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Diverse Helotiales associated with the roots of three species of Arctic Ericaceae provide no evidence for host specificity

John F. Walker, Laura Aldrich-Wolfe, Amanda Riffel, Holly Barbare, Nicholas B. Simpson, Justin Trowbridge and Ari Jumpponen

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

Author for correspondence:
Ari Jumpponen
Tel: +1 785 532 6751
Email: ari@k-state.edu

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Summary

- Ericoid mycorrhizal fungi differ in their abilities to use nitrogen sources and may be integral to maintaining fungal and plant diversity in ecosystems in which Ericaceae occur. In this study, we tested whether the fungal communities differ among three species of co-occurring Ericaceae.
- Fungi colonizing *Cassiope tetragona*, *Empetrum nigrum* and *Vaccinium vitis-idaea* roots in the Arctic tundra were characterized via culture-dependent and culture-independent techniques. The cultured fungi were tested for their ability to colonize *Vaccinium uliginosum* in laboratory-based assays.
- The pure-cultured Helotiales were grouped into eight clades and dominated by the *Phialocephala–Acephala* complex. Representatives of these clades, plus an unknown basidiomycete with affinity to the genus *Irpex* (Polyporales), colonized *V. uliginosum* intracellularly. The Helotiales detected by direct PCR, cloning and sequencing were assigned to 14 clades and dominated by members of the *Rhizoscyphus ericae* complex. Ordination analyses indicated that culture-dependent and culture-independent assays provided distinct views of root fungal communities, but no evidence for host specificity.
- These data suggest that ericaceous roots host diverse fungal communities dominated by the Helotiales. However, these fungal communities are unlikely to be controlled by fungal host preferences. The mechanisms maintaining high diversity in root-symbiotic communities remain to be elucidated.

Introduction

Plants within the family Ericaceae are common in heathland, tundra and boreal forest habitats. In Arctic tundra, where water saturation combined with low temperatures limits mineralization, and where decomposition is slower than new plant carbon (C) inputs (Nadelhoffer *et al.*, 1992), most nitrogen (N) remains in organic pools and inorganic N is sparsely available (Shaver & Chapin, 1980). One key to the success of the Ericaceae in this environment may be their proposed reliance on organic N sources (Michelsen *et al.*, 1996, 1998; Jonasson & Shaver, 1999; Walker *et al.*, 2010), which allows ericaceous plants to bypass competition for inorganic nitrogen (Kielland, 1994; Michelsen *et al.*, 1996; Jonasson & Shaver, 1999; Schimel & Bennett, 2004).

The diversity of plant communities in Arctic tundra is probably tied to organic N acquisition (McKane *et al.*, 2002). Nitrogen, with its numerous organic forms, can be considered as a complex niche dimension in organic tundra soils (Kielland, 1994). Ericoid mycorrhizal (ErM) fungi vary in their functional attributes and thus may be key to the diversity and coexistence of ericaceous plants in low-nutrient environments (Read, 1991, 1996; Cairney & Meharg, 2003). The ability to use various N sources differs between species of ErM fungi (Xiao & Berch, 1999), but has been shown to vary even between conspecific strains (Midgley *et al.*, 2004; Grelet *et al.*, 2009). This functional variability among root-associated fungi may be integral to maintaining plant niche differentiation and may contribute towards the maintenance of plant diversity in ecosystems in which Ericaceae occur. This is supported by the plant

community theory which postulates that plant coexistence requires stabilizing mechanisms that can partly be attributable to niche modification mediated by root-associated fungi (Bever *et al.*, 2010).

If plant diversity is indeed maintained by niche dimensions defined by the functional attributes of root-associated fungal communities, fungal communities among coexisting plants should differ compositionally and functionally. On the level of operational taxonomic units (OTUs), fungal communities that form arbuscular mycorrhizas (Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002; Aldrich-Wolfe, 2007; Li *et al.*, 2010) and ectomycorrhizas (Morris *et al.*, 2009; Tedersoo *et al.*, 2009b) with different hosts have been shown to be distinct, suggesting some form of host preference. Studies on the host preference of ErM fungi are few, especially in tundra ecosystems (but see Kjøller *et al.*, 2010). Kjøller *et al.* (2010) sampled four co-occurring Ericaceae in a sub-Arctic mire and found that the plants shared fungal communities, suggesting the absence of host preference. By contrast, Bougoure *et al.* (2007) showed that ErM communities in an open heathland differed from those in a native *Pinus sylvestris* forest and were distinct between *Calluna vulgaris* and *Vaccinium myrtillus* in the forest site. Similarly, Ishida & Nordin (2010) showed that *V. myrtillus* and *V. vitis-idaea* seemed to host different ErM communities in a habitat dominated by *Picea abies*. The considerable diversity and abundance of Ericaceae in tundra vegetation, the diversity of fungi that colonize the root systems of Ericaceae and the importance of these associations to nutrient acquisition necessitate a more complete characterization of these fungal communities, as well as their potential to diverge among different ericaceous hosts.

Our primary goal was to characterize fungal communities associated with the roots of three common co-occurring species in the Ericaceae in an Arctic tundra habitat, using culture-dependent and culture-independent methods. We chose three species (*Cassiope tetragona*, *Empetrum nigrum* and *Vaccinium vitis-idaea*) representing common genera in Arctic tundra to test whether or not the fungal communities associated with their roots diverged among hosts. We assessed the fungal communities from adjacent plants by culturing fungi from root samples and through the cloning and sequencing of DNA extracted from roots. To distinguish root-inhabiting fungi from others, common isolates obtained in pure culture were tested for their ability to colonize *Vaccinium uliginosum* in the laboratory.

Materials and Methods

Study site

Root samples were collected at the Arctic Tundra Long Term Ecological Research site near Toolik Lake, Alaska (USA), in the northern foothills of the Brooks Range. Three discrete circular plots (Table 1), with an approximate radius of 56 m (each measuring 1 ha), were located in two watersheds. The soils at the site consist of a 10–50-cm organic (peat) layer over a silty mineral soil and permafrost, and the vegetation is representative of tundra across the North Slope of Alaska (Shaver & Chapin, 1991), northern Canada and eastern Siberia (Bliss & Matveyeva, 1992). The three plant species selected for sampling form a major component of the circumpolar Arctic erect-shrub tundra vegetation (CAVM Team, 2003). During root sampling for DNA extraction in 2006 (see below), nine soil samples were taken randomly at each plot and analyzed for NH_4^+, NO_3^-, total N, total phosphorus (P) and pH at the Soil Testing Laboratory at Kansas State University, Manhattan, KS, USA. On the basis of these analyses, one of the three sites differed from the remaining two by more than two-fold in (P) and NH_4^+ availability and had significantly higher pH when measured in Shoemaker–McLean–Pratt (SMP) buffer (Table 1).

