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Root-Associated Ectomycorrhizal Fungi Shared by Various Boreal Forest Seedlings Naturally Regenerating after a Fire in Interior Alaska and Correlation of Different Fungi with Host Growth Responses[▽]

Elizabeth Bent,[1]* Preston Kiekel,[2] Rebecca Brenton,[1] and D. Lee Taylor[1]

Institute of Arctic Biology, University of Alaska—Fairbanks, Fairbanks, Alaska 99775,[1] and University of Washington, Seattle, Washington 98101[2]

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The role of common mycorrhizal networks (CMNs) in postfire boreal forest successional trajectories is unknown. We investigated this issue by sampling a 50-m by 40-m area of naturally regenerating black spruce (*Picea mariana*), trembling aspen (*Populus tremuloides*), and paper birch (*Betula papyrifera*) seedlings at various distances from alder (*Alnus viridis* subsp. *crispa*), a nitrogen-fixing shrub, 5 years after wildfire in an Alaskan interior boreal forest. Shoot biomasses and stem diameters of 4-year-old seedlings were recorded, and the fungal community associated with ectomycorrhizal (ECM) root tips from each seedling was profiled using molecular techniques. We found distinct assemblages of fungi associated with alder compared with those associated with the other tree species, making the formation of CMNs between them unlikely. However, among the spruce, aspen, and birch seedlings, there were many shared fungi (including members of the *Pezoloma ericae* [*Hymenoscyphus ericae*] species aggregate, *Thelephora terrestris*, and *Russula* spp.), raising the possibility that these regenerating seedlings may form interspecies CMNs. Distance between samples did not influence how similar ECM root tip-associated fungal communities were, and of the fungal groups identified, only one of them was more likely to be shared between seedlings that were closer together, suggesting that the majority of fungi surveyed did not have a clumped distribution across the small scale of this study. The presence of some fungal ribotypes was associated with larger or smaller seedlings, suggesting that these fungi may play a role in the promotion or inhibition of seedling growth. The fungal ribotypes associated with larger seedlings were different between spruce, aspen, and birch, suggesting differential impacts of some host-fungus combinations. One may speculate that wildfire-induced shifts in a given soil fungal community could result in variation in the growth response of different plant species after fire and a shift in regenerating vegetation.

In Alaska and adjacent boreal forest regions, the frequency, severity, and extent of fires are increasing due to climatic changes, and this trend is expected to continue and intensify in the future (14, 26). Major tree species found in developing postfire forests in interior Alaska include black spruce (*Picea mariana*), white spruce (*Picea glauca*), trembling aspen (*Populus tremuloides*), and paper birch (*Betula papyrifera*). Plant dispersal and physiological responses to soil, site, climatic factors, and other species will certainly influence which plant species survive and thrive in a given area. However, a likely influence that has only recently been given attention is the role of soil fungi in mediating these plant responses.

Despite their importance to the functioning of ecosystems, microorganisms are rarely explicitly considered in individual ecosystem or global process models; the interactions of soil microbes with plants are similarly ignored or are considered a "black box"(45). It is widely known that ectomycorrhizal (ECM) fungi, which can form associations with many boreal forest trees, have a profound effect on plant nutrition, plant health, soil structure, and even water uptake (11). It has been estimated that mycorrhizal fungi provide 61 to 86% of the nitrogen present in Arctic tundra plants (20). There is very little evidence concerning the positive or negative effects of particular fungal species on forest plant hosts under field conditions. Such information would be useful, when it is combined with ecological data on changing fungal and plant communities, in predicting future shifts in forest plant communities.

One aspect of plant-fungal interactions that is of great interest to ecologists is the establishment and functioning of common mycorrhizal networks (CMNs), or mycorrhizal hyphal networks that transfer nutrients between the root systems of two different individual plants. Mycoheterotrophic plants are provided with photosynthate via CMNs (29), and the establishment of functioning CMNs between different autotrophic plant species has been documented in a variety of studies (2, 19). For example, Nara (35, 36) demonstrated that the establishment of CMNs between pioneer species and subsequent colonizers was important in the success of the latter and also showed that the effect of CMNs on seedling nitrogen acquisition varied between different species of fungal symbionts. Simard et al. (44) showed that carbon can be transferred bidirectionally between saplings of a conifer (Douglas fir) and a deciduous hardwood (paper birch), probably via shared ECM fungi. There was preferential carbon flow to the conifer, particularly when it was shaded. Of perhaps greater relevance to nutrient cycling and

* Corresponding author. Present address: Department of Forest Mycology and Pathology, P.O. Box 7026, Swedish University of Agricultural Sciences (SLU), Ulls Väg 26A, SE-750 07 Uppsala, Sweden. Phone: 46 18 67 1579. Fax: 46 18 67 3599. E-mail: elizabeth.bent@mykopat.slu.se.

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competitive dynamics within plant communities would be exchange of nitrogen or phosphorus via CMNs. The predominant source of fixed nitrogen to boreal forests of interior Alaska is the symbiosis between alder species and nitrogen-fixing *Frankia* bacteria (52).

Very little is known about the composition of postfire ectomycorrhizal fungal communities in interior Alaska. To determine if CMNs could potentially form between reestablishing tree seedlings and nitrogen-fixing alder shrubs (*Alnus viridis* subsp. *crispa*), a survey of ectomycorrhizal root tips was conducted. Molecular methods were used to determine whether there were any ectomycorrhizal root tip-associated fungi often found in common on alder roots and the roots of black spruce (*P. mariana*), trembling aspen (*P. tremuloides*), or paper birch (*B. papyrifera*) seedlings taken from an area burned by wildfire 5 years previously. This study was designed to determine whether fungal species were shared among postfire tree species and thus evaluate the potential for CMNs but not to evaluate hyphal connections between tree species, which would be required to prove the existence of CMNs.

