).

To test which fire and host plant variables influenced the degree of similarity between seedling and shrub root-associated fungal communities, we used Random Forest regression trees. Random Forest is an ensemble decision tree method that uses an algorithmic approach to make predictions based on the input variables (Breiman 2001b). Random Forest is increasingly used in ecological studies because of its predictive accuracy when modeling data with complex interactions that may not meet the assumptions of parametric analysis (Breiman 2001b; Cutler *et al.* 2007). In comparison to other regression and classification tree methods, Random Forest does not over-fit the data and through the random selection of explanatory variables it reduces bias (Prasad *et al.* 2006). We utilized the Random Forest method because of its ability to accurately model small sample sizes with potentially noisy predictor variables and the ease of interpretation (Cutler *et al.* 2007; Diaz-Uriarte & de Andres 2006; Qi 2012).

Similarity between fungal communities was evaluated using the Sørensen dissimilarity distance for each seedling-shrub pair and analyzed in the R 3.0.2 package Random Forest (R Development Core Team, 2013, R Foundation for Statistical Computing, Vienna, Austria). We built 500 regression trees using a random sample of the 47 observations of paired fungal communities. At each node in the trees, two predictor variables were chosen at random from the seven explanatory variables (seedling age, site, seedling taxon, organic depth, vegetation composition, distance between seedling and shrub, and shrub taxon). For each predictor, Random Forest computes the prediction error (mean square error) of a tree in the forest from the permutation of the observed values for a variable in the out-of-bag data, data were not used to produce the given tree being evaluated at that time (Breiman 2001a). The difference between the classification of observed boot strap data and the classification of permuted data is averaged across all trees and divided by the standard error (Breiman 2001a; Cutler *et al.* 2007). We used partial dependence plots to evaluate the marginal effects of the most important variables predicting similarity between tree and shrub mycobiont communities. Partial dependence plots depict predicted dissimilarity in relation to each explanatory variable after accounting for the average effect of all other explanatory variables.

Relationships between fungal composition and seedling attributes

Our second research goal was to determine whether fungal composition or specific fungal taxa that associate with seedlings are related to measures of seedling performance (biomass and foliar nitrogen). We specifically tested whether fungal composition was correlated with C:N ratio of foliar tissue and biomass of seedlings using Canonical Correspondence Analysis (CCA, Ter Braak 1986). C:N ratio of foliar tissue from the current growing year reflects the growth strategy and nitrogen limitation of a plant at the time of harvest. We excluded all spruce seedlings from these analyses (n=8 seedlings) because their needles were not separated by annual growth increment and therefore could reflect growing conditions prior to the year of harvest. Foliar C:N ratio from the current growing year may be reflective of the importance of specific fungal taxa to seedling success. In the analysis we had one species matrix with 47 seedlings (rows), a reduced number due to the elimination of seedlings that only associated with rare ribotypes, and the nine ribotypes (columns), representing all ribotypes with over five percent coverage across these seedlings. With CCA we rescaled the axes using the Centered with Unit Variance method and optimized the representation of fungal taxa. We used Monte Carlo procedures with 999 randomizations to test whether there was a significant relationship between the environmental variables and fungal composition. We compared the species matrix to an environmental matrix with 47 seedlings (rows) and two columns (C:N and seedling biomass). We assessed correlations between specific fungal taxa and the canonical axes to determine if the presence and abundance of a particular fungal taxon was related to biomass or C:N ratio of foliar tissue.

Part of our second research goal was to evaluate the relative importance of fungal composition and shared fungal taxa on tree seedling performance in relation to the other abiotic and biotic explanatory variables. We used the Random Forest regression tree method again to identify important associations between seedling biomass and explanatory variables: fire severity, fungal and vegetation variables. We used partial dependence plots to evaluate the marginal effects of the most important variables predicting seedling biomass. We built 500 regression trees using a random sample of the 69 observations of seedling biomass. At each node in the trees, two predictor variables were chosen at random from the nine explanatory variables (seedling age, site, seedling taxon, organic

depth, vegetation composition, fungal composition, percent of shared fungi, distance between seedling and shrub, and shrub taxon).