Table 1 Properties of the three plots sampled for three co-occurring ericaceous hosts at Toolik Lake Long Term Ecological Research site in Alaska, USA

	Plot 1	Plot 3	Plot 4
Location	68°37′55N; 149°37′12W	68°37′57N; 149°34′27W	68°38′11N; 149°34′44W
Elevation (m asl)	726	752	732
Phosphorus (ppm)[1]	8.78 ± 9.22[b]	26.78 ± 9.00[a]	9.33 ± 6.44[b]
Total nitrogen (%)	0.57 ± 0.29[a]	0.79 ± 0.19[a]	0.76 ± 0.48[a]
NH_4^+–N (ppm)	9.72 ± 8.42[b]	20.35 ± 6.48[a]	8.33 ± 3.39[b]
NO_3^-–N (ppm)	0.45 ± 0.06[a]	0.69 ± 0.44[a]	0.57 ± 0.35[a]
pH (H_2O)	4.35 ± 0.25[a]	4.63 ± 0.51[a]	5.08 ± 0.95[a]
pH (SMP)[2]	5.62 ± 0.36[b]	6.26 ± 0.29[a]	5.79 ± 0.48[b]

[1]Bray 1 digestion.
[2]SMP, Shoemaker–McLean–Pratt buffer (2.5 ml l^{-1} triethanolamine, 1.8 g l^{-1} *p*-nitrophenol, 3 g l^{-1} potassium chromate and 2 g l^{-1} calcium acetate; Shoemaker *et al.*, 1961).
Values for soil parameters are mean ± standard deviation (n = 9); values followed by different letters differed statistically (Tukey's honestly significant difference test, α = 0.05). (Plot 2 was not included in this table because it was not sampled for the molecular assessment of the root associated communities in 2006.)

Isolation of fungi from Ericaceae

The shoots and attached roots of adjacent *Cassiope tetragona* D. Don, *Empetrum nigrum* L. and *Vaccinium vitis-idaea* L. were sampled in 2003. Three individuals of each species were sampled at each plot for a total of nine individuals per host species; only roots attached to identifiable shoots were used. All samples were processed within 24 h of the time of collection. Roots were cleaned of debris, rinsed with deionized water and excised. Excised roots were cut into 1-cm segments, rinsed in 70% ethanol and surface sterilized with 30% H_2O_2 for 30–60 s. A selection of different culture media was used to maximize the species richness detectable by culturing: corn meal agar (17 g l^{-1} of BBL Corn Meal Agar; Becton Dickson and Co., Sparks, MD, USA); tap water agar (20 g l^{-1} Bacto Agar; Becton Dickson and Co.); Hagem's agar (4 g l^{-1} malt extract, 1 g l^{-1} yeast extract, 5 g l^{-1} D-glucose, 0.5 g l^{-1} NH_4Cl, 0.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$, 0.5 g l^{-1} $FeSO_4$, 100 μl biotin (0.5 mg ml^{-1}), 100 μl thiamine HCl (1 mg ml^{-1}), 15 g l^{-1} Bacto Agar); and Hagem's agar amended with 50 mg l^{-1} benlate (added to inhibit the growth of nontarget fungi and to facilitate the recovery of slow-growing fungi). A random selection of five root fragments was plated on each of the four media, resulting in a total of 540 plated root segments. The plates were transported to Kansas State University by air in insulated ice chests. The mycelia that emerged from the root fragments were isolated, transferred into monocultures on Hagem's medium and maintained at room temperature. The pure culturing resulted in a total of 303 cultures.

Molecular characterization of fungal cultures

Fungal cultures were first sorted into groups on the basis of colony phenotype. Genomic DNA was extracted from each fungal culture using a cetyltrimethylammonium bromide (CTAB) protocol modified from Gardes & Bruns (1993) and amplified by PCR using ITS1F and ITS4 primers (Gardes & Bruns, 1993). The amplicons were digested with *Hinf* I and *Alu*I to test the fidelity of the morphological groups by restriction fragment length polymorphism of the internal transcribed spacer region (ITS-RFLP). Problematic or ambiguous groups were further digested with *Kpn*I, as the first digestion provided limited resolution for some of the established groups (e.g. those identified by sequencing as members of the *Phialocephala–Acephala* complex (PAC); Grünig *et al.*, 2008a,b).

Up to five representatives from each ITS-RFLP phenotype that were obtained from at least five root fragments were sequenced to confirm the accuracy of the RFLP phenotyping and to assign taxon affinities to the phenotypic groups (Table 2). Many PAC isolates showed length and RFLP polymorphisms as a result of a commonly occurring, *c.* 200-bp insertion in the ITS1 region (Vrålstad *et al.*, 2002). When this insertion was considered as a single mutation, the cultures shared ≥ 98% sequence identity and appeared to represent a single PAC group that may comprise a number of cryptic species (see Grünig *et al.*, 2004).

Resynthesis system

To identify fungi that colonize the Ericaceae intracellularly, one or two representatives of each ITS-RFLP phenotype were used to inoculate *V. uliginosum* seedlings in a sterile resynthesis system (Table 2). *Vaccinium uliginosum* was selected for these tests as its seeds were readily available for laboratory manipulations and germinated reliably. Seeds of *V. uliginosum* were surface sterilized for 1 min in 30% H_2O_2, germinated on 10^{-1} strength Murashige–Skoog (MS) medium (MP Biomedicals, Solon, OH, USA) and transferred to a split Petriplate resynthesis system, similar to that described in Xiao & Berch (1999), with 10^{-1} strength MS medium without a C source. Seedlings were allowed to establish for 2 wk before inoculation with a 6-mm plug from a fresh culture growing on Hagem's medium and incubated for 6–12 wk under fluorescent lights (photon flux *c.* 250 μmol m^{-2} s^{-1} photosynthetically active radiation at room temperature with a 12-h day length) to establish colonization. At harvest, plant roots were collected from the medium and half of the root system was prepared for microscopy by clearing for 3 min in the autoclave in 10% KOH. Cleared roots were screened for intracellular fungal complexes, distinct fungal coils and/or microsclerotia using differential interference contrast (Nikon Eclipse E600; Nikon Inc., Melville, NY, USA) and confocal (Zeiss LSM5 Pascal; Carl Zeiss Microimaging Inc., Thornwood, NY, USA) microscopy. The remaining roots were used to confirm that the fungal ITS-RFLP phenotype matched that used in the inoculation. A random selection of roots was cut into 1-cm segments and surface sterilized for 60 s in 30% bleach (1.75% sodium hypochlorite after dilution) and transferred onto Hagem's medium. Genomic DNA was extracted from the remaining roots and cultures obtained using CTAB and PCR amplified employing the ITS1F and ITS4 primers as described already. The *Hinf*I and *Alu*I RFLP digests were compared with the original inoculum cultures to confirm successful inoculation.