Our main hypothesis was that ectomycorrhizal fungi would be found in common on alder and the other sampled plant species. Secondary hypotheses were (i) that specific fungal species would be associated with increased or decreased plant biomass and (ii) that plants located closer to one another would support communities of fungi that are more similar to each other than plants located at greater distances from each other.

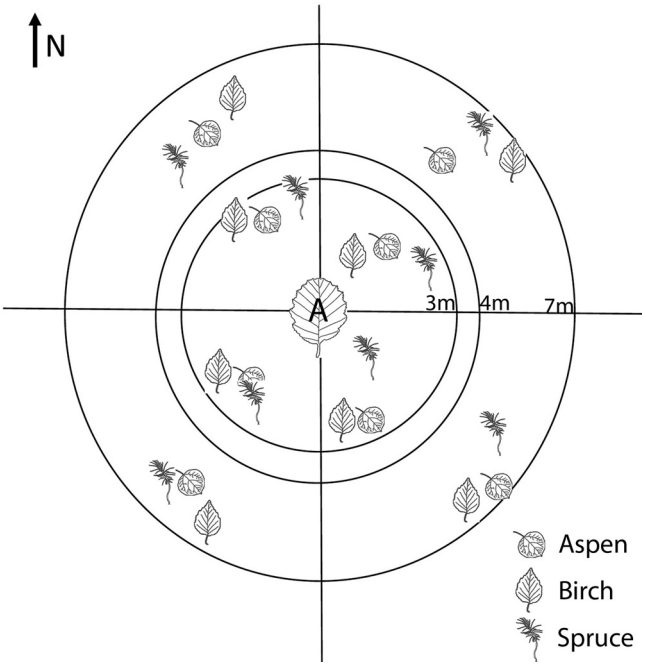

FIG. 1. Diagram of sampling scheme used to harvest plants in the vicinity of each alder (A, center). Quadrants were established on the basis of compass points (northeast, northwest, southeast, and southwest) and areas of various distances from alder (0 to 3 m and 4 to 7 m) identified. From each of these eight areas, one spruce, one aspen, and one birch seedling were located and harvested as described in the text. Samples depicted indicate locations of seedlings harvested relative to alder 1 (not to scale).

MATERIALS AND METHODS

Sample collection and processing. Previously, 90 interior Alaskan black spruce forest sites burned by three large fire complexes in 2004 had been established and characterized (22). Several of these were located in the Caribou-Poker Creeks Research Watershed, which is a Bonanza Creek Long Term Ecological Research area north of Fairbanks, Alaska. A 50-m by 40-m area (65.14793N, 147.47123W) adjacent to one of these sites (site BF83) was chosen for this study because it contained alder (*Alnus viridis* subsp. *crispa*) plants and was easily accessible. The burn severity for BF83 was rated moderate (22). Note that recruitment at site BF83 occurred only in the summer of 2005, after the fire, meaning that all seedlings studied at this site were 4 years old (T. Hollingsworth, personal communication). Recruitment at BF83 and the other sites studied by Johnstone et al. (22) is favored by bare soil and becomes rare once herbaceous and grassy vegetation has reestablished.

The locations of all alder plants at this site were mapped in 2008, and in 2009, three of these alders that were located more than 10 m apart were chosen. The area around each alder was divided into four quadrants, based on compass points: northeast, northwest, southeast, and southwest (Fig. 1). Each quadrant was then divided into two zones, based on distances from the main stem of the alder plant: 0 to 3 m (near) and 4 to 7 m (far). From each of these eight zones (Fig. 1), one black spruce (*P. mariana*), one trembling aspen (*P. tremuloides*), and one paper birch (*B. papyrifera*) seedling were identified. The locations of each of these seedlings were mapped, and then each entire seedling was removed, taking care to keep the root system as intact as possible. Seedlings were placed in plastic bags, along with a small quantity of water, and placed in a refrigerated room on the University of Alaska—Fairbanks campus within 2 h. A sample of roots was also collected from under the central alder plant. Twenty-five samples (8 spruce, 8 birch, 8 aspen, and 1 alder) were collected on three separate occasions, in July and August of 2009, for a total of 75 plant samples.

Seedlings were processed within 1 week. Roots were carefully washed under tap water to remove coarse debris and were then kept refrigerated and examined on the same day under a dissecting microscope. All the healthy ectomycorrhizal root tips that could be found on the sample were removed. These tips either could be traced back to the stem of the plant or, in the case of the alder root sample, could be traced to a root nodule. Of the total pool of root tips taken from each plant, 10 (or fewer, where fewer total root tips were observed) were randomly selected and placed in individual 0.6-ml microcentrifuge tubes. Each individual root tip was frozen in a small quantity of sterile nanopure water at −80°C and lyophilized.

Shoots were severed from roots and dried at 56°C for 3 to 4 days. Shoot dry weight and stem diameter were recorded.

DNA extraction and automated ribosomal intergenic spacer analysis (ARISA). Lyophilized samples of pooled root tips were suspended in 50 μl of sterile nanopure water, and then each was ground in a 0.6-ml microcentrifuge tube using a grinder and sterile pestle of appropriate size. Preliminary work demonstrated that there was no difference in the amount of amplified fungal DNA obtained from alder, paper birch, trembling aspen, or black spruce ectomycorrhizal root tip homogenates (ground root preparations) compared with the amount of genomic DNA that had been extracted from the same ground tissue using a commercially available kit, when a given volume of each preparation is similarly diluted (3). The amplification from root tip homogenates was optimal when they were diluted 1/100 (3). Therefore, immediately after they were ground, 1 μl of each root tip homogenate was diluted 1/100 in sterile nanopure water, and from this mixture, fungal internal transcribed spacer 1 (ITS1) and ITS2 sequences were amplified using fungus-specific PCR primers. Homogenates were then kept frozen at −20°C.