Ecological attributes of fungal taxa

Our study site is located at Arctic treeline and we wanted to know whether the root-associated fungal taxa that are compatible with seedlings and shrubs in our research sites have geographic ranges that extend into areas of predicted forest expansion. We documented the geographic locations of the top 100 GenBank hits with >90% coverage and >97% sequence similarity for each queried OTU that was shared by a seedling and its adjacent shrub. We restricted our reporting to boreal forest and Arctic tundra regions.

Results

Correlates with fungal composition

The sequences from the naturally occurring seedlings and adjacent shrubs were grouped into 53 OTUs, 17 of which were singletons. The majority (74%: 39 of OTUs) of the taxa detected in our sequencing effort belonged to the Basidiomycota (S. I. Table 3). The majority of the fungal sequences (75%) were EMF with a few additional dark septate endophytes (DSE), other endophytes, and ericoid mycorrhizal fungi (ERM). In the bulk of the Results and Discussion, we treat all these root-associated taxa together. The ARISA ribotypes from the seedlings and adjacent shrubs at Nome Creek and Finger Mountain yielded 94 ribotypes. Of the 94 ribotypes, 41 were matched with sequences and of those that were not matched only 9 occurred more than once in the dataset (S.I. Table 1). As with other molecular methods we obtained matches for the dominant taxa, but were less successful with the rare ribotypes.

Bray-Curtis ordinations resulted in a 3-D solution for the seedling fungal communities. The three axes accounted for 91.3% of the variation in EMF community structure, with axis 1 contributing 43.2%, axis 2 37.8%, and axis 3 10.3% (S.I. Figure 2). Axis 1 of the fungal ordination was not highly correlated with environmental or fire variables. Axis 2 was correlated with vegetation composition (r^2=0.29), and axis 3 was correlated with variables related to the depth of the organic horizon, a proxy for fire severity (r^2=0.22). Three axes accounted for 95.9% of the variation in vegetation community structure (axis 1 67.9%, axis 2 19.9%, and axis 3 7.2%).

Similarity of fungal communities between seedlings and shrubs

To investigate the potential for resprouting shrubs to provide inoculum to seedlings at treeline, we examined several lines of evidence. First, there was a significant positive association between the structure of the seedling fungal communities and the community of the closest shrub (Mantel Test r= 0.130, p=0.001). Second, we compared adjacent seedlings and shrubs with randomly paired (i.e. non-adjacent) seedlings and shrubs, and found that paired seedlings-and-closest shrubs had more similar fungal communities than random pairs ($T_{(61)}$ = 4.379, p=<0.0001). The Sørensen dissimilarity (0= identical communities, 1= no shared taxa) for adjacent seedling-shrub pairs was lower (0.816 ±0.031 S.E.) than for seedlings and shrubs that were paired at random (0.96 ±0.031 S.E). Third, on average, 31.53% (±0.045 S.E.) of the fungal taxa observed on seedlings were shared with the closest adjacent shrub. Finally, across both treeline sites more fungal taxa (37 out of 94 ribotypes) were observed on multiple host-plant species (i.e., they were apparent generalist mycobionts) than were accounted for as taxa that were shared between seedlings-shrub pairs (23 out of 94 ribotypes). Many of the EMF taxa that were shared by seedlings and the adjacent shrub were multi- or late-stage fungi, which are generally found on mature host plants of later successional seres and colonize from mycelia, from the genera *Russula*, *Lactarius*, *Cortinarius*, and *Boletus* (Table 1, S. I. Table 1, Dix 1995). When we consider successional stage within the context of fire severity, we found that the taxa that were indicators of low-severity burned patches belonged primarily to Russulaceae (in the genera

Russula and *Lactarius*), which are generally multi- or late-stage fungi (S. I. Table 1). The single ectomycorrhizal taxon that was an indicator of high severity, *Inocybe lacera*, was only observed on *Betula nana* shrubs (S. I. Table 1) and is classified as an early-stage taxon.