Root sampling for direct PCR

The three hosts used for the culture of root fungal communities were analyzed for root-associated fungi by direct PCR in 2006. At the same three plots, co-occurring individuals of *C. tetragona*, *E. nigrum* and *V. vitis-idaea* were selected at randomly chosen points in each plot, excavated with their intermingled root systems intact and stored for ≤ 12 h at 4°C. For each plant, nine roots connected to identifiable stems were excised and cleaned with tap water. A single, *c.*

Table 2 BLAST matches of fungal isolates used in the *Vaccinium uliginosum* studies *in vitro* based on ITS-RFLP groupings and sequencing

KSC#	Accession	Best BLAST match	Order	Phylum	Coverage	Similarity	Clade	Colonization
10054	EF026065	*Cryptosporiopsis ericae* (AY853167)	Helotiales	Asc.	100	99	12	N/A
10058	EF026066	*Cryptosporiopsis ericae* (AY853167)	Helotiales	Asc.	100	98	12	N/A
10066	EF026067	*Cryptosporiopsis ericae* (AY853167)	Helotiales	Asc.	100	99	12	ErM
10074	EF026068	*Cryptosporiopsis ericae* (AY853167)	Helotiales	Asc.	100	99	12	N/A
10184	DQ004264	*Cryptosporiopsis ericae* (EU880587)	Helotiales	Asc.	100	99	12	ErM
10060	DQ004260	*Cystodendron* sp. (DQ914672)	Helotiales	Asc.	93	95	14	ErM
10030	DQ004259	*Hymenoscyphus monotropae* (AF169309)	Helotiales	Asc.	92	97	13	ErM
10076	EF026069	*Hymenoscyphus monotropae* (AF169309)	Helotiales	Asc.	89	97	13	N/A
10210	EF026050	*Lachnum pygmaeum* (AJ430218)	Helotiales	Asc.	92	98	17	ErM
10301	EF026055	*Lachnum pygmaeum* (AJ430219)	Helotiales	Asc.	88	98	17	N/A
10498	EU026062	*Lachnum* sp. (EU794910)	Helotiales	Asc.	100	93	17	N/A
10293	EF026054	*Lachnum virgineum* (U59004)	Helotiales	Asc.	96	94	17	ErM
10198	EF026049	*Leptodontidium elatius* (FJ903294)	Helotiales	Asc.	97	92	8	ErM
10081	EF026070	*Meliniomyces variabilis* (EF093173)	Helotiales	Asc.	98	99	3	N/A
10083	EF026071	*Meliniomyces variabilis* (EF093178)	Helotiales	Asc.	97	99	3	ErM
10065	DQ004261	*Mollisia minutella* (DQ008242)	Helotiales	Asc.	92	98	7	ErM
10034	EF026057	*Phialocephala fortinii* (AY078143)	Helotiales	Asc.	98	99	6	Lightly melanized coiled, coarse hyphae
10100	DQ004262	*Phialocephala fortinii* (EF093159)	Helotiales	Asc.	100	98	6	N/A
10037	EF026059	*Phialocephala fortinii* (EU103612)	Helotiales	Asc.	97	98	6	N/A
10053	EF026064	*Phialocephala fortinii* (EU103612)	Helotiales	Asc.	96	97	6	Lightly melanized coiled, coarse hyphae
10133	DQ004263	*Phialocephala fortinii* (EU888624)	Helotiales	Asc.	100	100	6	N/A
10035	EF026058	*Phialocephala fortinii* (EU888625)	Helotiales	Asc.	97	98	6	N/A
10021	DQ004258	*Hypocrea pachybasioides* (AY240844)	Hypocreales	Asc.	99	99	–	N/A
10022	EF026051	*Hypocrea pachybasioides* (AY240844)	Hypocreales	Asc.	100	99	–	N/A
10023	EF026052	*Hypocrea pachybasioides* (AY240844)	Hypocreales	Asc.	100	99	–	N/A
10051	EF026063	*Hypocrea pachybasioides* (AY240844)	Hypocreales	Asc.	100	98	–	-
10007	EF026060	*Irpex lacteus* (EU273517)	Polyporales	Bas.	100	98	–	N/A
10008	EF026047	*Irpex lacteus* (FJ462768)	Polyporales	Bas.	100	99	–	N/A
10045	EF026061	*Irpex lacteus* (FJ462768)	Polyporales	Bas.	100	99	–	Loose, thin, hyaline coils
10011	EF026048	*Irpex lacteus* (FJ744594)	Polyporales	Bas.	98	98	–	N/A

Isolates were obtained from excised roots of three species in the Ericaceae: *Cassiope tetragona*, *Empetrum nigrum* and *Vaccinium vitis-idaea*. KSC# indicates reference collections for isolate and Accession indicates its GenBank accession number. Best BLAST matches with a complete binomial were selected for taxon assignment. Coverage and sequence similarity are in comparison with best matches in GenBank. Clade refers to the clade in Fig. 1, where the clade frequencies are given. Colonization column lists cultures with intracellular colonization (ErM, hyphal complexes typical of ericoid mycorrhiza; lightly melanized coiled, coarse hyphae; loose, thin, hyaline coils) or no colonization (–), or cultures that were not selected for the inoculation assays (N/A). Asc., Ascomycotina; Bas., Basidiomycotina.

1-cm segment of each root was dried in a desiccator, shipped with desiccant from Toolik Lake to Kansas State University and stored at −20°C. Eighty-one roots were sampled for each of the three hosts at each of the three plots for a total of 729 root segments.

DNA extraction and PCR amplification from roots

Genomic DNA was extracted separately from each of the 729 root segments using UltraClean Microbial DNA kits (MoBio Laboratories, Carlsbad, CA, USA) modified as described in Koide *et al.* (2005) and eluted in 50 μl of water. The fungal ITS region was amplified with ITS1F and ITS4 primers (Gardes & Bruns, 1993) in 50-μl reactions that contained 100 nmol of each of the forward and reverse primers, 2 μl of template DNA and 1X Platinum Blue PCR SuperMix (Invitrogen, Carlsbad, CA, USA). The PCR cycle parameters consisted of an initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min. All PCRs were performed in 96-well PCR plates on a MasterCycler (Eppendorf, Hamburg, Germany). Possible contamination was assessed by a negative PCR control in which the template DNA was replaced with sterile H_2O. No contamination was detected.