Ten-microliter reaction mixes were prepared containing 0.65 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.05 μM forward primer FAM-ITS1F (CTTGGTCATTTAGAGGAAGTAA [13] labeled on the 5′ end with 6-carboxyfluorescein [FAM; a fluorescein amidit; Applied Biosystems, Carlsbad, CA]), 0.05 μM reverse primer ITS4 (TCCTCCGCTTATTGATATGC [60]), 0.06 mg/ml bovine serum albumin, 0.15 μl JumpStart RED *Taq* (Sigma-Aldrich, St. Louis, MO), 1× JumpStart RED *Taq* buffer, and 1 μl of diluted root tip homogenate. Reaction mixes were prepared in 0.2-μl tubes and thermocycled in an MJ Research PTC-225 thermal cycler as follows: 96°C for 3 min and 35 cycles of 94°C for 30 s, 52°C for 30 s, and then 72°C for 3 min, followed by 72°C for 10 min.

Each PCR mixture was then diluted 1:1, and 1 μl of this was mixed with 14.25 μl of formamide and 0.75 μl of a size standard (MapMarker 1000 X-rhodamine; BioVentures Inc., Murfreesboro, TN). This mixture was heated to 95°C for 5 min and then immediately placed on ice, until it could be run through an ABI 3100

genetic analyzer (Pop6, 50-cm array, T-RFLP_1500 protocol, which allows longer reads; Applied Biosystems, Carlsbad, CA).

Amplicon cloning and sequencing. Fungal ITS region sequences were obtained by amplifying them via PCR, as described earlier, with the modification that the PCR primers were USER-ITS1F and USER-ITS4. These primers are identical to ITS1F and ITS4, respectively, except that each has an additional 8 bases added for use with the USER enzyme and vector pNEB205a (New England BioLabs, Ipswich, MA). The resulting primer sequences are as follows, with the additional bases underlined: USER-ITS1F, GGAGACAUCTTGGTCATT TAGAGGAAGTAA; USER-ITS4, GGGAAAGUTCCTCCGCTTATTGATA TGC. These PCRs were run concurrently with the above-described reactions for ARISA, so that root homogenates would not have to be frozen and then thawed. After PCR, the reaction mixtures were kept at 4°C until after the ARISA results could be analyzed to identify amplicons of interest. These were always peaks separated from all other peaks by at least 50 bases, representing a range of amplicon sizes and plant species (i.e., amplicons of similar size were isolated from each plant species where possible).

Five microliters of each reaction mix was electrophoretically separated for 45 min on an agarose gel in TBE (Tris-borate-EDTA) buffer (0.8% agarose, 100 V, 40 min). Gels were stained with ethidium bromide and visualized using a Kodak Gel Logic 200 imaging system to verify the presence of bright bands for the amplicons of interest. A larger volume of reaction mix (8.5 μl) was then electrophoretically separated under identical conditions, and the amplicons of interest were carefully excised with a scalpel. To reduce cross-contamination, these samples were run in every other lane. Only new, fresh buffer was used, and this was changed after each run. Gel slices were kept frozen in microcentrifuge tubes at −20°C until use.

Amplicons were recovered from the gel slices using a Qiagen gel extraction kit, following the manufacturer's instructions, save that DNA was eluted into 30 μl of 1/10-strength kit buffer EB. Amplicons were ligated into pNEB205A as follows: 5 μl of amplicon DNA was mixed with 0.5 μl each of USER enzyme and pNEB205A (New England BioLabs, Ipswich, MA), and the mixture incubated for 15 min at 37°C and then 15 min at room temperature.

Ligation reaction mixtures were then placed on ice and used within 3 h for the transformation of *Escherichia coli*. The entire ligation reaction mixture was added to 8 μl of competent *E. coli* cells (One Shot MAX Efficiency DH5α-T1R competent cells; Invitrogen Corporation, Carlsbad, CA) in a 2-ml microcentrifuge tube. Cells were transformed using heat shock and incubated in 200 μl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) for 30 min. Volumes of 50 or 100 μl were then plated on AIX medium (Luria-Bertani [LB] agar with 100 μg/ml ampicillin, 50 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [Sigma-Aldrich, St. Louis, MO], 12.5 μg/ml isopropyl β-D-thiogalactoside [Sigma-Aldrich, St. Louis, MO]) and incubated for 16 to 18 h at 37°C. One white colony was used to inoculate 2 ml of LB broth–100 μg/ml ampicillin in a disposable 12-ml culture tube, and this was incubated for 16 to 18 h at 37°C with 200 rpm shaking. The resultant cells were harvested, and plasmids were extracted using a Qiagen miniprep kit, following the manufacturer's instructions, except that plasmid DNA was eluted in 30 μl of 1/10-strength kit buffer EB. Plasmid DNA was then diluted to 100 ng/μl in sterile nanopure water and sent to a facility for forward and reverse sequencing (Murdock DNA Sequencing Facility, University of Montana, Missoula, MT; http://murdocklab.dbs.umt.edu).

Sequence analysis. Sequences were assembled from forward and reverse reads using only the highest-quality chromatogram peaks (a minimum Phred score of 20) and Geneious software, version 4.8.5 (9). Primers were left on the ends of sequences and vector was trimmed off, to give the length of each amplicon. Alignments of sequences of similar length were made to determine pairwise similarity, which was used as a guide to determining the boundaries of ARISA peak bins, or operational taxonomic units (OTUs). Sequences with greater than 97% pairwise similarity were considered to belong to the same OTU. Sequences were aligned using the Clustal-based alignment tool in Geneious (default settings) or the Clustal W algorithm in MegAlign (DNAStar Lasergene software package, version 8.1.4) and compared to GenBank database sequences using the Linnaeus BLAST querying tool (default settings, save that only 100 top hits were requested).

In cases where BLAST searches gave ambiguous identifications (i.e., the best matches are to unidentified environmental sequences), we carried out phylogenetic analyses as follows: top-matching sequences from GenBank were compared with the full and curated databases at http://borealfungi.alaksa.edu and aligned with our query sequence using the default settings in the Muscle program (version 3.7) (10). Alignments were trimmed and improved manually in the Se-Al program (version 2.0a11) (42), and maximum-likelihood trees were inferred under the GTR+G+I model in the Garli program (version 0.951) (61). Trees were inspected to identify clades containing our unidentified query sequences together with well-identified fungi.