We were interested in quantifying the importance of variables that predict the similarity between fungal composition of seedlings and nearby resprouting shrubs. Random Forest analysis indicated that shrub taxon was the most important variable, followed by distance (cm) between the seedling and adjacent shrub, vegetation composition, organic depth, seedling taxon, site, and seedling age (Figure 1a). *B. nana* shrubs appeared to have slightly less similar fungal communities compared to the similarity between seedlings and either *Salix spp.* or *A. uva-ursi* (Figure 1b)*.* As physical distance increased between seedlings and shrubs, there was a marked decline in the similarity between fungal communities, especially as the distance approached 50 cm (Figure 1c). The effect of distance on fungal similarity reached a plateau beyond a seedling-to-shrub distance of 75 cm. These results suggest that shrubs less than 50-75 cm from a tree seedling had greatest effect on the seedling's mycobiont community. Fungal communities were more similar in patches of tundra that burned at high fire severity with shallow residual organic depths (Figure 1d). Seedling species, site, and age appeared to exert little influence on the similarity between fungal communities of paired shrubs. These Random Forest results were supported by the MRPP results, which indicated that composition did not vary by seedling species (spruce, tree birch, aspen; A= 0.029, p=0.073), whereas mycobiont composition did vary by shrub species (dwarf birch, willow, bearberry; A= 0.042, p= 0.024).

Geographic ranges of shared fungi

From our GenBank sequence comparisons, we found that the majority of the sequenced fungi (11 out of 17 taxa) that we observed as shared by naturally established seedlings and adjacent shrubs at treeline also occur in Arctic tundra (Table 1), suggesting the presence of compatible mycobionts for boreal tree seedlings beyond current treeline.

Relationships between fungal composition and seedling attributes

Fungal composition was significantly correlated with both seedling C:N ratio and biomass (CCA Monte Carlo test C:N species-environment correlation=0.608, p=0.050 and biomass species-environment correlation=0.444, p=0.050, S.I. Figure 3). Axis 1 explained 4.7 % and axis 2 explained 2.6% of the variance in composition. Although the CCA explained a low percentage of the variance in fungal composition, the Monte Carlo randomization test indicates that the eigenvalues for axis 1 (C:N) and axis 2 (biomass) were higher than the range expected by chance (Supporting Information Table 4). When we examined correlations between abundances of specific taxa and the axes of the ordination, we found that seedlings with lower foliar C:N were correlated with a higher abundance of one taxon, *Russula nitida* (ribotype 66, Pearson r=0.450, r^2=0.202). This taxon was shared between aspen and spruce seedlings and birch shrubs. Biomass was not correlated with the abundance or presence of any single taxon, suggesting that the effects of fungal composition on seedling biomass and C:N ratio reflected the combined effects of the mycobiont community.

We were interested in the importance of fungal composition in predicting seedling biomass as compared to other fire, environmental, and host-plant variables. With Random Forest we can assess the marginal effects of each variable by evaluating the seedling biomass response to a given factor while holding the other variables constant. Analysis with Random Forest showed that seedling age was the most important variable predicting seedling biomass, followed by site, fire severity (organic depth), fungal composition, and vegetation composition (Figure 2a). The relationship to age is expected, and simply shows that the seedlings were growing over time; the other correlates are of greater biological interest. The distance between a seedling and resprouting shrub, seedling taxon, percentage fungi shared with a resprouting shrub and the shrub taxon all had less important influences on seedling biomass. Seedling biomass had a bimodal response to fire severity. Seedlings are predicted to have higher biomass at organic depths between three and five cm (high severity) and then again at organic depths greater than 17 cm (low severity; Figure 2b). Seedling biomass increased

when the percentage of shared fungi surpassed 20% (Figure 2c). The apparent dip in effect of shared fungi on biomass between zero and 20% shared may be an artifact of the very small sample size of seedlings that shared 20% of their fungal taxa (n=2).

Discussion

The effects of belowground species interactions on plant community assembly have been a central question in community ecology over the last several decades (Connell 1971; Janzen 1970; Perry *et al.* 1989; van der Heijden & Horton 2009). Our results are consistent with studies in other ecosystems showing that fungi associated with established vegetation may contribute to seedling establishment (Horton *et al.* 1999; Nara 2006b). We found the EMF and other root-associated fungal communities that associate with naturally established tree seedlings and shrubs were similar, particularly when tree seedlings established near *Salix* sp. or *Arctostaphylos uva-ursi*, following severe fire that consumed much of the organic soil horizon. Further, we found multi- and late-stage EMF taxa that likely colonized seedlings through vegetative mycelial spread of EMF that survived wildfire on resprouting shrubs. While it is possible that these fungi colonized both shrubs and seedlings after the fire via propagules, this seems less likely given that late-stage fungi are ineffective in colonizing seedlings from spores (Deacon *et al.* 1983; Fleming 1983; Fox 1983), mycelial colonization is faster and more vigorous than colonization from spores, and shrub roots survived the fire. We found that seedling mycobiont community structure was positively correlated with shrub mycobiont community structure, and that seedling mycobiont composition was in turn correlated with seedling biomass and foliar C:N ratio. Moreover, the occurrence of a shared EMF taxon, *Russula nitida,* was negatively correlated with foliar C:N. These results complement findings from studies during primary succession that reported species-level effects of EMF on the nitrogen economy of seedlings (Nara 2006a). We also view our results as a minimum estimate of the importance of mycobiont sharing between seedlings and adjacent shrubs. This is supported by our observation that many of the root-associated taxa detected in our study were host plant generalists. In many cases where we did not detect a given taxon