Cloning and sequencing of PCR products

PCR products from the separate DNA extracts were purified using the MinElute PCR purification kit (Qiagen, Valencia, CA, USA). The concentration of DNA was determined

with an ND1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and 20 ng of DNA from each PCR was pooled for each experimental unit (single host species within a plot) to minimize PCR bias. The pools of mixed PCR products were cloned using the TOPO-TA cloning system with pCR®2.1 vector and Oneshot® TOP10 chemically competent *Escherichia coli* (Invitrogen), as described in the manufacturer's manual, for a total of nine clone libraries (one for each host in each plot). The clone libraries were each combined with an equal volume of 60% glycerol, flash frozen in liquid N_2 and shipped for robotic colony picking, plasmid preparation and sequencing at the University of Washington High Throughput Genomics Unit (Seattle, WA, USA). Ninety-six clones from each of the clone libraries were randomly sampled and sequenced using the vector's T3 and T7 priming sites for reads in both directions across the ITS region, for a total of 864 possible sequences. Rarefaction analysis (Supporting Information Fig. S1; Colwell, 2006) indicated that this level of sampling did not saturate the resident richness. In addition, a majority – on average 83.2% – of the OTUs occurred only once in each experimental unit.

Sequence analysis and OTU designation of cloned sequences

Sequences of cloned PCR products were downloaded and imported into Sequencher (GeneCodes, Ann Arbor, MI, USA). Terminal vector contamination was removed using Sequencher's trimming function and the reads in two directions were used to construct a single contig. The sequences were assigned to OTUs using the alignment software CAP3 (Huang & Madan, 1999) at 97% similarity with a minimum overlap of 100 bp. The penalties for caps and mismatches were left at default settings. Sequence data for the cultured isolates and environmental PCR are available at GenBank (DQ004258–DQ004264; EF026047–EF026071; EU026062; HQ260025–HQ260315).

Frequencies were calculated for OTUs assigned at 97% sequence similarity (Table S1). As these sequences were broadly distributed across the Kingdom Fungi and most of the isolates that were able to colonize the roots had affinities with the Helotiales, we did not attempt a universal alignment, but selected those OTUs that were assigned to the Helotiales in BLAST analyses (Zhang *et al.*, 2000). We used two different strategies to establish taxon affinities for our cloned sequences. First, all OTUs were assigned to their best matches in BLAST (Zhang *et al.*, 2000). Second, because the first BLAST analyses included a large proportion of uncultured matches, we performed an Entrez-limited search to minimize the number of environmental samples. Using this second strategy, a representative of each nonsingleton OTU and all singletons were assigned to genera, families and orders.

Assignment of isolates and cloned OTUs to clades within the Helotiales

Because the majority of the isolates that colonized *V. uliginosum* intracellularly were identified as species within the Helotiales, and because alignment of all OTUs across multiple orders within the Ascomycota and Basidiomycota would not be feasible, we restricted further sequence analysis to OTUs within the Helotiales. Representatives of the OTUs that were determined to belong to the Helotiales were aligned in Sequencher against a broad selection of culturable and unculturable species within the Helotiales from previously published studies (Vrålstad *et al.*, 2002; Grünig & Sieber, 2005; Bougoure *et al.*, 2007; Grünig *et al.*, 2009). Alignments were analyzed in Geneious (Biomatters Ltd, Auckland, New Zealand) using neighbor joining (NJ) with a Tamura–Nei correction. The robustness of the acquired topologies was assessed by 1000 bootstrap replicates. On the basis of these analyses, we assigned our sequences to monophyletic clades and estimated the isolate or sequence frequencies within those clades for each of the hosts and each of the plots. Occasionally, a number of CAP3-assigned OTUs clustered within the same clade; these OTU frequencies were summed within that clade to provide a single estimate of the clade frequency. Such OTUs may represent intraspecific variability or potential cryptic species (see Grünig *et al.*, 2004).

Statistical analyses

Each host in each plot represents an experimental unit in both the isolate-based and PCR-based assays. To visualize the differences in the Helotiales associated with Ericaceae between the two assay techniques and among the three hosts, we analyzed the OTU frequency data for the fungal communities in PC-ORD (version 4.1; McCune & Mefford, 1999) using the isolate dataset and the direct PCR dataset separately as well as in combination. In addition, the entire clone library dataset – including non-Helotiales OTUs – was analyzed for differences in communities among the hosts. For each of the four analyses, pairwise distances were estimated using the Sørensen index and analyzed with nonmetric multidimensional scaling (NMS; Mather, 1976). The optimal number of dimensions (k) was determined on the basis of a Monte Carlo simulation at each level of dimensionality by comparing 40 runs with empirical data against 50 randomized runs with a step-down in dimensionality from 6 to 1 and a random seed starting value. Separate analyses of the isolate and direct PCR datasets failed to produce a solution that decreased stress in comparison with a randomized dataset, suggesting that these data contained minimal or no structure. Accordingly, we present only the results of the combined analyses using the optimal, two-dimensional solution ($k = 2$). Axis score data were near-normally distributed and

variances were equal across the experimental units (Levene's test; $P > 0.05$), meeting the assumptions for ANOVA. To test whether or not fungal community composition differed between the hosts or the two methods of community analysis, the axis scores for each of the two NMS axes were analyzed using a two-way ANOVA with 'method' and 'host' as independent variables. The ANOVAs were performed in JMP (v. 7.0.2; SAS Institute Inc., Cary, NC, USA).

Results

Fungal communities associated with Ericaceae based on culturing

Of the 303 isolates obtained, 153 belonged to ITS-RFLP phenotypes that included the 12 isolates that were capable of colonizing *V. uliginosum* in our resynthesis system (Table 2). Isolate 10051 (EF026063) with BLAST affinity to *Hypocrea pachybasioides* (AY240844) failed to colonize *V. uliginosum*. The remaining cultures were represented by fewer than five cultures or did not allow unambiguous assignment to an ITS-RFLP phenotype. A total of 148 of these 153 cultures was assigned to eight clades within the Helotiales (Fig. 1; Table 2). On the basis of the frequencies of those isolates able to colonize *V. uliginosum* intracellularly in the laboratory, the ErM fungal community of the three ericaceous hosts at the sites at Toolik was dominated by fungi assigned to a clade that represented PAC (Clade 6; 67.2%), followed by isolates assigned to a clade that included members of the genus *Pezicula* and *Cryptosporiopsis ericae* anamorphs (Clade 12; 20.7%). The remaining fungi occurred at low frequencies and had probable affinities with the genus *Lachnum* (Clade 17), grouped in a clade with *Hymenoscyphus monotropae* (Clades 13 and 14) or formed sister clades to *Mollisia* (Clade 7) and environmental samples (Clade 8). The isolates that colonized *V. uliginosum* but did not belong to Helotiales showed affinities to *Irpex lacteus* (Polyporales, Basidiomycota).