Primers were removed from sequences prior to submission to GenBank.

Statistical analyses. Each ARISA peak size bin, or ribotype, was considered to represent a separate fungal taxon. An OTU consists of a collection of amplicons that have greater than 97% sequence similarity to one another, and ideally, each ribotype will contain a single OTU. The abundance of each ribotype was represented by the percentage of root tips from each sample that produced a corresponding ARISA peak. Bray-Curtis dissimilarity was calculated from the taxon abundance data for each pair of plant samples taken in the vicinity of one of the three sampled alders using the R package vegan (37). Samples from the vicinity of different alders were not compared, and alders were not compared with each other, except for ordination and Mantel analyses (see below).

To test whether tree species were equally heterogeneous with respect to fungal population, we conducted *t* tests using tree type as the between-subjects predictor, with the criterion being the dissimilarity index (Bray-Curtis). We statistically corrected for variance attributable to multiple observations on individual trees (i.e., the entire data set was used in the equation to increase sample size [*N*], and then variance attributable to individual trees was statistically removed). We also statistically adjusted for samples of the referent alder, rather than conduct separate analyses for each sample, in order to increase sample size.

To test whether fungi are clumped or spread evenly across the sampled area, we used regression to determine if spatial distance is predictive of pairwise dissimilarity. Again, the regression analysis was adjusted to account for the repeated-measures structure of analyzing pairwise comparison data.

We then conducted more detailed analyses to further characterize fungal patchiness. We tested whether or not some of the specific fungal ribotypes displayed clumped distributions. We examined the overlap in population of the 15 most abundant fungal ribotypes among all pairs of trees. Overlap was defined dichotomously as shared versus not shared for each ribotype. In order to assess the unique effect of each fungal ribotype, after adjustment for the effect of other ribotypes present, a single regression model was used to test if distance can be predicted by sharing of each fungus. We statistically accounted for repeated measurement of multiple trees, as well as separate referent alder samples. For each fungal group, the regression therefore tests the hypothesis that its shared presence between two tree roots is contingent on distance between the trees, irrespective of the shared presence of other fungi.

We wished to determine whether any of the most abundant fungal ribotypes (major ribotypes, or those containing more than 1% of the total observations) tended to be present more often on larger plants, suggesting a possible role in plant growth promotion or health. In order to determine if specific root-associated fungi may be associated with changes in plant size, the presence of all the major fungal ribotypes was used in regressions as a predictor of plant shoot weight and stem diameter. Data from each plant species were separated, due to the innate differences in size between seedlings of birch, aspen, and spruce. Moreover, it is expected that a given fungus may facilitate growth on one species but not another. Presence or absence data for all major fungal ribotypes were included in the models, to statistically correct for each others' variance. However, because the full model for a particular species results in a very small *N* and because these are exploratory analyses, stepwise regression was used to weed out unproductive predictors.

Finally, to confirm and further refine findings on the basis of presence or absence of each fungus, replicate regressions were conducted on the abundance of fungi. This results in four stepwise regressions for each of the three species: presence of the fungi and abundance of the fungi by shoot weight and stem diameter.

To confirm the results of the first three analyses, we also conducted a Mantel test of combined data from all three sampled alders using PC-ORD software (version 5.23) (28), where community distance was calculated as Bray-Curtis dissimilarity and physical distance was represented as a Euclidean distance matrix. To provide a visual representation of how alder, aspen, birch, and spruce sample communities relate to one another, we used PC-ORD to conduct a nonmetric multidimensional scaling (NMS) ordination of Bray-Curtis distances, with seedlings of the same species pooled for each of the three sampling areas (i.e., around the three focal alders). Fungal ribotypes occurring on fewer than two samples were removed, and the proportional occurrences of the remaining taxa (0 to 1) were arcsine transformed.

Nucleotide sequence accession numbers. The sequences from this study were submitted to GenBank and may be found under accession numbers HM164553 to HM164680.

