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Long-term experimental manipulation of climate alters the ectomycorrhizal community of *Betula nana* in Arctic tundra

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Abstract

Climate warming is leading to shrub expansion in Arctic tundra. Shrubs form ectomycorrhizal (ECM) associations with soil fungi that are central to ecosystem carbon balance as determinants of plant community structure and as decomposers of soil organic matter. To assess potential climate change impacts on ECM communities, we analysed fungal internal transcribed spacer sequences from ECM root tips of the dominant tundra shrub *Betula nana* growing in treatments plots that had received long-term warming by greenhouses and/or fertilization as part of the Arctic Long-Term Ecological Research experiment at Toolik Lake Alaska, USA. We demonstrate opposing effects of long-term warming and fertilization treatments on ECM fungal diversity; with warming increasing and fertilization reducing the diversity of ECM communities. We show that warming leads to a significant increase in high biomass fungi with proteolytic capacity, especially *Cortinarius* spp., and a reduction of fungi with high affinities for labile N, especially *Russula* spp. In contrast, fertilization treatments led to relatively small changes in the composition of the ECM community, but increased the abundance of saprotrophs. Our data suggest that warming profoundly alters nutrient cycling in tundra, and may facilitate the expansion of *B. nana* through the formation of mycorrhizal networks of larger size.

Keywords: Arctic, *Betula nana*, climate change, ectomycorrhiza, fungi, internal transcribed spacer, rRNA genes, shrub expansion, tundra

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Introduction

Arctic regions have warmed rapidly in recent decades (Kaufman *et al*., 2009) highlighting their relevance as sensitive indicators of global climate trends. Although general, Arctic warming is spatially nonuniform. In the western North American Arctic, where regional rates of warming are among the highest globally, temperature gains reached 0.1 °C yr^{-1} over the past 35 years (Anisimov *et al*., 2007). This warming is associated with marked changes in terrestrial ecosystems, including increased microbial activity leading to increased plant N availability (Chapin, 1983; Nadelhoffer *et al*., 1992; Aerts, 2006) and faster carbon (C) turnover in Arctic soils (Hobbie & Chapin, 1998; Shaver *et al*., 2006). Differences in the relative productivity of tundra species have led to ecosystem-scale changes in plant community composition characterized by the expansion of shrubs (Hobbie, 1996; Sturm *et al*., 2001) and the consequent decline of mosses and evergreen ericaceous species. Shrub growth affects positive feedbacks that enhance ecosystem change and lead to greater climate forcing (Sturm *et al*., 2001). Beneath shrub thickets, increased local snow-trapping in winter, increased soil insulation, higher winter and spring-time soil temperatures, and increased rates of nutrient mineralization lead to local conditions that further favour shrub growth and expansion onto tussock tundra (Sturm *et al*., 2005; Weintraub & Schimel, 2005).

In low-Arctic tussock tundra, *Betula nana* is among the species most responsive to climate change. It is the strongest competitor for soil N (Bret-Harte *et al*., 2008), increasingly dominates fertilized tussock tundra in long-term experiments (Chapin *et al*., 1995; Shaver *et al*., 2001; Mack *et al*., 2004), and shows positive growth responses to warming treatments (Chapin *et al*., 1995). *B. nana* is obligately symbiotic with ectomycorrhizal (ECM) fungi in nature (Molina *et al*., 1992), allocates a high proportion of C belowground to rhizomes, roots, (Chapin *et al*., 1980) and mycorrhizal structures (Hobbie & Hobbie, 2006), and utilizes mycorrhizal networks to rapidly reallocate C among neighbours according to

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need (J. R. Deslippe & S. W. Simard, unpublished results). A model based on the natural abundance of ^{13}C and ^{15}N in plant and fungal tissues from Alaskan tundra estimated that 61–86% of the nitrogen (N) in plants is filtered through mycorrhizal fungi (Hobbie & Hobbie, 2006). These characteristics suggest an important role for *B. nana*'s symbiotic ECM fungi in mediating its response to climate change factors. ECM fungi have been shown to be important determinants of plant response to ecosystem change through their dual role as drivers of decomposition processes (Read & Perez-Moreno, 2003) and as the main nutrient harvesting structures of plants (Smith & Read, 1997).

There is growing evidence to suggest that mycorrhizal fungi respond strongly to climate change factors. Enhanced soil nutrient availability is frequently associated with negative impacts on ECM communities (Treseder, 2004). For example, ECM community diversity declined sharply over an increasing N-deposition gradient in boreal Alaska (Lilleskov *et al*., 2002a), and net N mineralization rates have been negatively correlated to ECM fungal richness in the field (Parrent *et al*., 2006). Likewise, the proportion of *B. nana* root tips colonized my ECM fungi declined after 3 years of fertilization treatment (Urcelay *et al*., 2003). Despite significant increases in ECM mycelial production after 14 years of fertilizer additions and warming treatments in low-Arctic tundra, these gains were offset by much greater increases in above ground plant biomass, leading the conclusion that ECM fungal biomass in warming and fertilization treatment plots represented the *status quo* or even a decline relative to plant biomass (Clemmensen *et al*., 2006). By contrast, warming treatments without nutrient additions tend to show positive impacts on ECM biomass (Treseder, 2004), though these are rarely corrected for total plant biomass gains as in the former study (Clemmensen *et al*., 2006). For example, warming led to increased ergosterol content of hair roots of ericoid mycorrhizal plants (ERM) in sub-Arctic Sweden (Olsrud *et al*., 2004), and to an increase in *Salix arctica* root-associated fungal biomass, at a Canadian high-Arctic site (Fujimura *et al*., 2008).