Fungal communities associated with Ericaceae based on direct PCR

The 864 clones yielded 515 high-quality reads. The number of successfully sequenced clones ranged from 44 to 91 (59 ± 15; mean ± 1 SD) per library (96 possible). These clones were assigned to 80 nonsingleton OTUs and 224 singletons for a total of 304 fungal OTUs. Before NJ analysis, we assigned the fungal OTUs to taxa using BLAST taxonomies after queries with two different strategies: one to identify the best match in the database, and the other to provide a taxon identifier whilst avoiding the uncultured or environmental sequences (Table S1). Both strategies provided taxon lists strongly dominated by ascomycetes. In the analyses that included all GenBank accessions, 54.7% of the reads were assigned to Ascomycota, 3.7% to Basidiomycota and one sequence (0.2%) to Spizellomycetales (Chytridiomycota). A large proportion of reads (41.1%) could only be assigned to Kingdom Fungi as a result of the prevalence of unidentified environmental sequences in GenBank ('uncultured fungi', 'uncultured mycorrhiza' or 'fungal endophyte').

In the Entrez-limited BLAST analyses used for taxon assignments, the sequenced fungi also represented mainly ascomycetes (86.3%), but included a number of basidiomycetes (13.5%) and one chytridiomycete (0.2%). The ascomycetes were distributed across 24 orders, but the majority of sequences belonged within the Helotiales (129 OTUs at 97% sequence similarity comprising 47.9% of the sequences; Fig. 2a). Additional common ascomycetes included members of the Hypocreales (10 OTUs at 97% sequence similarity comprising 9.3%) and Chaetothyriales (23 OTUs at 97% sequence similarity comprising 7.9%), or ascomycetes that remained unassigned to orders (*incertae sedis*). The non-Helotialean fungi largely represented root-associated parasites or pathogens (e.g. *Nectria*, Hypocreales (9.1%)), but some (*Capronia*, Chaetothyriales (7.5%)) showed high similarity (85–96%) to fungi previously isolated and shown to form ErM coils *in vitro* (Allen *et al.*, 2003). We also detected five basidiomycete orders dominated by members of the Sebacinales (5.8% of all sequences) and Agaricales (2.7%; Fig. 2b). The former includes fungi that can form ericoid mycorrhizas and ectomycorrhizas, whereas the latter includes a wide variety of fungi ranging from saprobes to root-associated symbionts.

In the NJ analyses, the cloned Helotialean sequences were assigned to a total of 14 clades, six of which (Clades 3, 6, 7, 8, 12, 17) included sequences from both the isolate and the clone datasets (Fig. 1). The majority of the cloned Helotialean sequences were assigned to a clade containing *Rhizoscyphus ericae* (Clade 1; 26.8% of all Helotiales sequences in our clone library dataset), which was absent among the sequences derived from the isolates. This was followed by two clades (Clades 2 and 10) that grouped as sisters to environmental sequences and comprised 20.1% and 18.1% of all Helotiales, respectively, and PAC (Clade 6) that comprised 9.4% of all Helotiales. The remaining clades within the Helotiales comprised < 10% of our Helotialean samples and were clustered mainly within other cultured and uncultured environmental samples or represented their sister clades.

Analyses of host preference

Neither the isolate-based nor the clone-based datasets provided evidence for the divergence of fungal communities across host taxa. With the exception of the combined dataset, the NMS ordinations failed to produce a resolution better than that produced randomly. In the combined analysis, the first two NMS axes represented 74.2% and 19.1%