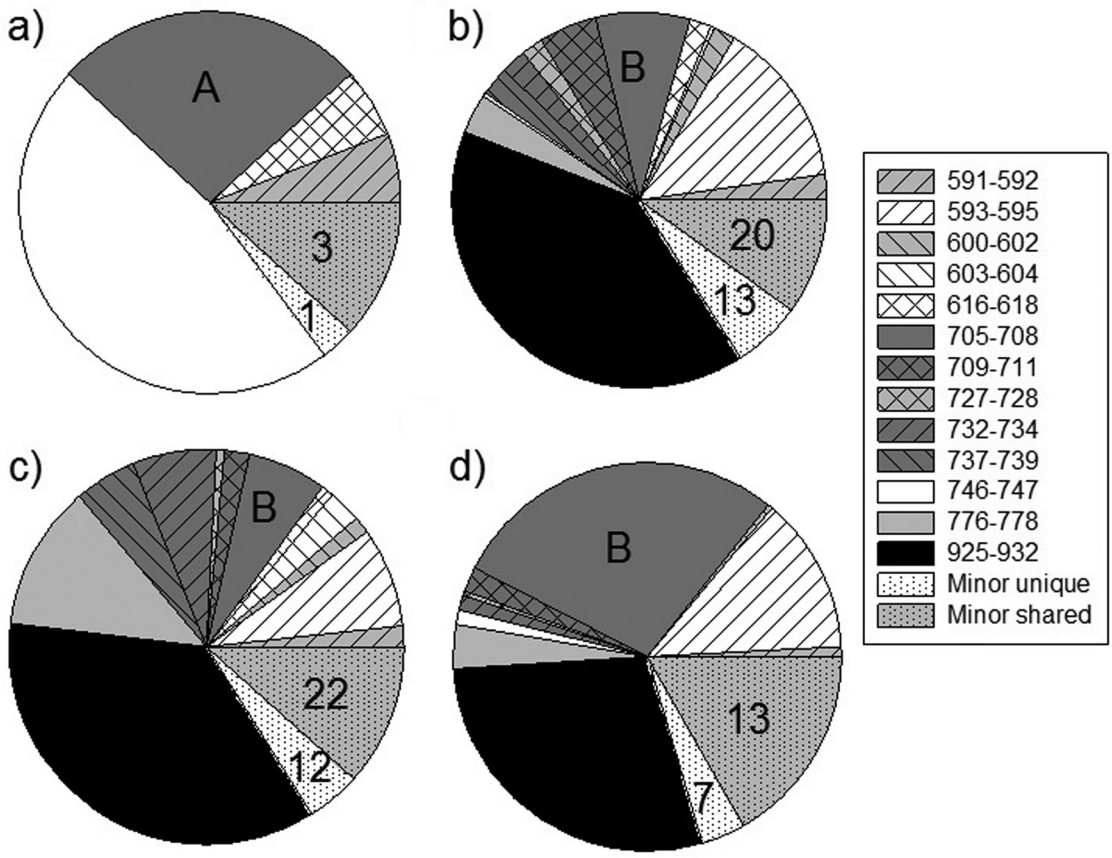

FIG. 2. Proportions of each plant species' total observed ARISA peaks per fungal ribotype for alder (a), aspen (b), birch (c), and spruce (d) seedling roots. Minor ribotypes are defined as those containing less than 1% of the total peaks observed for all species. The letter A or B on pie slices corresponding to 705 to 708 bp indicates different OTUs (see Table 1 and text for details). Numbers on minor ribotype pie slices indicate how many ribotypes are contributing to the size of the slice.

RESULTS

Quality of ribotype data used in community analyses. Seventy-one fungal ribotypes were identified. Sequenced amplicons were used to determine the boundaries of ribotypes where possible. In a few cases, it was necessary to divide fungi with the same ARISA peak size into different OTUs on the basis of the sequencing results (described in more detail below). Where no sequences were present, ribotype boundaries were placed at no more than 2 bp apart, which is the width of the majority of sequenced ribotypes that contained a single OTU.

Of the 71 ribotypes, 13 of them contained more than 1% of the total number of peaks observed in all plant species and so are considered major ribotypes. These accounted for 80 to 85% of all the peaks observed on the roots of any of the plant species sampled (Fig. 2). The majority of the observations in the remaining, or minor, ribotypes were shared among plant samples from more than one species, though minor ribotypes unique to one plant species were also observed (Fig. 2).

Table 1 summarizes the sequence data used to define the boundaries of the ribotypes. Sequences were not available for every peak detected in this study, and so some ribotypes remain unidentified. Identifications for ribotypes are given in Table 1. Of the 15 ribotypes for which more than one sequence is available, 13 of them contain a single OTU, or contain sequences with greater than 97% sequence similarity (Table 1). The remaining two ribotypes split into two overlapping OTUs. In the case of ribotype 705 to 708 bp, all the sequences from alder (A) form one OTU with high pairwise sequence similarity, while all the sequences from alder, birch, or spruce form a second OTU with high pairwise sequence similarity (B) (Table 1). Sequences from A and B were sufficiently different for these OTUs to be considered distinct (Table 1).

In the case of ribotype 925 to 932 bp, all of the 27 amplicons falling into this size range have high pairwise sequence similarity to Helotiaceae fungi in the *Pezoloma* complex (synonyms *Rhizoschyphus*, *Meliniomyces*, and *Hymenoscyphus*), but the minimum pairwise sequence similarity was 95.9% (Table 1). There was no obvious separation of amplicons into subgroups on the basis of size or source plant species, but the sequences did form two subgroups with greater than 97% minimum pairwise sequence similarity among them (A and B in Table 1). The ribotype 925 to 932 bp must therefore be considered an umbrella group for at least two fungal OTUs, both with ITS region sequences similar to those contained in various unidentified Helotiales fungi. The large size of the amplicons in this species complex is due to the presence of an intron at the 3′ end of the small subunit, as has been previously observed in this clade (57). This rapidly evolving and highly divergent in-

TABLE 1. Sequence data from cloned fungal amplicons identified in ribotypes

Amplicon size range (bp) of ribotype	Minimum % similarity among sequences	No. of sequences from ribotype	RA[b] from ribotype	Best taxonomic match (BLASTn) of RA	% similarity of RA to best taxonomic match	GenBank accession no(s). of best taxonomic match
591–592	99.7	2	HM164567	*Articulospora tetracladia*	95.6	EU998918
593–595	98.5	10	HM164556	*Cadophora finlandica*/*Meliniomyces bicolor*	97.6/97.3	AB543058/EF093183
603–604	99.2	4	HM164571	*Phialocephala fortnii*	99.7	AY394921
613–614	99.8	2	HM164573	Helotiaceae cf. *Phialophora*[a]	93.5	AY394893
616–618	NA[c]	1	HM164575	Helotiaceae cf. *Phialophora*[a]	97.1	FJ475666
636–637	99.7	3	HM164582	*Wilcoxina rehmii*	98.8	AF266708
683–684	99.6	4	HM164589	*Piloderma* spp.	99.7	FJ553335
705–708A (alder)	99.3	5	HM164604	*Tomentella sublilacina*/*Theleophora terrestris* complex	99.7	GQ398249
705–708B (not alder)	97.6	15	HM164616	*Thelephora terrestris*	99.1	GQ267490
705–708 A+B	95.0	20				
709–711	99.6	4	HM164596	*Tomentella atramentaria*	96.8	AF266708
732–734	100	3	HM164618	*Russula* spp.	99.6	AJ534906
737–739	99.4	6	HM164625	*Clavulina cinerea*	98.9	EU118616
746–747	98.9	7	HM164627	*Alnicola* (*Naucoria*) *escharoides*	98.2	GQ398420
776–778	98.7	5	HM164640	*Lactarius pubescens*	99.0	AY606953
804–805	99.5	3	HM164645	*Paxillus involutus*	99.7	EU819416
903–904	99.7	3	HM164649	*Phialocephala sphaeroides*	99.9	AY524844
925–932A	97.5	7	HM164659	*Pezoloma* complex[a]	99.6	FJ553656
925–932B	98.2	20	HM164673	*Pezoloma* complex[a]	98.5	FJ553303
925–932A + B	96.1	27				

[a] Identified using a curated database of fungal sequences from the University of Alaska, not BLAST.
[b] RA, representative accession.
[c] NA, not available (less than one pair of sequences).

tron sequence may contribute to the difficulty in separating species by a precise ARISA peak size.