Currently, our ability to predict the response of ECM and plant communities to climate change factors is hampered both by the few detailed descriptions of the members of these communities as well as our limited understanding of the ecological role of many fungal species. There are 5000–6000 fungal species that form ECM, and these are phylogenetically diverse, spanning three phyla (Smith & Read, 1997; Agerer, 2006). It is unlikely that the response of the ECM community to climate change factors is monotonic. In their characterization of ECM communities across an N deposition gradient, Lilleskov *et al*. (2002a) found *Paxillus involutus* and *Lactarius theiogalus* to be 'nitrophillic', increasing linearly with net nitrification rate in soils, while some ECM taxa, such as *Laccaria bicolor* and *Hebeloma* spp., were found only at higher N sites. Conversely, several ECM fungi, including *Cortinarius* spp. and *Cenococcum geophillum* were 'nitrophobic'. Still other ECM species, such as *Tomentella sublilacina*, reached greatest abundance at sites with intermediate nitrification rates, where nutrient availability was higher but where soil pH had not yet been strongly reduced by N-pollution.

Patterns in the natural abundance of N isotopes ($^{15}N:^{14}N$ ratios, expressed as $\delta^{15}N$) in plants, soils, and fungal sporocarps have provided insight to the ecological role of many ECM taxa. N isotopes are useful markers of the mycorrhizal role in plant N supply because discrimination against ^{15}N during creation of transfer compounds within mycorrhizal fungi leads to low $\delta^{15}N$ in plants and high $\delta^{15}N$ in fungi, relative to N in soil organic matter (Hobbie *et al*., 2005, see Hobbie & Hobbie, 2008, for a review). In low N environments, plants allocate a high proportion of C belowground to ECM and ERM fungi (Högberg *et al*., 2003) and are strongly dependent on the proteolytic functions of their symbionts for N (Hobbie & Hobbie, 2008), leading to an 8–10‰ depletion of ^{15}N plant tissues relative to fungal tissues (Hobbie *et al*., 2005). As N availability increases and plants are released from N-limitation, foliar N concentration increases linearly with $\delta^{15}N$ (Hobbie *et al*., 2000), suggesting that plants access proportionally less N from their fungal symbionts, and leading to a negative relationship between foliar $\delta^{15}N$ and ECM fungal biomass (Hobbie & Colpaert, 2003). This relationship has proven useful in accessing plant, ECM and ERM response to climate change factors. For example, leaves of ERM *Vaccinium* spp. became more depleted in ^{15}N with exposure to elevated CO_2 (Olsrud *et al*., 2004). Likewise, $\delta^{15}N$ values of the foliage of the two dominant ECM species *S. arctica* and *Dryas integrifolia*, were significantly depleted following warming at a high-Arctic site (Deslippe, 2004). By contrast, long-term fertilizer additions increased mean $\delta^{15}N$ values for *B. nana* in moist acidic tundra (MAT) from -6.32 ± 0.15‰ to -2.52 ± 0.21‰ (Gough *et al*., 2000). These findings suggest that, under N-limiting conditions, climate change factors that promote plant photosynthesis, such as elevated temperature and CO_2, will lead to increased C allocation to ECM and ERM fungi; however, the opposite pattern can be expected from climate change factors that enhance soil nutrient availability.

In addition to field-based studies that provide evidence linking ECM taxa with ecological functions, several morphological attributes may suggest roles for species or groups (Agerer, 2001). For example, several families in the order Boletales have structurally

advanced rhizomorphs and are associated with the efficient transport of water and nutrients in soils (Agerer, 2006). The wide variability of $\delta^{15}N$ enrichment in ECM fruiting bodies, which is frequently observed in nature, may reflect species-specific ecological roles for ECM fungi in regards to N cycling. For example, $\delta^{15}N$ signatures of sporocarps were recently correlated to hyphal growth patterns in ECM species at four sites that differed in latitude and N availability (Hobbie & Agerer, 2010). Of the hyphal characteristics measured, hyphal hydrophobicity, the ability to form rhizomorphs, and the extent and pattern of hyphal growth, called 'exploration type', were found to most closely correlate with ^{15}N signatures. These hyphal characteristics may occur in common because ECM species with high biomass types may alone have the C necessary to produce more complex hyphal patterns, such as rhizomorphs, whose function in long distance transport of water and nutrients would be enhanced by hydrophobity. ECM that form high-biomass exploration types had high $\delta^{15}N$ values, and dominated low-N sites, while those characterized by lower biomass forms had low $\delta^{15}N$ values and were more prevalent at sites with higher N availability (Hobbie & Agerer, 2010).

The objective of this study was to determine how climate change factors will affect the ECM community of a dominant tundra shrub. Specifically, we tested the hypothesis that long-term fertilizer addition and warming by greenhouses would alter the composition of the mycorrhizal community of *B. nana* in low-Arctic tussock tundra. Given the evidence suggesting generally positive effects of warming on ECM fungi and generally negative effects of increased nutrient availability, we predicted that warming and fertilization treatments would lead to different ECM community compositions. While fertilization should result in ECM taxa characteristic of sites with high N availability, warming should lead to ECM taxa adapted to N limitation. We used a molecular approach to achieve the high taxonomic resolution necessary to detect ECM species changes among treatments.

Methods

Study site

The study site was located on a gentle (<5°), north-facing slope in MAT near Toolik Lake, Alaska, USA (68°38′N, 149°34′W, elevation 780 m). MAT forms on old glacial surfaces (>11 000 years BP) and supports 'heathland'-type tundra plant communities. Regular patterns of vegetation occur in this plant community as a result of the perennial, rhizomatous growth of the tussock-forming sedge, *Eriophorum vaginatum*. The dominant deciduous dwarf shrub, ECM *B. nana*, occupies hollows between the sedge-tussocks in mixture with mid-canopy ericaceous ERM species, such as *Ledum palustre* and *Vaccinium* spp., and arbuscular or nonmycorrhizal species such as *Rubus chamaemorous* as well as herbs. Both plurocarpus and acrocarpus mosses are common and form a continuous ground cover that can exceed 40 cm depth in some areas. Soils are characterized by a thick organic horizon that is 10–20 cm deep and with a pH of 4.4 (Nadelhoffer *et al*., 1991). Mineral horizons are essentially unweathered, pale grey in colour, permafrost affected, and show only occasional evidence of cryoturbation. Maximum thaw depth occurs in late July, when the active layer typically comprises the entire organic horizon and the top 5–10 cm of the mineral soil.