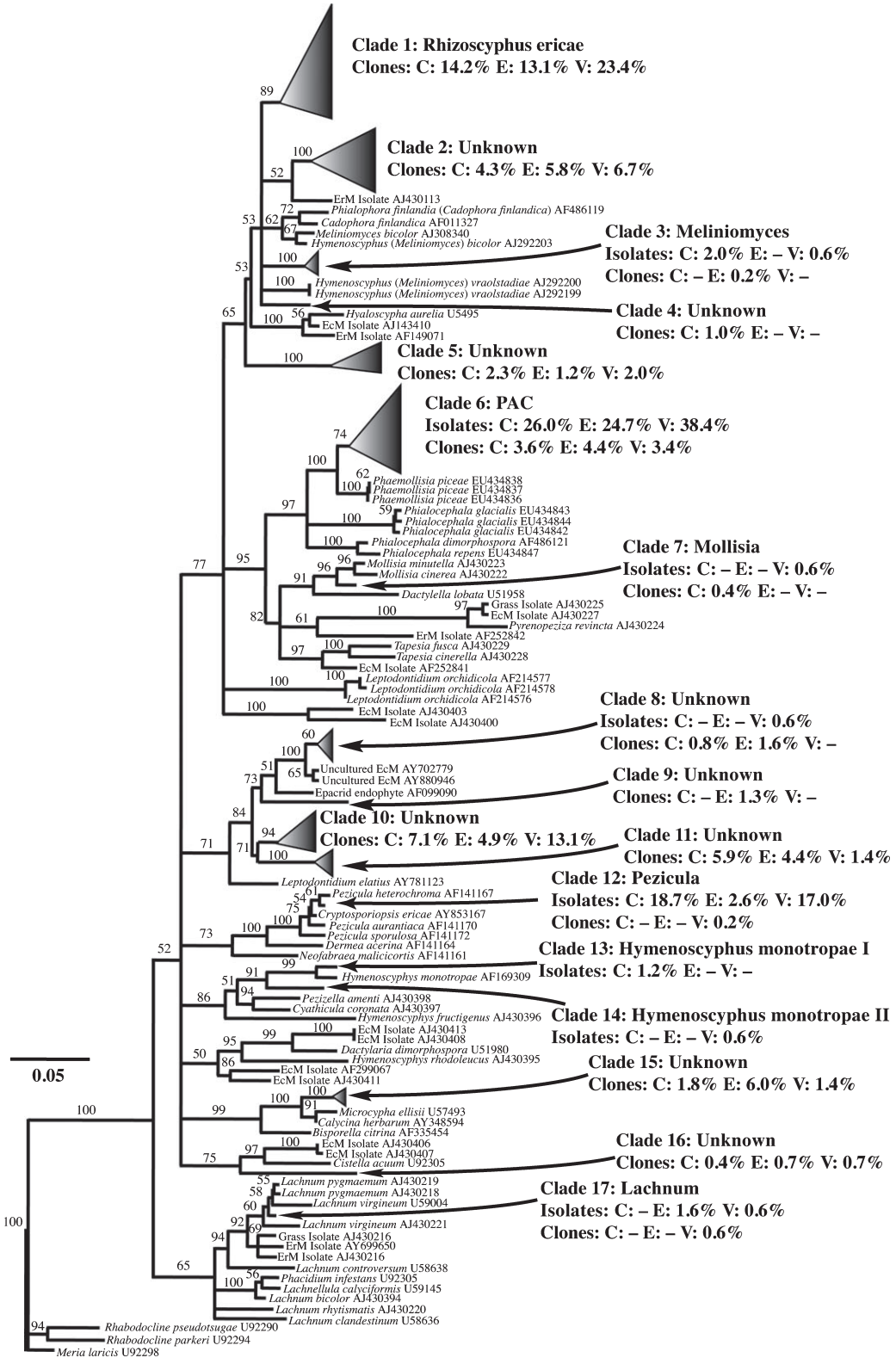

Fig. 1 Neighbor-joining cladogram with the assignment of Helotiales into 17 clades. The frequencies of the clones and isolates from *Cassiope tetragona* (C), *Empetrum nigrum* (E) and *Vaccinium vitis-idaea* (V) are shown. Dashes following the host taxon identifiers indicate that the clade was not detected in that host. The numbers above the clades indicate the proportion of bootstrap replicates (1000) that included that clade. Where the GenBank taxonomy conflicts with revisions, the revised current name for the accession is given in parentheses. EcM, ectomycorrhizal; ErM, ericoid mycorrhizal; PAC, *Phialocephala–Acephala* complex.

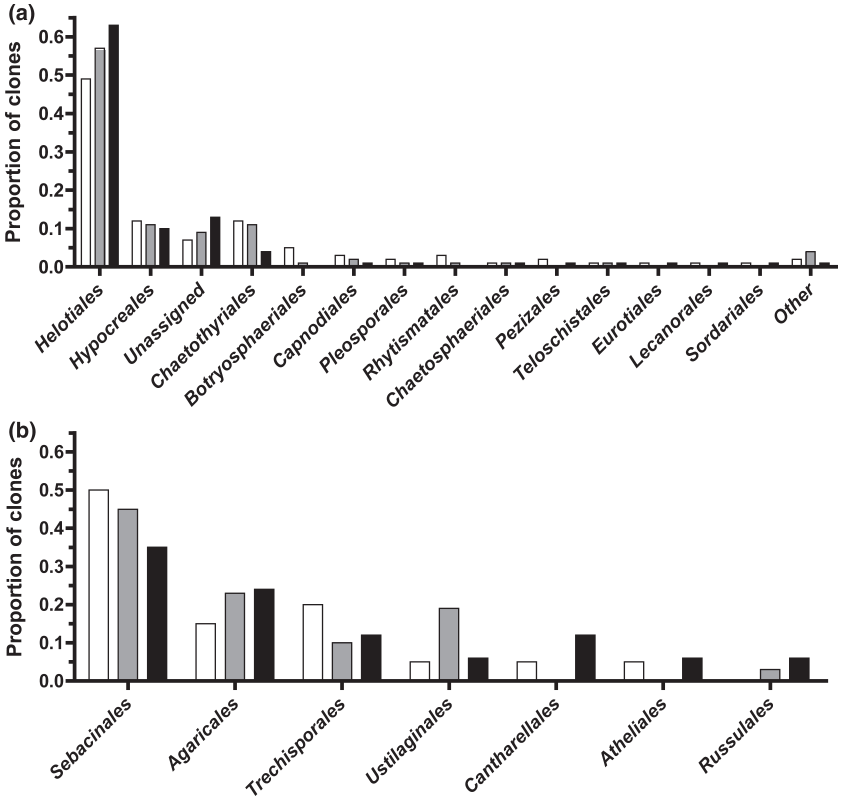

Fig. 2 Rank order of the ascomycete (a) and basidiomycete (b) orders observed in the clone libraries from three ericaceous hosts (*Cassiope tetragona*, white bars; *Empetrum nigrum*, gray bars; *Vaccinium vitis-idaea*, black bars) from tussock tundra near Toolik Lake, Alaska, USA. Ascomycetes comprised 86.3% of all cloned sequences, whereas basidiomycetes comprised 13.5%. To better illustrate the low-frequency orders, the proportions are shown relative to either the ascomycete or basidiomycete pools.

of the variance (93.3% in total). Although the scores for the isolate and clone communities differed on both axes (ANOVA: $F_{1,18} > 55$; $P < 0.0001$), there were no differences among the hosts (Fig. 3). In addition, there were no significant interactions between the method of analysis and the hosts.

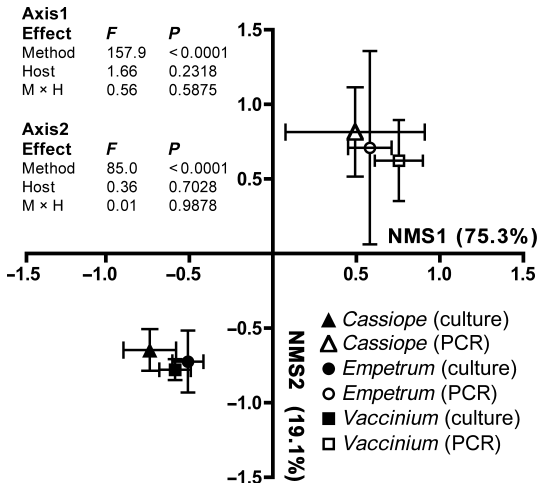

Fig. 3 Nonmetric multidimensional scaling (NMS) ordination of the Helotiales associated with the roots of three ericaceous hosts (*Cassiope tetragona*, *Empetrum nigrum* and *Vaccinium vitis-idaea)* in tussock tundra near Toolik Lake, Alaska, USA. Symbols indicate mean ± standard deviation. Helotialean communities did not differ among host species, but did differ depending on the technique used for detection (inset with the ANOVA tables for Axis 1 and Axis 2).