Two ribotypes that were identified as belonging to the genus *Phialophora* (613 to 614 bp and 616 to 618 bp; Table 1) had 91% sequence similarity, indicating that they are appropriately separated.

Community data analyses. Despite the imperfections in the ribotype data, each ribotype was considered to represent a separate fungal taxon. Bray-Curtis dissimilarities were calculated for pairs of samples taken from the vicinity of one of the alders (alder 1, alder 2, or alder 3).

After adjustment for repeated measurements on specific tree samples, alder has a detectably higher mean Bray-Curtis dissimilarity index for its fungal populations (mean [M] = 1.01, standard error of mean [SEM] = 0.04) than nonalder trees [M = 0.68, SEM = 0.01, $t(735) = 7.05$, $P < 0.001$]. No detectable differences were found for aspen, birch, or spruce. This suggests that aspen, spruce, and birch are more likely to share fungal taxa with each other than with alder. The preferred NMS ordination solution encompassed two dimensions and had low stress (3.16) and instability (<0.000001). Fungal communities clearly clustered by plant species (Fig. 3), suggesting that each species has a somewhat distinct spectrum of fungi. However, the communities of birch and aspen were very close in ordination space, and those associated with spruce overlapped somewhat with those of birch and aspen, while the alder community was completely distinct. Communities did not group by sampling area (data not shown). Three minor ribotypes were observed to be shared between alder and deciduous species (610 to 611 bp, aspen; 662 to 664 bp and 903 to 904 bp, both aspen and birch), though it is unknown if the sequences associated with these rarely observed peaks are identical or not. A major ribotype predominantly associated with alder (476 to 474 bp) was also observed in low abundance on aspen and spruce, though again, it is unknown if the sequences associated with aspen and spruce are identical to those from alder.

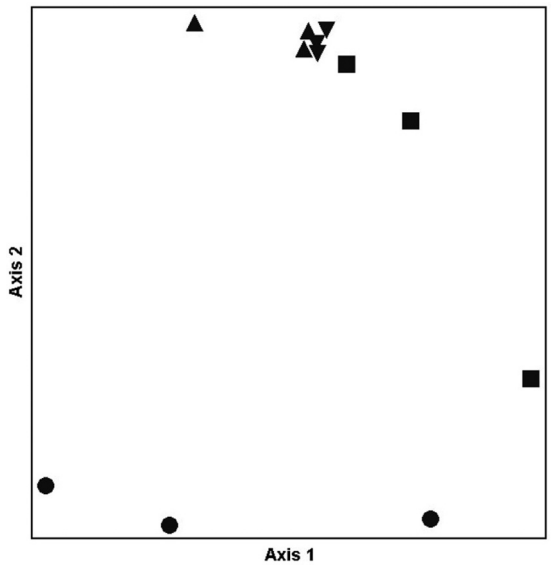

FIG. 3. Result of an NMS ordination where fungal root tip community data from seedlings of the same species are pooled for each of the three sampling areas around each focal alder, as described in the text. Different plant species are represented by different symbols: ▼, aspen; ▲, birch; ■, spruce, and ●, alder. Note that the alder communities are distinct from those of the other three plant species.

TABLE 2. Relationships between presence or abundance of specific fungal taxa on roots and size of aspen, birch, and spruce shoots

Fungal ribotype (bp)	Tentative fungal identifier[a]	Plant species	Plant size indicator	Presence/abundance	β wt (valence)[b]	*P* value
620–622	None	Aspen	Shoot wt	Presence	0.61 (+)	<0.001
				Abundance	0.67 (+)	<0.001
705–708	*Thelephora terrestris*	Aspen	Shoot wt	Presence	0.43 (+)	0.004
				Abundance	0.34 (+)	0.043
709–711	*Tomentella atramentaria*	Aspen	Shoot wt	Presence[c]	0.41 (+)	0.007
603–604	*Phialocephala fortnii*	Aspen	Shoot wt	Presence[c]	−0.32 (−)	0.032
732–734	*Russula* sp.	Birch	Shoot wt	Presence	0.41 (+)	0.045
				Abundance	0.60 (+)	0.002
600–602	None	Spruce	Stem diam	Presence	0.50 (+)	0.011
				Abundance	0.50 (+)	0.011
727–728	None	Spruce	Stem diam	Presence	−0.38 (−)	0.043
				Abundance	−0.38 (−)	0.043

[a] As summarized from Table 1.
[b] The size of the β weight indicates the strength of the relationship between plant size and fungal presence or abundance. The valence indicates a positive (larger plants) or negative (smaller plants) relationship with increased fungal presence or abundance.
[c] These indicator findings were not replicated with abundance data.

Does the physical distance between samples influence root fungal community composition? One would expect plants that are close together to support fungal populations that are more similar than those from plants located farther apart. After adjustment for the repeated-measures structure in the analysis predicting fungal population dissimilarity from distance, no statistically detectable relationship was found. This null finding is found whether analysis includes the referent alder as a covariate or separate analyses are conducted for each of the three alder samples (all P values were >0.4). There was no effect of distance on the probability of two fungal communities in a sample set being similar. The results of this analysis are supported by the nonsignificant ($P = 0.3676$) result of the Mantel test which examined the relationship between community dissimilarity and physical distance between samples. However, we note that our likely undersampling of fungal diversity and distribution may have made it difficult to detect spatial structure in this data set.