Experimental treatments

The study treatments were established in 1988 and are maintained as part of the Arctic Long-Term Ecological Research (LTER) experiment. Warming and fertilizer treatments with ambient controls are replicated four times in a randomized block design in the MAT. Fertilizer treatments consist of $10\,g\,m^{-2}$ additions of N as NH_4NO_3, and $5\,g\,m^{-2}$ additions of P as P_2O_5, both applied as pellets immediately after snowmelt annually. Warming is accomplished passively with greenhouses constructed of 0.15 mm polyethylene fixed on permanent wooden frames that are 2.5×5 m and 1.5 m in height. The uneven microtopography of the study site allows for air circulation from the base the greenhouse walls. Greenhouses reduce the photosynthetically active radiation by approximately 20% over the growing season, but have no significant effect on soil water content, and previous shading experiments indicate that plant production and biomass are unresponsive to light reductions of 50–64% at this site (Chapin *et al*., 1995). Soil temperature in treatment plots tends to be cooler than in control plots in summer, because of increased shading by higher plant biomass, but warmer than ambient control plots in winter, due to increased insulation by snow trapped by plants. Mean annual temperature differences among treatments in 2007 are shown in Table 1.

B. nana growth responded strongly to the experimental treatments (Shaver *et al*., 2000). Fertilizer addition resulted in significant increases in total aboveground biomass, but smaller and nonsignificant increases in rhizome biomass, and these changes were further amplified by warming. As a result, fertilization and warming strongly altered biomass allocation in *B. nana*; from a ratio of above to belowground biomass of 1 : 4 in control plots, to nearly 1 : 1 in fertilizer plots, and 2 : 1 in warming + fertilizer plots. Warming alone resulted in a 55% increase in above ground biomass of *B. nana* (see Fig. S1, supporting information).

Root sampling and nucleic acid extraction

ECM roots of *B. nana* were sampled on 28 July 2007, a time that corresponds to maximum annual aboveground biomass. To obtain root samples, soil cores were sampled from three randomly selected locations in each of the four replicate plots

Table 1 Mean annual change (treatment – ambient control) in air temperature within the plant canopy and soil temperature at three depths

	Mean annual temperature change by treatment (°C)		
Measurement location	Fertilizer	Warming	Warming + fertilizer
Canopy	1.89*	2.09*	3.30*
Soil at 10 cm depth	0.65*	1.76*	2.46*
Soil at 20 cm depth	0.28*	1.60*	2.13*
Soil at 40 cm depth	−0.042	1.26*	1.72*

Significance of treatment effects were determined by comparing the control and treatment plot data using paired *t*-tests.
*Significant treatment effect ($\alpha = 0.05$). Data are from the Arctic LTER (Shaver & Laundre, 2007).
LTER, Long-Term Ecological Research.

per treatment and control (48 cores total). Soil cores were 5 cm in diameter and 20–30 cm in depth from surface to mineral soil, as roots of *B. nana* rarely penetrate into the C-horizon. Each soil core was packaged separately in a clean, airtight, plastic bag, and placed immediately on ice. Soil cores were maintained at 4 °C until processed for ECM roots.

Each soil core was placed in a 2 mm soil sieve and washed well in tap water. Morphological differences allowed the firm-textured, light-brown fine roots of *B. nana* to be unambiguously distinguished from the pale yellow, spongy roots of *Salix pulchra*, which was the only other ECM species present in the treatment plots. Fine roots of *B. nana* were removed, floated in a shallow tray, cut into 2 cm sections, and pooled by plot. Root sections were selected at random and placed under a dissecting microscope. At least 1000 root tips per plot (mean = 1120; total = 17 927) were assessed for colonization by ECM fungi. Root tips were identified as ECM if a fungal mantle enveloped >50% their length and no root hairs were present. For each core, three randomly selected subsamples comprised of 10 root tips each were removed for DNA extraction. Individual root tips were measured with a micrometer and cut with a microscalpel to 2.0 mm, in an attempt to equally sample ECM fungal species in DNA extractions. Root tips were placed in sterile microfiche tubes and lyophilized at −50 °C in a Labconco lyophilizer (Kansas City, MO, USA). Lyophilized root tips were stored at −80 °C until DNA was extracted.

Lyophilized root tips were placed in Lysing Matrix E® (MP Biomedicals) tubes with 400 μL of Buffer AP1 and 4 μL RNase A stock solution (Qiagen, Mississauga, ON, Canada) and processed at maximum speed for 30 s in a FastPrep® instrument (MP Biomedicals, Solon, OH, USA). Subsequently, DNA extraction followed the Qiagen plant miniprep kit protocol (DNeasy Plant handbook, Qiagen). As a qualitative assessment of DNA quality, a 5 μL aliquot of each soil extract was run on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The three root tip extractions per plot were then pooled for subsequent analysis.

PCR and sequencing

Polymerase chain reactions targeted the internal transcribed spacer (ITS) of the fungal rRNA operon. PCRs used the forward primer ITS1F (Gardes & Bruns, 1993), and the reverse primer, ITS4 (White *et al*., 1990). PCR reactions consisted of 50 ng genomic DNA, 20 μmol dNTPs, 10 nmol primers, ×10 PCR Buffer, 50 μmol $MgCl_2$, 15 μg BSA, and 2.5 U of Taq DNA polymerase (Fermentas, Burlington, ON, Canada) in a final volume of 25 μL. The thermocycler programme consisted of an initial denaturing temperature of 95 °C for 5 min followed by 30 cycles of: denaturing at 94 °C for 1 min, annealing at 55 °C for 50 s, and extension at 72 °C for 50 s, followed by a final extension period of 7 min at 72 °C. Subsequently, a 'reconditioning' step, designed to eliminate PCR artifacts formed in late-stage, template-limiting cycles of PCR, was used (Thompson *et al*., 2002). Here, 5 μL of initial PCR product were added as template to 45 μL of fresh PCR reagents and subjected to three additional thermal cycles. PCR products were purified using MiniElute purification columns (Qiagen). Cleaned PCR products were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and 100 ng of DNA from each PCR reaction was pooled according to treatment; for a total of four cloning reactions. Pooled templates were cloned using a TOPO TA Cloning® kit with pCR®II-TOPO® vector and DH5α™ competent cells (Invitrogen, Burlington, ON, Canada) with blue/white screening. Clones were plated, picked and sequenced at the Genome Sciences Centre (Vancouver, BC, Canada) using the vector specific primers M13for and M13rev.