with paired seedlings and adjacent shrubs, study-wide that taxon occurred on multiple host species and thus the potential for sharing may be greater than what we observed (see Supporting Information Table 1 for list of host plants associated with each fungal taxon). These patterns of fungal similarity suggest that in fire-disturbed treeline sites, establishment near a resprouting shrub may provide mycobionts with which young seedlings can associate. Furthermore, given the late-successional classification of some of these EMF taxa, it seems likely that they survived the fire disturbance rather than recolonizing from spores in this short time period. The survival of host shrub roots certainly occurred, and would potentially provide refugia for these fungi. In toto, there was significant similarity between seedling and shrub communities, and the shrubs are the most likely sources of inoculum for these fungi after fire.

Our sequence analysis of the geographic distributions of fungi shared by seedlings and shrubs showed that these EMF, ERM, and DSE taxa have broad distributions across northern biomes, suggesting that mycobionts capable of colonizing boreal tree seedlings are present beyond the current range limit of boreal trees. These results illustrate that ECM and ERM tundra vegetation may play roles in the provision of mycobionts to expanding boreal tree species at range margins, but also might provide patches with suitable inoculum well beyond treeline in tundra after wildfire.

Our analysis of taxa that occur with both seedlings and resprouting shrubs revealed that multi- and late-stage fungi were present post-fire in addition to some early-stage taxa. The colonization of seedlings by late-stage EMF after fire contrasts with findings from the temperate zone where post-fire seedlings are primarily colonized by members of the resistant propagule community, including early-stage genera such as *Wilcoxina*, *Tuber*, and *Rhizopogon*, which are distinct from taxa associated with mature vegetation at the same sites (Baar *et al.* 1999; Grogan *et al.* 2000; Taylor & Bruns 1999). The exception to the replacement of late-stage fungi by early-stage fungi occurs in cases where host trees survive (Stendell *et al.* 1999), which is parallel to survival of shrubs at Arctic treeline. Ultimately, the successional status of EMF correlates with functional traits related to nutrient capture from increasingly complex substrates over succession (Last *et al.* 1987). In a sister study, we also observed the persistence of multi- and late-stage taxa after fire on resprouting shrubs in tundra and similarly

concluded that the later successional-stage mycobionts survived the wildfire (Hewitt *et al.* 2013). In both studies, we interpret the presence of multi- and late-stage EMF on resprouting shrubs and seedlings as demonstrating taxonomic resilience after fire and suggesting functional resilience as well.

The detection of early-, multi-, and late-stage EMF taxa may indicate heterogeneity in inoculum source after fire. After soil combustion associated with wildfire, we would expect root densities to be reduced in a patchy manner related to fire severity. In temperate ecosystems, high root densities are associated with root colonization by mycelial spread whereas low root densities are associated with root colonization by spore dispersal (Peay *et al.* 2011). High-severity burned patches may, therefore, foster the germination of early-stage, spore-dispersed taxa; in contrast, lower-severity patches and resprouting vegetation with higher root densities appear to support the maintenance of multi- and late-stage taxa that likely colonize roots through mycelial spread. Consistent with this framework, we found that *I. lacera*, an early-stage, spore-dispersed fungus (Nara *et al.* 2003), was the only EMF taxon that was a significant indicator of high-severity sites. In contrast, the three EMF taxa that were significant indicators of low-severity sites were all in the genera *Russula* and *Lactarius*, two genera whose members are often classified as multi-stage or late-stage fungi (Smith & Read 2008).