Are any of the dominant fungi more likely to be shared between samples that are close together? Patchiness for each fungal ribotype was tested with a repeated-measures-adjusted multiple-regression test, predicting distance from the sharedness of each fungus. Only one ribotype, 630 to 631 bp, showed a clumped distribution, where the beta parameter estimate in the regression equation (β) is equal to −2.19, the standard error (SE) of β is equal to 1.11, $t(720)$ is −1.98, and P is 0.048. The negative valence of β indicates that greater distance is associated with absence of the fungal OTU.

There was therefore no influence of distance on the probability of peaks from the major fungal OTUs being shared between two plants for the majority of fungal OTUs tested. The exception was 630 to 631 bp, an OTU which remains unidentified and which was not present on any of the alders.

Is there a relationship between specific fungi and plant size? The statistically detectable relationships between the presence or abundance of specific fungal taxa with plant size for each plant species are given in Table 2. Negative β values indicate that the fungi potentially inhibit growth, and positive β values indicate that the fungi potentially facilitate growth. Each tree species was supported or hindered by different fungal groups.

Aspen and birch had detectable relationships between specific fungi and shoot weight but not stem diameter (Table 2). For spruce, the reverse is true: stem diameter could be predicted from the presence or abundance of specific fungi (Table 2), but shoot weight was unaffected.

The differential responses of trees to fungi is illustrated by ribotype 705 to 708 bp, which was a common fungus on birch, aspen, and spruce (Fig. 2) but which was correlated with increased growth only on aspen.

DISCUSSION

Fungal genera identified in this study. Fully sequenced amplicons were obtained for most major ribotypes (defined as containing at least 1% of the total peak observations) and some of the minor groups (Table 1). Most of the ribotypes contain sequences that are highly similar to ones from known genera of ectomycorrhizal fungi (*Alnicola*, *Clavulina*, *Lactarius*, *Paxillus*, *Piloderma*, *Russula*, *Tomentella*, *Theleophora*, *Wilcoxina*; Table 1).

Of the ribotypes predominantly found on alder, sequences of ribotype 746 to 747 bp were predominant on alder (Table 1) and closely matched sequences of *Naucoria escharoides* (since renamed *Alnicola escharoides* [33]). Fungi from this genus have been found to be host-specific symbionts of alder in other studies (49), making it unlikely that the few amplicons from this ribotype found on aspen and spruce (Fig. 2) belong to the same fungal species. Ribotype 705 to 708 bp, which is the second most abundant "taxon" in this study, is actually composed of two groups of sequences of overlapping size. It is notable that one of these groups (group A, *Tomentella* spp.) is comprised solely of sequences from alder root tips, while the other (group B, *Thelephora terrestris*) consists of sequences from aspen, birch, and spruce roots (Table 1). Sequences from ribotype 705 to 708 bp of group A and ribotype 705 to 708 bp of group B have a minimum of 95% similarity (Table 1). This is consistent with the observation that some *Tomentella* spp. are associated with alders and that alder-associated ectomycorrhizal basidiomycetes are host specific (49).

The third most abundant ribotype in this study was 593 to

595 bp, identified as having equal sequence similarity to *Cadophora finlandica* and *Meliniomyces bicolor*, both members of the *Pezoloma ericae* (*Hymenoscyphus ericae*) species aggregate (17). ARISA peaks of 593 to 595 bp were not observed on alders but were present on the other three plant species, particularly spruce (Table 1). Interestingly, *Meliniomyces variabilis* and *M. bicolor* isolated from pine ectomycorrhizas were found to form functional ericoid mycorrhizas with *Vaccinium vitis-Vaccinium idaea*, supporting the idea that these isolates are capable of more than one type of association with a broad range of hosts (15).

Two small ribotypes contained members of the genus *Phialocephala*, known to contain dark septate endophytes (24, 50). A *Phialocephala*-related fungus was found to form ectomycorrhizas on pine (but not aspen [34]). Dark septate endophytes which enhance plant growth have been observed (1, 53), but there are also reports of negative and neutral effects (24). It has been suggested that the effect of dark septate endophyte colonization can vary along a continuum from parasitism to mutualism, much as the effect of mycorrhizal symbioses can vary (24). Dark septate fungi are more frequently recorded on ECM roots after fire and other disturbances (7, 56).

The ribotype 591 to 592 bp is notable for the fact that it is shared between alders and the other three plant species in this study, albeit in low abundance. This group contained sequences with low percent similarity (95.6%) to *Articulospora tetracladia*, an aquatic hyphomycete. *A. tetracladia* has been identified to be a root endophyte of the carnivorous bog plant *Drosera rotundifolia* (41), and while it is possible that this fungus was present as an endophyte, it is also possible that it merely contaminated the surface of the sampled root tips. Among the identified major ribotypes, few were shared between alder and other plant species, and it is not clear that shared ribotypes contain similar sequences (see next section).

Other sequences were not identifiable to the genus level on the basis of BLAST searches. In fact, the most abundant ribotype in this study (925 to 932 bp) consists of at least two species of organisms that were both identified as helotialean fungi in the *Pezoloma* complex (*Pezoloma*/*Rhizoscyphus*/*Meliniomyces*/*Hymenoscyphus*) that have overlapping amplicon sizes (Fig. 2; Table 1). It is notable that, unlike ribotype 705 to 708 bp, no distinction could be made between the two OTUs (A and B) identified on the basis of the source plant species. Because different members of this complex have been identified to be ericoid, ectomycorrhizal, endophytic, or pathogenic, assumptions about the ecological role of these fungi are tenuous (47, 48).

Two other ribotypes were identified as uncultured Leotiomycetes (613 to 614 bp) and uncultured Sordariomycetes (616 to 618 bp) using BLAST searches, but these family-level identifications could be unreliable due to taxonomic confusion and the high probability of the presence of misidentified sequences in GenBank. Our subsequent attempts to improve the identification of these sequences using the smaller curated database at the University of Alaska led to the identification of both of these groups as Helotiaceae cf. *Phialophora*, a genus which contains ectomycorrhizal fungi (58). Sequences from these ribotypes had 91% similarity to each other, indicating that they contain different OTUs.