Phylogenetic analyses

Bidirectional sequence reads were assembled using the ContigExpress function of Vector NTI 10.3 (Invitrogen), manually checked for base-calling errors, and trimmed of the vector sequence. The Bellerphon server (Huber *et al*., 2004) was used to detect chimeric sequences in each of the fungal libraries. Phylogenetic affiliations of fungal ITS sequences were determined using the Fungal ITS Pipeline (Nilsson *et al*., 2009), which uses multiple alignment programmes to align ITS sequences, and HMMER (Eddy, 1998), a hidden Markov model algorithm to run similarity searches of the query sequences using a local installation of NCBI-BLAST (Altschul *et al*., 1997). The Fungal ITS Pipeline groups like sequences based on 50% identity of the 15 closest NCBI-BLAST hits. Sequence groups and singletons were assigned to taxa based on the following parameters: sequences with >97% identity over >90% of the query sequence were considered species-level matches; sequences with 93–97% identity or sequence groups where there was species level incongruence among member's top BLAST hits, were assigned to genera; sequences with matches below 93% were considered family-level; and those below 83% were considered order-level. The 1060 fungal ITS and rRNA gene sequences generated in this study were deposited in GenBank under the accession numbers GU997732 to GU998791.

Phylogenetic analyses were based on MAFFT version 6 (Katoh, 2008) alignments of fungal sequences. Alignments were visually

Table 2 Relative δ^{15}N value, study site, and reference for mycorrhizal fungal taxa found in this study

Taxon	Relative δ^{15}N value	Study site	References
Cenococcum sp.	High	Boreal, Alaska	Lilleskov *et al*. (2002b)*
Cortinarius sp.	High	Toolik Lake, AK	Clemmensen *et al*. (2006)
Laccaria bicolor	Low	Boreal, Alaska	Lilleskov *et al*. (2002b)
Laccaria sp.	Low	Toolik Lake, AK	Hobbie & Hobbie (2006)
Lactarius sp.	Medium	Toolik Lake, AK	Hobbie & Hobbie (2006)
Russula sp.	Low	Boreal, Alaska	Lilleskov *et al*. (2002b)
Thelephora terrestris	Low	Laboratory study	Hobbie & Colpaert (2003)*,†

*δ^{15}N values are for hyphae only.
†As reported in Hobbie (2005).

checked for accuracy and manually edited in BIOEDIT (Hall, 1999) version 7.0.5.3. Distance matrices for fungal sequence sets were made using the DNAdist function of BIOEDIT. Rarefaction of sequence libraries was used to assess treatment effects on ECM diversity. Rarefaction curves were calculated in Mothur (Schloss *et al*., 2009) with an operational taxonomic unit delimitation of 0.03 and plotted in SIGMAPLOT 11.0 (Systat Software Inc., Chicago, IL, USA). Phylogenetic trees were constructed using the neighbor-joining/UPGMA method in BIOEDIT and visualized using FIGTREE version 1.3.1 (Rambaut, 2007).

Hyphal characteristics

ECM fungi that could be identified at least to the genus level were categorized according to their hyphal characteristics (presence of rhizomorphs, hyphal hydrophobicity, exploration type) based on previously published reports for the taxon as summarized in Agerer (2006) and Hobbie & Agerer (2010). The presence of rhizomorphs and hydrophobic hyphae are believed to enhance the ability of an ECM fungus to transport water and dissolved nutrients through soils while longer distance exploration types are formed by high-biomass ECM species which are likely to place greater C-demands on their host plants. Where data on the δ^{15}N values of affiliated genera were available, putatively ECM fungi were further characterized by their relative ^{15}N enrichment (Table 2). ECM fungi with greater ^{15}N enrichment degrade organic matter to access N-containing compounds and tend to form longer distance exploration types (Hobbie & Agerer, 2010). Because of site-specific effects on the isotope signatures of ECM fungi, we utilized a relative scale of δ^{15}N values (Lilleskov *et al*., 2002b; Hobbie, 2005), and wherever possible selected fungal isotope values reported for Toolik Lake or other N-limited sites in Arctic and boreal regions. For sporocarp data from Toolik Lake, AK, the relative scale of high, medium and low, corresponded to δ^{15}N values of >7‰, 3–7‰, and <3‰, respectively. Table 2 summarizes the data sources utilized for assigning fungal taxa to relative δ^{15}N enrichment categories.

Statistical analyses

Paired *t*-tests were used to assess treatment effects on soil temperatures compared with the control plots. A one-way ANOVA was used to assess treatment effects on the proportion of *B. nana* root tips colonized by ECM fungi. Barlett's and Levene's tests were used to test for heteroscedascity among treatments. Tukey's *post hoc* tests were used to assess significant differences among pairs of treatments. These statistical analyzes were performed using STATISTICA version 8.0. (StatSoft Inc., Tulsa, OK, USA). Clone library size was normalized to that of the control. The frequency of clones affiliated with phylogenetic groups and hyphal characteristics were compared with the control values using χ^2-tests with Yates correction. χ^2-tests were performed using a purpose-made Excel® spreadsheet obtained from http://udel.edu/~mcdonald/statchigof.html.