In addition to investigating the identities and ecological attributes of root-associated fungi shared by naturally established seedlings and nearby resprouting shrubs, we wanted to know which fire, host-plant and environmental factors may influence whether compatible fungi associate with both seedlings and shrubs. Shrub taxon and distance to the nearest shrub were the two most important variables predicting similarity between seedling and shrub-fungal communities, followed by vegetation composition and fire severity. These results emphasize the role of highly stochastic processes (dispersal, fire severity) in shaping fungal communities on seedlings. For example, if a tree seed randomly germinates and establishes close to an individual *Salix* in a highly burned patch, the tree and shrub will likely share a substantial fraction of mycobionts. These findings are also consistent with observations of high spatial heterogeneity in EMF, ERM, and DSE composition (Bjorbækmo *et al.* 2010; Kjøller *et al.* 2010; Mundra *et al.* 2015; Taylor *et al.* 2010; Walker *et al.* 2011; Zhang *et al.* 2009). We were surprised that shrub taxon was an important predictor because none of the host

shrubs are known to have particularly narrow fungal specificities (Molina *et al.* 1992; Ryberg *et al.* 2009; Timling *et al.* 2012). We expected them to support generalist mycobiont taxa that associate with seedlings. However, it appears that *B. nana* supports mycobiont communities that are somewhat less similar to boreal seedling mycobionts than do *Salix* sp. and *A. uva-ursi*. Our field data support results from Reithmeier and Kernaghan (2013) who carried out a growth chamber study in which inoculation with soils collected from below *Salix* sp. and *A. uva-ursi* shrubs resulted in greater EMF root colonization of black spruce seedlings than did soils from below *B. nana*.

It is intuitive that distance to the nearest host plant influences fungal communities because the colonization of a seedling root system by EMF mycelium that are already established (Newman 1988) is a more effective process than the combined steps of spore dispersal, germination, and root colonization. In our study, we observed that distance to the potential inoculum source on the scale of less than one meter influenced the composition of the seedling mycobiont community, while in other studies distances of several meters affected fungal composition of seedlings (Dickie & Reich 2005). Spatial constraints on fungal colonization potential for the other mycobiont groups observed in our sequence dataset (DSE and ERM) appears to be lower even with small genet size (Grelet *et al.* 2010; Trowbridge & Jumpponen 2004)

The similarity between seedling and shrub mycobionts increased as residual organic depth decreased, which might be explained by reduced competition and priority effects leading to greater mycobiont overlap under conditions of greater fire severity and removal of the organic layer (Barker *et al.* 2013; Dahlberg 2002) (Kennedy *et al.* 2009). Our previous observations after tundra fire also suggested that EMF fungi were resilient to high-severity fire disturbance and strong competitors for colonizing new roots (Hewitt *et al.* 2013). These observations add a level of complexity to the scenario described previously where fire-severity yields a spatially heterogeneous landscape of root-associated fungi with varying successional status and dispersal strategies (spores vs. mycelial).

We found compelling evidence that fungal composition is an important driver of seedling biomass independent of whether the shrub and seedling communities were highly similar. Age, site,

fire severity, and fungal composition were the top variables predicting seedling biomass. Site and fire severity are correlated variables ($r^2=0.60$), which contributes to the similarity in their ranked variable importance scores and suggests that, although some other site factors may influence seedling biomass, fire-severity is likely the main driver. Previous research in the boreal forest has shown that residual organic depths strongly influence seedling establishment and successional trajectories (Johnstone & Chapin 2006; Johnstone *et al.* 2010a). Our results support those findings and also highlight for the first time the importance of fungal symbionts to naturally established treeline seedling biomass.

Fungal composition was also an important correlate of the nitrogen economy of seedlings in our CCA analysis. In particular, *Russula nitida* was significantly correlated with lower seedling foliar C:N ratios. This taxon has been detected in inorganic, nitrogen-rich soils in the boreal forest (Toljander *et al.* 2006), suggesting that it might be effective at mobilizing labile sources of nitrogen, made more available after fire, to host plants. On the other hand, the correlation between fungal abundance of this species and lower foliar C:N ratios may indicate fungal preference for inorganic N hotspots that also improve seedling performance. Our findings are consistent with those of studies during primary succession, where fungal species-level effects on plant C:N ratio were detected (Nara 2006a). Although the important role of EMF in providing mineral nutrients to host plants is well established, these observations are often made under artificial conditions and compare acquisition by non-mycorrhizal and mycorrhizal plants. Our results suggest important roles of EMF species for nutrient acquisition of *naturally* established seedlings colonized by complex communities of native fungi.