Community data analyses. Alder ectomycorrhizal root tips appeared to harbor fungal communities distinct from those of spruce, aspen, and birch. Both the Bray-Curtis dissimilarity data and the data from sequenced amplicons in ribotype 705 to 708 bp support this. There are shared ribotypes with sizes of 591 to 592, 616 to 618, and 746 to 747 bp between alder and the other plant species (Fig. 2), as well as among some minor ribotypes (610 to 611 bp, 662 to 664 bp, and 903 to 904 bp), but (i) without sequenced amplicons from both plant species, it should not be assumed that the similarly sized amplicons have identical sequences in alder versus the other plant species, and (ii) with the exception of ribotype 746 to 747 bp, these ribotypes represented only a small portion of the total sampled ectomycorrhizal root tips in this study. Given the fact that ribotype 746 to 747 bp was identified to be the alder-specific fungus *Alnicola escharoides* (Table 1) on the basis of sequences from alder, it is unlikely that the few amplicons of this length obtained from spruce and aspen belong to the same species. It is clear that the majority, if not all, the fungi associated with ectomycorrhizal root tips in alders are distinct from those in spruce, aspen, and birch (Fig. 2).

Our results agree with those of previous studies that have shown that other alder species are unusual in forming associations with relatively few ECM fungal species (30–32, 39, 40, 49). Furthermore, many of the ECM fungi recorded in association with alder on the basis of surveys of aboveground sporocarps are specialized to alder. The specificity seems to be reciprocal. It is conceivable that selection has acted to restrict the occurrence of CMNs between alder and other ECM hosts, as this might facilitate excessive loss of nitrogen. Additionally, alder may prefer fungi that provide maximum returns of phosphorus but little or no N, which might constitute an ineffective association for other ECM hosts.

It is also equally clear that there are many shared fungi among spruce, aspen, and birch seedlings, raising the possibility that though these plant species may not be linked via CMNs with alder, they may be linked via CMNs with each other.

Distance between sampled plants did not have any effect on overall fungal community composition of root tips, as measured by Bray-Curtis dissimilarities, or on the probability that the majority of fungal OTUs were shared between two plants. One exception was noted, that of ribotype 630 to 631 bp, an unidentified group, which is significantly ($P < 0.05$) more likely to be shared between two plants that are closer together. Thus, it appears that infective propagules were widely and evenly distributed following the fire in 2004, although it should be noted that samples were taken where plants were found and not in a regular grid, and so the sampling design of this experiment may not be optimal for detecting spatial relationships. It is also possible that the even distribution of fungi in this experiment is an effect of high ECM diversity on plant roots and limited sampling of root tips. The apparent even distribution of fungi could be due either to a large bank of resistant propagules that survived soil heating and drying (46) or to rapid postfire dispersal. Most other studies of spatial structure in ectomycorrhizal communities have revealed clumped distributions of taxa, attributed to either stochastic dispersal or the competitive dynamics of growing fungal genets (27). However, most spatial studies have been carried out in mature forests rather than following a recent disturbance. The ECM fungal

community in this study was dominated by ascomycete taxa, including dark septate and ericoid mycorrhiza-related members of the Helotiaceae, while highly clumped patterns have been observed more often in late-successional, basidiomycete-dominated systems (27).

Plant size seemed to be affected by the presence or abundance of specific fungi (Table 2). Each plant species seemed to be affected by different groups of fungi: greater aspen shoot mass was associated with ribotype 705 to 708 bp (*Thelephora terrestris*) and ribotype 709 to 711 bp (tentatively identified to be *Tomentella* spp.), while greater birch shoot mass was associated with ribotype 732 to 734 bp (*Russula* spp.), and larger spruce stem diameters were associated with ribotype 600 to 602 bp, an unidentified fungal taxon. Negative associations were also observed: smaller aspen shoot mass was associated with the presence of ribotype 603 to 604 bp (*Phialocephala fortnii*, a dark septate endophyte), while smaller spruce stem diameters were associated with ribotype 727 to 728 bp, an unidentified fungal taxon.

The association of different fungi with increased plant growth for each plant species in this study supports the idea that plant species may respond differently to a given fungal community. It is known that the presence of particular mycorrhizal species in a given soil can facilitate the establishment of plant species that benefit from that fungus in that soil (4, 8, 18, 25, 35, 36, 51, 55, 59). There are also many examples of relationships between mycorrhizal fungal infection by specific fungal species and the growth rate and/or competitive dynamics of particular plant species (6, 12, 36, 38, 43, 54, 55) and ECM or plant ability to acquire N and other nutrients (16, 21, 35). Previously, Nara (35) demonstrated that different fungal inoculants can have various effects on seedling growth and N acquisition under field conditions. Recently, observations of differential ^{15}N uptake between spruce seedlings colonized by different ectomycorrhizal fungal species at different levels have been observed under field conditions (23), further supporting the idea that a given plant host interacts differently with the various species of fungi present on its roots. While differential growth responses to particular ECM fungi have been observed in numerous greenhouse studies, exceptionally few studies have documented such patterns with naturally occurring fungi in the field, making our results noteworthy.

In summary, while it seems most unlikely that alder forms abundant, direct nutritional links with other plant species via CMNs, our results support the possibility that black spruce (*P. mariana*), trembling aspen (*P. tremuloides*), and paper birch (*B. papyrifera*) form CMNs with one another in postfire habitats of interior Alaska. Different fungal taxa appeared to be linked to greater plant growth for aspen, birch, and spruce, supporting the idea that plant species may respond differently to a given fungal community. One may speculate that wildfire-induced shifts in a given soil fungal community (as reviewed in reference 5) could result in variation in the growth response of different plant species after fire and a shift in regenerating vegetation. This possibility warrants further investigation.

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