Results

We found no significant differences in the proportion of *B. nana* root tips colonized by ECM fungi among experimental treatments ($P = 0.750$); mean percent colonization was 68% in this study (Table 3). However, analysis of the clone library composition for the four treatments revealed that a significantly smaller proportion of clones derived from the warming + fertilizer treatment were of mycorrhizal origin ($P = 0.00036$, Table 3). Similarly, there was a trend towards fewer mycorrhizal-affiliated clones in the fertilizer treatment than in the control. The vast majority of clones that were not affiliated with mycorrhizal taxa in this study were yeasts, although a few (<1%) basiomycetous mycelium forming saprotrophic fungi were also observed. Supporting information Table S1 provides a complete list of the fungal taxa observed in this study.

Rarefaction analysis of ECM fungi-affiliated clones revealed that warming and fertilization treatments led to opposite changes in the diversity of the ECM fungal community. ECM fungal diversity was significantly higher in clone libraries constructed for treatments that received warming (warming; warming + fertilizer) compared with those that did not (control; fertilizer; Fig. 1a). Conversely, ECM diversity was significantly

Table 3 Characteristics of root tips sampled in this study: range of sample size and proportion colonized by ECM, reported as mean ± one standard error

	Characteristics of root tips		Characteristics of clone libraries		
Treatment	Root tips examined per plot	Proportional colonization by ECM	Clone library size	Proportion of mycorrhizal sequences	*P*-value
Control	1035–1209	0.61 ± 0.06	248	0.90	–
Fertilizer	791–1207	0.64 ± 0.07	248	0.75	0.093
Warming	1043–1184	0.67 ± 0.09	308	0.95	0.58
Warming + fertilizer	1101–1226	0.71 ± 0.02	256	0.61	0.00036

One-way ANOVA revealed no significant differences among treatments. Characteristic of clone libraries constructed for ITS region of rRNA genes of root-tip-associated fungi of *Betula nana*. The proportion of mycorrhizal sequences was compared with that of the control where the level of significance was determined by χ-tests with Yates correction.
ECM, ectomycorrhiza; ITS, internal transcribed spacer.

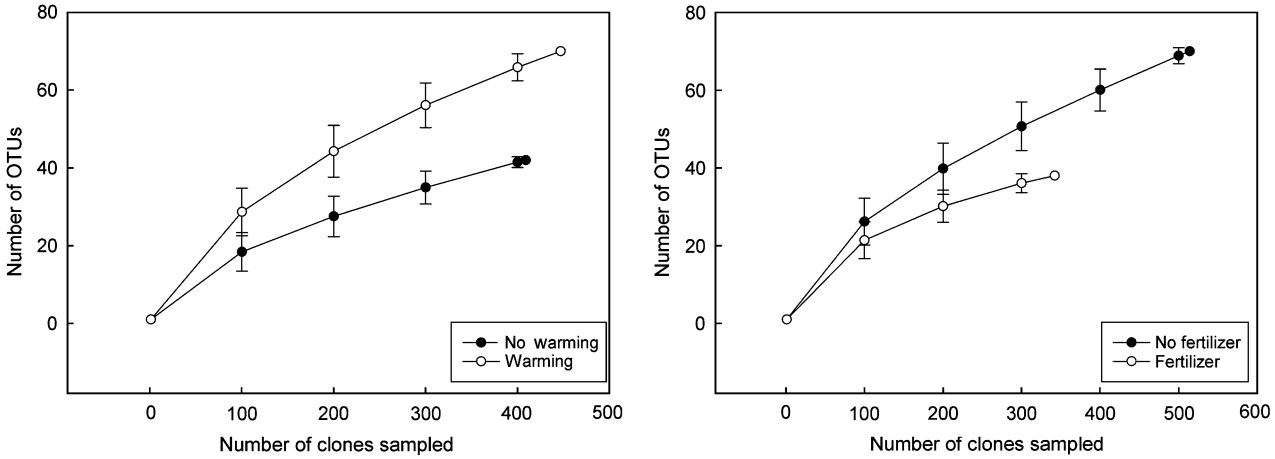

Fig. 1 (a) Rarefaction curves for ectomycorrhiza (ECM)-affiliated clones in libraries constructed for treatments that received warming (warming; warming + fertilizer) and those than did not (control; fertilizer), (b) Rarefaction curves for ECM-affiliated clones in libraries constructed for treatments that received fertilizer (fertilizer; warming + fertilizer) and those that did (control; warming).

lower in clone libraries constructed for treatments that received fertilizer (fertilizer; warming + fertilizer) than those that did not (control; warming; Fig. 1b). Limited by the relatively few saprotroph-affiliated sequences in clone libraries, rarefaction analysis revealed no clear effects of warming and fertilization treatments on the diversity of saprotrophic fungi.

Analysis of the compositions of the clone libraries revealed that control and fertilizer treatments were similar at the family level, except that the clone library for the control was unique in containing sequences affiliated with the Clavulinaceae (Fig. 2). Clavulinaceae-affiliated clones made up 5% of the control library, but were absent from all other treatment libraries and this change was statistically significant ($P = 0.0026$). By contrast there were large changes in the composition of clone libraries in the warming and warming + fertilizer treatments. Most notable were the large differences in clones affiliated with the Cortinariaceae. Cortinariaceae-affiliated clones increased by a factor of 15 in the warming treatment over the control, and this change was highly significant ($P = 3.1 \times 10^{-24}$). While clones affiliated with the Cortinariaceae doubled in the warming + fertilizer treatment compared with the control, this change was not statistically significant ($P = 0.21$). These changes were accompanied by significant reductions in the number of Russulaceae-affiliated clones in the warming and warming + fertilizer treatments ($P = 0.047$ and 1.5×10^{-8}, respectively), as well as a significant reduction in the number of Helotiaceae-affiliated sequences in the warming treatment ($P = 5.9 \times 10^{-4}$). Interestingly,