There are many ways the nurse plants could provide inoculum and influence seedlings. Shrubs provide litter and influence the soil environment through shading, water uptake, rhizodeposition, etc. However, one of the most direct routes of influence, and most likely in our view, is that mycelial spread from colonized nurse plant roots led to colonization of seedling roots. This could create common mycorrhizal networks. However, our ITS data can neither confirm nor rule out direct physiological connections through mycelial networks in the case of fungal species shared by shrubs and seedlings. Additional experiments that manipulate fungal identity versus connection to a

resprouting shrub via mycorrhizal hyphae, a CMN, (sensu Booth 2004) would provide important insights into the direct and indirect effects of shrubs as sources of inoculum and of particular fungi in facilitating seedling biomass accrual and establishment success in this post-fire treeline system.

Geographic sequence comparisons indicated that mycobionts of tree seedlings at treeline after fire also occur on tall and dwarf shrubs in tundra. Similarly, Timling *et al.* (2014) found that 73% of all fungal taxa observed in the southern-most extent of Arctic tundra, the region most likely to undergo conversion from tundra to boreal forest, were detected in the boreal forest. Furthermore, there is continuity between the understory composition in the boreal forest and tundra plant communities (Hollingsworth *et al.* 2006), particularly with regard to ECM hosts such as *Salix* sp. and *Dryas* sp. Together, this evidence suggests that compatible inoculum is present on the landscape pre-fire, and, that post-fire resprouting shrubs are a source of compatible inoculum for establishing tree seedlings. Moreover, resprouting shrubs host EMF and other root-associated fungi that support seedling biomass accrual and are correlated with seedling nutrient status. Based on our results, we would expect that in shrub tundra, where there are abundant ECM host shrubs, ECM nurse shrubs may facilitate forest expansion and exert important feedbacks to the climate system.

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Data Accessibility

DNA Sequences: GenBank accessions: KU176235-KU176321.

Environmental and fire-severity data pertaining to our research sites are archived with the Bonanza Creek LTER http://www.lter.uaf.edu/data/data-detail/id/342.

Seedling biomass, foliar C:N, fungal community composition, and variables pertaining to distance between focal plants and soil characteristic are archived with the Long Term Ecological Research Network DOI: 10.6073/pasta/ce3825796e5c852ba2acf6ef91ac386b

Author Contributions

R.E.H and D.L.T. designed the research, R.E.H and F.S.C. III performed field studies, R.E.H and D.L.T. performed molecular and bioinformatics work, R.E.H and D.L.T. contributed reagents and analytical tools, R.E.H. analyzed data, R.E.H., D.L.T, T.N.H., and F.S.C III wrote paper.

TABLES

Table 1: Distribution of GenBank matches in the boreal forest and beyond treeline in Arctic tundra for fungi shared by seedlings and adjacent shrubs. The top 100 GenBank matches at the species level (>90% coverage of ITS1-5.8s-ITS2) gene region with >97% sequence similarity) were considered. Ribotypes with multiple OTU assignments and those that did not have an OTU assignment (ribo51, ribo25, ribo15) were excluded. We recognize that some taxa, e.g. *Cortinarius* sp., generally require higher stringency for species level matches, i.e. 99% similarity; for OTU 36 sequence matches in the boreal region match at 99% and those in the Arctic match at 98% similarity.

OTU	# of seedling-shrub pairs	Name	Biome		Host Plant					
			Arctic tundra	Boreal Forest	*Arctosaphylos uva-ursi*	*Betula nana*	*Betula neo-alaskana*	*Picea glauca*	*Populus tremuloides*	*Salix* sp.
OTU2	7	*Thelephora terrestris*		+	+	+	+	+	+	+
OTU37	6	*Russula vinososordida*	+			+	+		+	+
OTU29	4	*Meliniomyces bicolor*	+	+		+	+		+	+
OTU57	3	*Lactarius rufus*		+		+	+		+	+
OTU34	2	*Inocybe lapponica*	+						+	+
OTU9	2	*Russula nitida*	+	+		+		+	+	
Singleton14	1	*Gyoerffyella rotula*	+	+		+		+	+	
OTU11	1	*Meliniomyces variabilis*	+	+		+		+		
Singleton6	1	*Amphinema byssoides*		+		+			+	