Discussion

Fungal communities in roots of three members of the Ericaceae in Arctic tundra

We used culture-dependent and culture-independent assays to characterize fungal communities associated with ericaceous plants that co-occur in Arctic tundra. We expected that these communities would include a substantial proportion of members of the Helotiales, comprising (among others) a number of root-associated ectomycorrhizal, ErM and endophytic ascomycetes (Wang *et al.*, 2006; Tedersoo *et al.*, 2009a). The assignment of molecular OTUs into functional guilds is often problematic. Therefore, it is possible that our ericoid plant roots may have hosted taxa that span more than one well-defined root-associated guild as shown by Vrålstad (2004). To address this, we included a resynthesis experiment which indicated that the majority of the most commonly isolated fungi were capable of colonizing *V. uliginosum*.

Approximately one-half of the isolates cultured from roots of the three species were assigned to ITS-RFLP phenotypes within the Helotiales, all of which included isolates that colonized *V. uliginosum* intracellularly. It is possible that the isolates that occurred in numbers below our threshold (ITS-RFLP phenotypes with at least five isolates) may have included additional groups capable of forming associations with *V. uliginosum*. As these pure culture synthesis

experiments are labor intensive and time consuming, we concentrated on the most frequently cultured fungi.

Clone libraries obtained through the amplification of DNA directly from the roots of the three species were also dominated by members of the Helotiales (48% of all sequences), emphasizing their importance in the fungal communities associated with the Arctic Ericaceae. Although the ecological roles of fungi detected in these culture-independent assays often remain uncertain, the culture-based assays suggest that many of the Helotialean fungi form mycorrhizal symbioses with the Ericaceae (see also Bergero *et al.*, 2000, 2003). Other than the predominance of members of the Helotiales, the two assay methods provided incongruent community views (Fig. 3) as has been observed previously (Allen *et al.*, 2003; Bougoure & Cairney, 2005). Only six of the 17 clades (3, 6, 7, 8, 12, 17) were detected by both methods. Overall, the compositions of these ascomycetous communities are similar to those reported by Tedersoo *et al.* (2009a), who targeted ascomycetous communities associated with the ectomycorrhizal roots of various hosts in Tasmania. Similar to the communities that we detected via direct PCR from ericaceous roots, those communities included a substantial number of Helotiales, but also a group of Chaetothyriales (*Cladophialophora–Exophiala–Capronia*) that may include facultative endophytes, putative ErM associates and fungi isolated from a variety of substrates (Narisawa *et al.*, 2002; Allen *et al.*, 2003), and members of the Hypocreales that include probable root-associated parasites or pathogens.

In addition to the ascomycetes, our clone libraries included a smaller number of basidiomycetes (13.5% of all sequences). These basidiomycetous communities were dominated by Sebacinales (a total of 25 OTUs comprising 40.0% of basidiomycetous sequences) that have been reported to fill a number of ecological roles ranging from endophytes and ericoid mycorrhizas to ectomycorrhizas (Walker & Parrent, 2004; Setaro *et al.*, 2006; Selosse *et al.*, 2007). Sebacinales detected in our study appear to comprise a less dominant component in the root-associated fungal communities of Ericaceae than they do in the understory Ericaceae in temperate coniferous forests (Allen *et al.*, 2003) or in a sub-Arctic mire (Kjøller *et al.*, 2010). Although not as frequently detected, Sebacinales appear to be a common component of this Arctic Ericaceae-associated community. The remaining detected basidiomycete orders included fungi with a variety of ecological roles, such as potential ectomycorrhizal fungi (Russulales), plant pathogens (Ustilaginales), saprobes or members of orders that include combinations of these roles (Agaricales, Atheliales).

Vaccinium uliginosum colonization *in vitro*

Although the colonization of *V. uliginosum* roots tended to be low after up to 12-wk of incubation (data not shown), we were able to use a resynthesis system to identify fungi belonging to distinct clades within the Helotiales that demonstrated the potential to establish ErM associations. Although some of these clades represent known mycorrhizal associates of Ericaceae (e.g. *Meliniomyces* – Clade 3; *Pezicula–Cryptosporiopsis* – Clade 12), some grouped only with environmental sequences (Clades 8, 9 and 16) or with presumed ErM associates (*Lachnum* spp. – Clade 17; *H. monotropae* – Clades 13 and 14). Two ITS-RFLP groups were unexpected. An ITS-RFLP group with a putative affinity to *Irpex lacteus* (Basidiomycota) was observed to form loose hyaline coils in *V. uliginosum* roots. In addition, representatives of an ITS-RFLP group assigned to a clade in PAC did not form the microsclerotia typical of dark septate endophytes (DSEs; Jumpponen & Trappe, 1998), but instead produced lightly pigmented, coarse coils. Although unusual, neither result is unprecedented. Zhang *et al.* (2009) reported that, among the 17 RFLP phenotypes that they observed, those that exhibited intracellular colonization of *Rhododendron* had BLAST affinities to *Phialocephala fortinii* and to an unknown basidiomycete. We compared our basidiomycetous cultures with those of Zhang *et al.* (2009) and determined that they shared < 90% sequence identity (BLAST queries against all accessions reported in Zhang *et al.* (2009): EU888587–EU888694). Taken together, these results suggest that the diversity and taxonomic affinities of fungi that form symbiotic associations with Ericaceae may be greater than previously believed (see also Usuki & Narisawa, 2005).

The ability of PAC to form ericoid mycorrhizas warrants further discussion. Although this complex is better known as a group of endophytes that form melanized structures when colonizing roots (DSEs; Jumpponen & Trappe, 1998), they appear to be capable of forming complexes indicative of ericoid mycorrhizas (Zhang *et al.*, 2009). However, PAC-related fungi probably represent cryptic species (Grünig *et al.*, 2004) that differ in their colonization morphologies, as exemplified by the intracellular microsclerotia in Ericaceae colonized by *P. fortinii* (Vohnik *et al.*, 2003). The morphology of the DSE colonization may vary among fungal strains (Mandyam & Jumpponen, 2008), hosts may be colonized by different strains or cryptic taxa with distinct morphologies, or the host may partially control the colonization morphology. The latter is supported by studies which indicate that a given fungal strain may be capable of forming distinct ectomycorrhizal or ErM morphologies when colonizing hosts of different mycorrhizal habits (Villarreal-Ruiz *et al.*, 2004; Vrålstad, 2004).