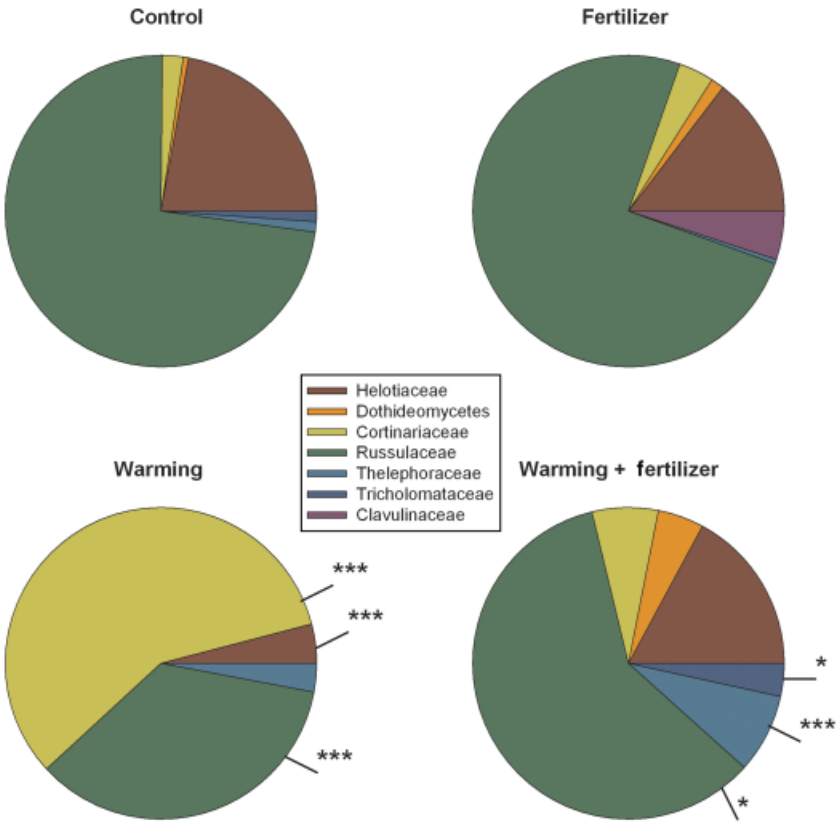

Fig. 2 Proportion of clones affiliated with ectomycorrhizal fungal families in clone libraries constructed from *Betula nana* root tips from the four treatments. Significant treatment effects, relative to the control are denoted by symbols as follows: $^{*}P<0.05$, $^{***}P<0.001$.

the proportion of Thelephoraceae-affiliated sequences increased in all treatments compared with the control, but this change was only statistically significant in the warming + fertilizer treatment ($P = 2.4 \times 10^{-4}$). Clones affiliated with the Tricholomataceae occurred only in the two treatments where fertilizer was added, and their numbers were significantly greater than in the warming + fertilizer treatment than the control ($P = 0.023$).

Seventy percent (597/858) of clones affiliated with mycorrhizal taxa could be identified at least to genus level, compared with 73% (777/1060) of clones overall. When only clones identified to genus or species levels were considered, control and fertilizer treatment libraries remained similar in composition, with no significant differences in the proportion of clones affiliated with different genera or species ($P > 0.05$, for all tests). By contrast, there were large differences in the composition of the clone library of the warming treatment and, to a lesser extent, the warming + fertilizer treatment. The significant increase ($P = 0.0014$) in sequences affiliated with *Cortinarius* remained an important effect of the warming treatment. Interestingly, *Cortinarius favrei* identified from sporocarps was the most common species observed in the control plots, and the only species that fruited in the warming treatment (see supporting information Table S1 for a complete list of fungal sporocarps collected in this study). The change in fungal community composition with warming was also characterized by a significant reduction in the number of clones affiliated with *Rhizocyphus ericae* ($P = 0.0012$), and with *Lactarius* spp. ($P = 0.0014$). Clones affiliated with the genus *Pseudotomentella* occurred only in the warming treatment, and this increase was significant ($P = 0.023$). Sequences affiliated with *L. bicolor* occurred only in the clone libraries for treatments that received fertilizer additions. For a graphical summary of the composition of fungal clone libraries for sequences that could be identified at least to genus see supporting information Fig. S2. Our phylogenetic analyses confirmed that the warming-induced shift in ECM community structure, which was predominantly characterized by the large increase in *Cortinarius*-affiliated sequences and the consequent reduction of *Russula*-affiliated sequences, represented an exchange of mutually exclusive sets of fungal ITS phylotypes (see supporting information Figs S3 and S4).

Table 4 Proportion of clones from four treatment libraries affiliated with mycorrhizal fungal genera with different hyphal characteristics

	Hyphal characteristic							
		Exploration type				Relative δ^{15}N value		
Treatment	Presence of rhizomorphs	Contact/short-distance	Medium-fringe	Medium-smooth	Hydrophobic hyphae	High	Medium	Low
Control	0.51	0.44	0.06	0.5	0.52	0.11	0.69	0.20
Fertilizer	0.51	0.47	0.03	0.5	0.51	0.03	0.79	0.17
Warming	0.72***	0.06***	0.44***	0.5	0.72***	0.64***	0.35**	0.01***
Warming + fertilizer	0.54	0.28	0.05	0.66	0.56	0.16	0.68	0.16

Only sequences that could be identified to genus level are included. Significant differences among treatment and control libraries are assessed using χ^2-tests with Yates correction, symbols: $^{**}P<0.01$; $^{***}P<0.001$.

When ECM clones were categorized according to the hyphal characteristics of their closest taxonomic identity, a strong pattern emerged. We found that the warming treatment was dominated by clones affiliated with mycorrhizas that have hydrophobic hyphae, form rhizomorphs, have longer distance explorations types, and tend to be enriched in δ^{15}N relative to other mycorrhizal species (Table 4). By contrast, the control, fertilizer and warming + fertilizer treatments had approximately equal proportions of clones affiliated with taxa that form rhizomorphs as those that lack rhizomorphs. Moreover, these treatments had similar numbers of clones affiliated with contact, short distance, and medium distance-smooth exploration types and, compared with the warming treatment, far fewer clones affiliated with mycorrhizal taxa that exhibit the medium-fringe hyphal growth pattern. Likewise, clone libraries for the control and two fertilizer treatments were dominated by mycorrhizal taxa that have been shown to have intermediate δ^{15}N values; these treatments also had a high proportion of taxa with low δ^{15}N values.