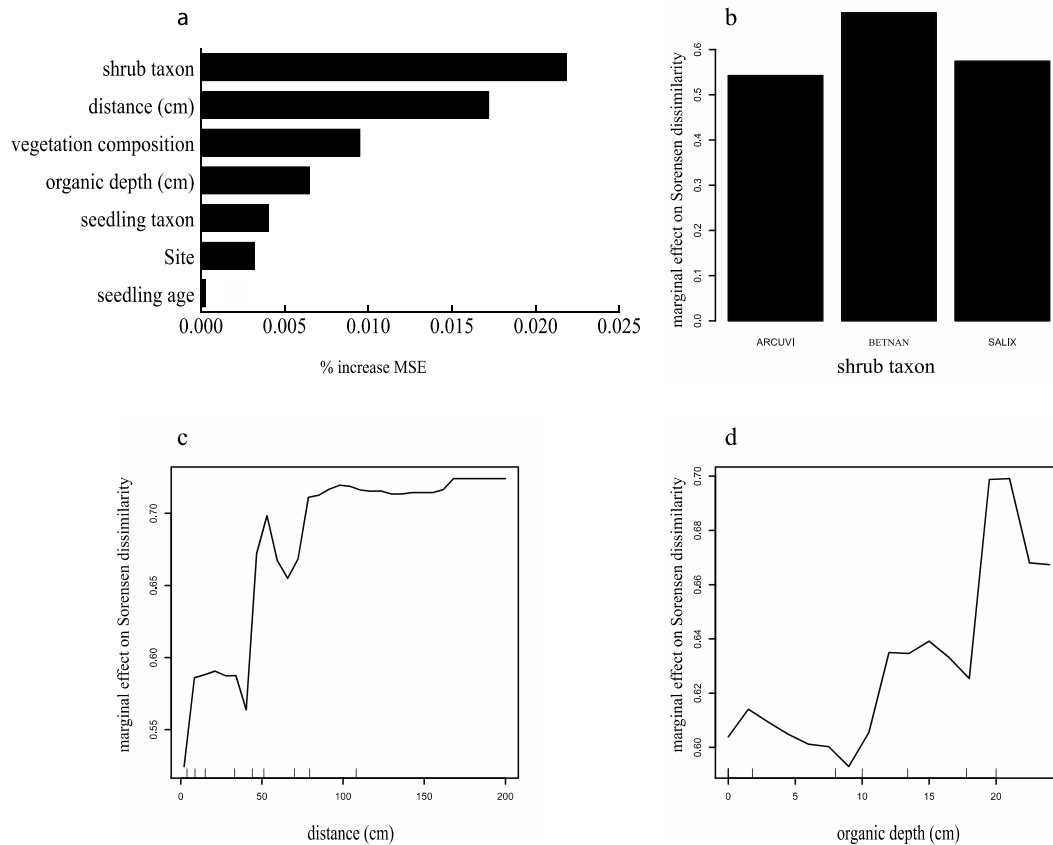|										
OTU36	1	*Cortinarius* sp.	+	+		+	+		+	+
OTU19	1	*Tylospora asterophora*		+	+		+		+	
OTU25	1	Ceratobasidiaceae		+		+			+	
OTU12	1	*Tomentella terrestris*	+	+		+		+	+	
OTU33	1	*Lactarius glyciosmus*	+	+					+	+
OTU6	1	*Lactarius vietus*	+	+		+	+		+	
OTU28	1	*Boletus edulis*		+	+				+	
OTU7	1	*Phialocephala fortinii*	+	+		+		+		