Helotiales associated with Ericaceae in the Arctic tundra

The isolate groups characterized by ITS-RFLP that included strains able to colonize *V. uliginosum* were assigned to eight

clades and were dominated by isolates assigned to PAC (Clade 6) and *Pezicula* spp. (*Cryptosporiopsis* anamorph – Clade 12). Fungal communities isolated from *Rhododendron fortunei* in China (Zhang *et al.*, 2009) were also strongly dominated by RFLP phenotypes assigned to *P. fortinii*. In that study, 112 of 220 isolates (50.9%) belonged to *P. fortinii* RFLP types. Although the data by Zhang *et al.* (2009) and Bougoure *et al.* (2007) suggest substantial variability in the fungal communities across spatially discrete sampling units, our data in combination with theirs support a broad distribution of PAC around the Northern Hemisphere and its common occurrence in Ericaceae. Similarly, fungi that show affinities or are closely related to *C. ericae* (with probable *Pezicula* spp. teleomorphs; Verkley, 1999) are commonly detected in the roots of Ericaceae in culture-based assays regardless of the study location (e.g. Sigler *et al.*, 2005; Zijlstra *et al.*, 2005).

The clone libraries were similarly dominated by sequences assigned to the Helotiales, which represented 14 distinct clades in our NJ analyses (Fig. 1). Although the nutritional modes of many of these fungi remain unknown, they include taxa that form ericoid mycorrhizas (e.g. *R. ericae* complex) and additional groups that group with other environmental sequences from ericoid mycorrhizas and common root-associated endophytes (PAC). Our isolates included no *R. ericae,* despite this taxon dominating the clone libraries and being commonly isolated from plant species in the Ericaceae. Root-associated fungal communities in the Arctic probably include a large proportion of PAC-related fungi that may mask the slow-growing *R. ericae*, indicating a methodological bias resulting from culture-based community analysis (Allen *et al.*, 2003).

Evidence of host preferences for fungal associates

In addition to characterizing fungal communities associated with three genera of Ericaceae in the Arctic tundra, we aimed to test whether or not fungal communities differed between host species. A total of seven clades (1, 2, 5, 6, 11, 15 and 16) detected by cloning and sequencing occurred in all three hosts. Six clades (3, 4, 7, 9 12, 17) detected by cloning and sequencing occurred solely in one of the three hosts. Neither the fungal communities determined by culturing nor those determined by cloning and sequencing could be distinguished between the three host species, thus supporting our null hypothesis of no host preference for fungal communities. This was also true for the full clone dataset not restricted to sequences with affinities to the Helotiales, suggesting rather homogeneous fungal communities in ericaceous hosts in this tundra habitat. Our findings are congruent with those of Kjøller *et al.* (2010), who targeted four co-occurring Ericaceae in a sub-Arctic mire and found no evidence for community divergence among the hosts. By contrast, Bougoure *et al.* (2007) reported distinct fungal communities in *V. myrtillus* and *C. vulgaris* in pine forest sites in Scotland, and Ishida & Nordin (2010) observed distinct communities in *V. myrtillus* and *V. vitis-idaea* in boreal forest stands dominated by Norway spruce. It is possible that the observed differences in the root-associated communities are confounded by microscale variability in soil. Dumbrell *et al.* (2010) sampled 425 plants representing 28 species and concluded that, compared with the abiotic controls (soil pH), the host specificity effects among arbuscular mycorrhizal fungi were negligible. For our direct molecular assessment of the fungal communities, we sampled three intermingled Ericaceae root systems, thus minimizing spatial variability among the experimental units. Further research is needed to determine the relative importance of hosts and soil conditions in controlling the community assembly of root-associated fungi.

Fungal communities that colonize the roots of co-occurring plants can be expected to diverge if differences in fungal functional attributes maintain the diversity within the ecosystem (Read, 1996; Cairney & Meharg, 2003). In an extensive review aiming to explore the soil microbial communities as mechanisms that maintain plant community structure and diversity, Bever *et al.* (2010) concluded that microbially mediated plant niche modifications may indeed be necessary for plant coexistence. Previous studies have proposed that plant diversity in Arctic tundra is maintained by capabilities to acquire N from different organic forms (McKane *et al.*, 2002). For the Ericaceae studied here, as well as those studied by Kjøller *et al.* (2010), it appears likely that, although fungal species may differ in their functional attributes, these differences do not determine the hosts with which they form associations. Consequently, a niche model based on host identity may be inappropriate for the characterization of the community assembly of ErM fungal species in this system (see also Dumbrell *et al.*, 2010 for arbuscular mycorrhizal fungi). Our findings suggest that, if fungal communities drive plant niche differentiation in the Arctic tundra, this differentiation is not correlated with fungal community composition. However, the ErM fungi are necessary for N acquisition for the Ericaceae (Xiao & Berch, 1999; Whittaker & Cairney, 2001; Walker *et al.*, 2010). On this basis, we propose that, if N acquisition is the primary mechanism for ericaceous plant coexistence, the differences in N use largely emerge from intraspecific rather than interspecific variability.

The importance of intraspecific variability in fungal community assembly is poorly understood. One fungal strain may form ectomycorrhizas or ericoid mycorrhizas when colonizing hosts of different mycorrhizal habits (Villarreal-Ruiz *et al.*, 2004; Vrålstad, 2004). However, it is presently unclear whether conspecific fungal strains may vary in their abilities to colonize different species of hosts that share a mycorrhizal habit. As both intra- (Midgley *et al.*, 2004; Grelet *et al.*, 2009) and interspecific (Xiao & Berch, 1999) variabilities in

nutrient acquisition have been documented, clear niche differentiation may operate on a subspecies' level. Although we consider that strain-level niche separation is unlikely, further studies with subspecies' resolution, as well as studies estimating the relative importance of intra- and interspecific variability, may be necessary to further elucidate these issues.

Conclusions

The Arctic ErM communities were dominated by ascomycetes with affinities within the order Helotiales, but included a basidiomycetous component dominated by Sebacinales. Our analyses suggest that a number of associations remain to be characterized between the Helotiales and Ericaceae. Pure culturing and direct PCR from root samples identified distinct fungal communities, confirming earlier results indicating a bias towards rapidly growing species in culture-based views of community composition. Neither pure culture nor direct PCR assays of fungal communities in the three host species provided any support for host preference. Given that the host may not be an important driver for the composition of root fungal communities in the Arctic Ericaceae, other mechanisms for the maintenance of diversity in these communities should be explored.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Rarefaction curves of fungal operational taxonomic units (OTUs) associated with three Ericaceae hosts (*Cassiope tetragona*, *Empetrum nigrum*, *Vaccinium vitis-idaea*).

Table S1 Taxon and clade assignments of the operational taxonomic units (OTUs) detected via direct PCR, cloning and Sanger sequencing

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