Discussion

The proportion of *B. nana* root tips colonized by ECM fungi did not differ from control levels after 18 years of warming and/or fertilization, agreeing with Clemmensen *et al*. (2006), but contrasting with another study reporting nearly a 50% reduction after 3 years of fertilization at this site (Urcelay *et al*., 2003). The reasons for this discrepancy are unknown. Given that the percent ECM colonization was similar in control plots from our study (61%) and theirs (60%, Urcelay *et al*., 2003), the previously observed reduction in ECM colonization with fertilization may reflect a short-term response of the ECM community of *B. nana*, whereby initial reductions in plant C allocation belowground resulted in less mycorrhization. Our study suggests that, over time, *B. nana* and its ECM community acclimate to fertilizer amendments and mycorrhization returns to original levels.

Although percent ECM colonization was unchanged by treatments, fertilizer additions were associated with significantly fewer clones of mycorrhizal origin, particularly when fertilizer was combined with warming. We selected only live ECM root tips and standardized their size, thus, the decreased abundance of clones affiliated with mycorrhizal taxa in the fertilizer treatments likely reflects an increased abundance of saprotrophic relative to ECM fungal biomass on roots. This suggests that fertilization enhanced growth of saprotrophic fungi, and that this effect was stronger when fertilizer was combined with warming. The reduction of ECM diversity in fertilizer treatments was also partly driven by this increase in saprotrophic sequences, as witnessed by the lower number of ECM-clones sampled in the fertilizer treatments in the rarefaction curves. It is interesting to note that methods used to estimate fungal biomass in fine roots that do not distinguish between saprotrophic and mycorrhizal fungi, such as ergosterol content, may be prone to overestimate ECM biomass where the ratio of saprotrophic to mycorrhizal fungi increases with experimental treatments.

The fundamentally different trajectories of ECM fungal diversity with warming or fertilizer treatments, confirm our prediction that these treatments apply different selective pressures on the ECM community of *B. nana*. This finding agrees with the contrasting responses of microbial communities to warming and fertilizer treatments in a Swedish sub-Arctic heath (Rinnan *et al*., 2007), and suggests that direct fertilization of tundra ecosystems does not simulate the increase in mineralization rates that result from warming (Hobbie, 1996).

The most dramatic response we observed was the large and significant increase in the proportion of clones

affiliated with the Cortinariaceae, and *Cortinarius* in particular, in the warming treatment. *Cortinarius* spp. are strongly proteolytic (Lilleskov *et al*., 2002a, b), a characteristic that is reflected in their generally high δ^{15}N values (Hobbie, 2005). *Cortinarius* spp. have rhizomorphs, hydrophic hyphae, and they exhibit a medium distance-fringe exploration type of relatively high biomass. These characteristics suggest that *Cortinarius* is adapted to N-limited conditions, and thrives on high C inputs from its host to fuel the production of biomass and extracellular enzymes that act on soil organic matter. Congruently, Lilleskov *et al*. (2002a) found that *Cortinarius* increased linearly with decreasing nitrification rates in boreal Alaska. The strong increase in *Cortinarius*-affiliated sequences and the strong positive biomass response of *B. nana* in warming treatments (Chapin *et al*., 1995; Shaver *et al*., 2000) suggests that the N derived from the proteolytic capabilities of *Cortinarius* spp. may be important in sustaining *B. nana*'s increased growth with warming. Although no foliar δ^{15}N values are available for *B. nana* in warming plots in the MAT–LTER, δ^{15}N values for *Salix reticulata*, the dominant ECM species in moist nonacidic tundra, showed significant declines with warming (Gough & Hobbie, 2003), supporting the idea that ECM plants in Arctic tundra increasingly depend on their fungal symbionts for N with warming. The increased populations of ECM fungi with longer-distance exploration types may also enhance connectivity among *B. nana* individuals and facilitate nutrient transfer through mycorrhizal networks. Mycorrhizal networks dominated by rhizomorph-forming species are considered better facilitators of nutrient transfer than those dominated by shorter-distance exploration types (Simard *et al*., 2002). Interestingly, warmer soil and air temperatures are associated with increased C transfer to *B. nana* through mycorrhizal networks in this ecosystem (J. R. Deslippe & S. W. Simard, unpublished results) suggesting that warming affects the C-dynamics of *B. nana* and its ECM over multiple time-scales.

Warming may enhance the growth of high-biomass ECM fungi partly by altering the physiology of their host plants. ECM tundra plants, including *B. nana*, are known to respond to warmer temperatures by producing leaves with significantly higher C to N ratios, when nutrients constrain growth (Tolvanen & Henry, 2001; Welker *et al*., 2005). In culture studies of ECM plants, belowground allocation of C ranges from 27% to 68% of net primary productivity (NPP). Of this C, the proportion allocated to ECM fungi is a linear function ranging from 1% to 21% of total NPP, and is highest at lowest nutrient concentrations and at lowest plant growth rates (Hobbie, 2006). Thus, as warming acts to enhance photosynthesis while N limitation constrains growth, ECM plants may increasingly allocate excess C to ECM fungi, fuelling the growth of high biomass types capable of degrading protein, and supplying their host with limiting N.

Concurrent with the significant increase in *Cortinarius*-affiliated sequences with warming was the decline in taxa that have small genet sizes characterized by contact, short, or medium distance-smooth exploration types, including significant reductions in affiliates of *R. ericae, Lactarius* spp., and members of the Russulaceae. *Russula* spp. typically have low δ^{15}N values (Hobbie, 2005), and although δ^{15}N values of *Lactarius* spp. range from low to medium values, they have shown limited ability to degrade protein in culture studies (Abuzindah & Read, 1986). Thus, the decline of populations affiliated with these ECM taxa in the warming treatment provides further support for the idea that *B. nana* allocates more C to ECM with proteolytic capabilities in response to warming.