Figures

Figure 1 a-d: a) Variable importance scores resulting from plots from Random Forest regression trees predicting the Sørensen dissimilarity between the root-associated fungal communities of seedlings and adjacent shrubs. Variable importance scores are derived from the increase in the mean square error (% increase MSE) when a variable is permuted. b-d) Partial dependence plots depict predicted Sørensen dissimilarity in fungal communities after accounting for the average effect of all other explanatory variables: b) shrub taxon, c) physical distance between the seedling and shrub, and d) residual organic soil depth after wildfire. ARCUVI=*Arctostaphylos uva-ursi*, BETNAN=*Betula nana.* On the y-axis 0= identical communities, 1= no shared taxa.
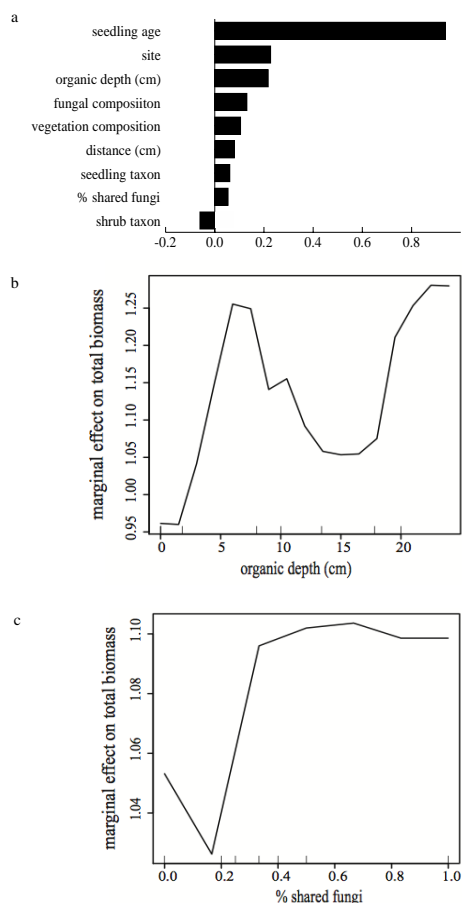
Figure 2 a-c: a) Variable importance scores resulting from Random Forest regression trees predicting seedling biomass. Variable importance scores are derived from the increase in the mean square error (% increase MSE) when a variable is permuted. b-c) Partial dependence plots depict predicted biomass in relation to each explanatory variable after accounting for the average effect of all other explanatory variables: b) residual organic soil depth after wildfire and c) percentage of shared fungi occurring on a seedling and adjacent shrub pair.

S.I. Appendix A: Detailed description of molecular methods

S.I. Appendix B: Sensitivity analysis to determine the effect of removing ribotypes with more than one Operational Taxonomic Unit (OTU) assignment.

S.I. Figure 1: A map of our study region with sampling sites at Finger Mountain and Nome Creek, where we sampled naturally established tree seedlings at post-fire treeline sites. Both Finger Mountain and Nome Creek are within burn scars from wildfires in 2004, shown as orange polygons.

S.I. Figure 2: Bray-Curtis ordination biplot of fungal composition associated with naturally established seedlings at treeline after wildfire. The direction and magnitude of the vectors portray important correlations ($r^2 > 0.200$) with vegetation composition and the depth of the organic soil remaining after fire.

S.I. Figure 3: Canonical Correspondence Analysis ordination of dominant fungal ribotypes in environmental space using LC (linear combination) scores. Biplot vectors show direction and magnitude of the correlation between species composition and foliar C:N and biomass for seedlings harvested at Finger Mountain and Nome Creek.

S.I Table 1: Fungal ARISA ribotypes detected on the root systems of seedlings and adjacent resprouting shrubs. Direct and clones sequence identities (OTUs) were directly matched to ribotypes by fragment length. ARCUVI=*Arctostaphylos uva-ursi*, BETNAN=*Betula nana*, POPTRE=*Populus tremuloides*, BETNEO=*Betula neo-alaskana*.

S.I. Table 2: Spearman correlations to detect collinearity between variables. Significant correlations are in bold.

S.I. Table 3: Operational Taxonomic Units (OTUs) detected on the root systems of seedlings and adjacent shrubs at Nome Creek and Finger Mountain. Phylum B= Basidiomycota; A= Ascomycota. Guild EMF= ectomycorrhizal fungus, DSE= dark septate endophyte, ERM= ericoid mycorrhizal fungus. Reference and Identity (%) is the sequence similarity between the query sequence and the hit sequence from the GenBank or UNITE database. Coverage is the percentage of the top match sequence that aligns with the query sequence.

S.I Table 4: Monte Carlo test results for Canonical Correspondence Analysis of EMF composition versus seedling biomass and C:N ratio.