Eighteen years of fertilization caused few changes in the composition of the ECM community of *B. nana*, suggesting that this *Russula* spp.-dominated community is well positioned to take advantage of higher nutrient conditions when they occur. Forming ECM with *Russula* spp. has the advantage of costing less C than symbioses with higher biomass ECM fungi, yet still provides *B. nana* with better access to labile soil N. The small size and profuse growth of hyphae in soils often lead to as much as 60 times the absorptive area of hyphae relative to fine roots (Simard *et al*., 2002), and given this difference, hyphal N uptake should dominate root uptake in tundra and forest soils (Hobbie & Hobbie, 2008). The persistence of a fungal community with a high affinity for inorganic N fits with *B. nana*'s characterization as the strongest competitor for soil N (Bret-Harte *et al*., 2008) and likely predisposes *B. nana* to increasingly dominate tussock tundra in long-term fertilization experiments (Chapin *et al*., 1995; Shaver *et al*., 2001; Mack *et al*., 2004).

Both fertilizer treatments increased the presence of *L. bicolor* sequences. *Laccaria* spp. have been characterized as 'nitrophilic' (Lilleskov *et al*., 2002a), 'nonprotein' (Abuzindah & Read, 1986) fungi. Congruently, sporocarp production by *Laccaria proxima* increased significantly in response to N fertilization of a *Pinus sylvestris* stand in the Netherlands (Termorshuizen, 1993). *L. bicolor* utilizes three assimilatory pathways for ammonium and possesses highly active aspartate and alanine aminotransferases (Iftikhar *et al*., 1990). Pure cultures of *L. bicolor* can sustain exponential growth on ammonium (Lilleskov *et al*., 2002b) as well as aspartate or alanine (Iftikhar *et al*., 1990), but grow poorly on bovine serum albumin as their sole N source (Lilleskov *et al*., 2002b). When *L. bicolor* forms ECM, one of its

ammonia transporters is greatly upregulated (Martin *et al*., 2008), suggesting that mycorrhization enhances the potential for N uptake in this species. *Laccaria* spp. show no host specificity (Molina *et al*., 1992) and are known to fruit prolifically from relatively few ECM root tips, suggesting a high allocation of resources to reproduction (Lilleskov *et al*., 2002a and references therein). These characteristics suggest that *Laccaria* spp. may be opportunistic, competing effectively with other soil organisms for inorganic N and responding quickly to increased N by sporulating and colonizing new hosts. The increase in *L. bicolor*-affiliated clones with fertilization treatments fits with this species' role as an opportunistic nitrophile.

The relatively small change in ECM community composition observed with fertilizer treatment is consistent with other studies showing little effect of N fertilization on belowground ECM communities (Wallenda & Kottke, 1998; Peter *et al*., 2001; Treseder *et al*., 2007). Although ECM morphotype richness often declines with increasing N availability (Read & Perez-Moreno, 2003), all studies that have found ECM communities to respond strongly to increasing N availability were conducted on conifer-associated ECM communities (Lilleskov & Bruns, 2001). Along an N-deposition gradient in Europe, there were stronger declines in diversity of conifer-associated than deciduous-associated ECM taxa (Arnolds, 1991). Likewise, while a negative relationship between ECM morphotype richness and soil inorganic N was found in *Picea abies* stands in Europe, a weaker and positive relationship between these variables was found in *Fagus sylvatica* stands (Taylor *et al*., 2000). These findings may suggest that the ECM communities of deciduous plants are more stable with increasing N than are the ECM communities of conifers (Lilleskov & Bruns, 2001), which may reflect greater relative belowground C allocation in deciduous ECM plants compared with conifers (Simard *et al*., 1997; Steele *et al*., 1997; Coleman *et al*., 2000) or greater baseline levels of N availability in many deciduous than coniferous forests (Jerabkova *et al*., 2006).

We demonstrate that 18 years of warming and fertilizer treatments led to contrasting responses of the ECM community of *B. nana*. Warming led to a higher diversity of ECM fungi and was associated with a significant increase in fungal taxa with proteolytic capacity, particularly *Cortinarius* spp., and a reduction in fungal taxa with high affinity for labile N, especially *Russula* spp. By contrast, fertilization led to a reduced diversity of ECM fungi and a greater proportion of saprotrophic fungi associated with root tips. Although reduced, the composition of the ECM fungal community remained relatively stable to fertilizer additions, but with a significant increase in the nitrophillic species *L. bicolor*. Our data shed light on the unique roles of ECM taxa in tundra nutrient cycling and suggest that climate warming elicits changes in the composition of ECM communities that enhance decomposition of soil organic matter and may increase the connectivity of *B. nana* individuals through mycorrhizal networks of larger size. These changes should act to enhance N acquisition and nutrient redistribution to *B. nana*, further facilitating shrub expansion onto Arctic tundra.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Betula nana* biomass in response to 9 years of fertilizer and warming treatments; GH = warming, NP = fertilizer. Above ground biomass is the sum of stem, leaves and inflorescences. Root biomass was not determined. Significant factor effects as determine by two-way ANOVA are indicated; $^{***}P<0.001$, and letters above bars denote means that are significantly different as determined by a Tukey's post hoc test. All data is from the Arctic LTER data archive, file # 1982_2000gs81tusbm (Shaver *et al*. 2000).
Figure S2. Proportion of clones affiliated with ECM fungi genera in clone libraries constructed for *Betula nana* root tips from the four treatments. Significant treatment effects are denoted by symbols as follows; $^{*}P<0.05$, $^{**}P<0.01$.
Figure S3. Unrooted phylogram of the 171 Cortinariaceae-affiliated sequences obtained in this study and two *Russula* sequences, one Russula-affiliated clone from the current study, and one *Russula* sequence downloaded from Genbank.
Figure S4. Unrooted phylogram of the 330 *Russula* spp.-affiliated sequences obtained in this study and two *Cortinarius* sequences, one *Cortinarius*-affiliated clone from the current study, and one *Cortinarius* sequence downloaded from Genbank.
Table S1. Fungi identified in association with plant roots and from sporocarps at the study site near Toolik Lake Alaska.

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