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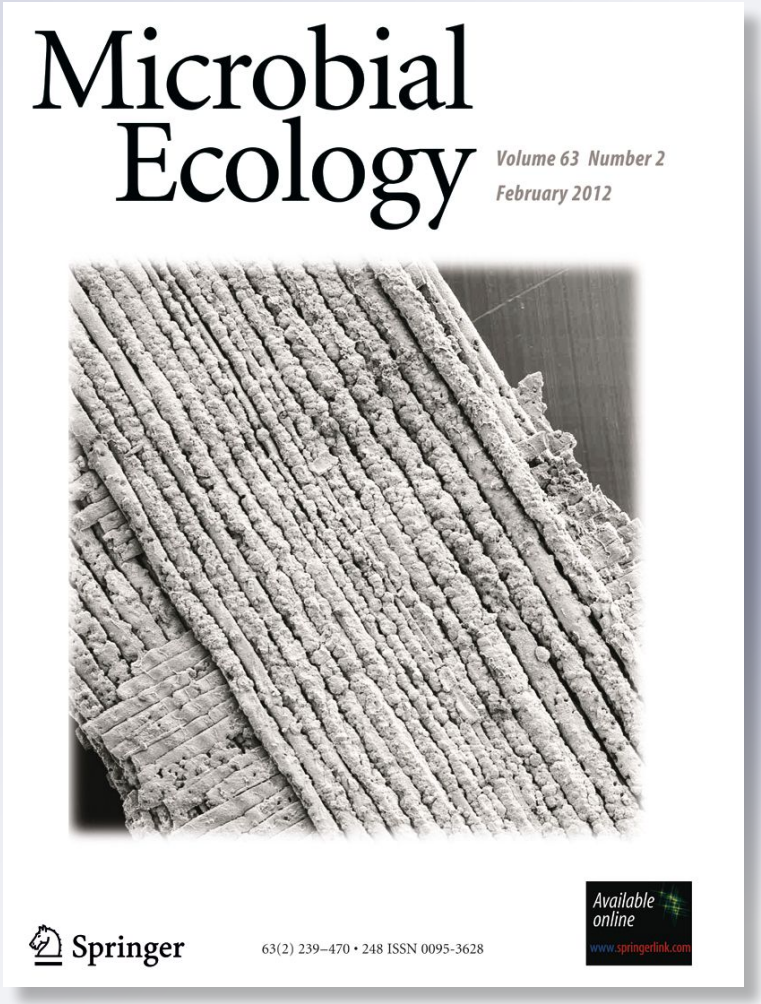
Microbial Ecology

ISSN 0095-3628
Volume 63
Number 3

Microb Ecol (2012) 63:682-693
DOI 10.1007/s00248-011-9968-7



Springer

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PLANT MICROBE INTERACTIONS

Host Identity Impacts Rhizosphere Fungal Communities Associated with Three Alpine Plant Species

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Received: 24 June 2011 / Accepted: 9 October 2011 / Published online: 27 October 2011

Abstract Fungal diversity and composition are still relatively unknown in many ecosystems; however, host identity and environmental conditions are hypothesized to influence fungal community assembly. To test these hypotheses, we characterized the richness, diversity, and composition of rhizosphere fungi colonizing three alpine plant species, *Taraxacum ceratophorum*, *Taraxacum officinale*, and *Polemonium viscosum*. Roots were collected from open meadow and willow understory habitats at treeline on Pennsylvania Mountain, Colorado, USA. Fungal small subunit ribosomal DNA was sequenced using fungal-specific primers, sample-specific DNA tags, and 454 pyrosequencing. We classified operational taxonomic units (OTUs) as arbuscular mycorrhizal (AMF) or non-arbuscular mycorrhizal (non-AMF) fungi and then tested whether habitat or host identity influenced these fungal communities. Approximately 14% of the sequences represented AMF taxa (44 OTUs) with the majority belonging to *Glomus* groups A and B. Non-AMF sequences represented 186 OTUs belonging to Ascomycota (58%), Basidiomycota (26%), Zygomycota (14%), and Chytridiomycota (2%) phyla. Total AMF and non-AMF richness were similar between habitats but varied among host species. AMF richness and diversity per root sample also varied among host species and were highest in *T. ceratophorum* compared with *T. officinale* and *P. viscosum*. In contrast, non-AMF richness and diversity per root sample were similar among host species except in the willow understory where diversity was reduced in *T. officinale*. Fungal community composition was influenced by host identity but not habitat. Specifically, *T. officinale* hosted a different AMF community than *T. ceratophorum* and *P. viscosum* while *P. viscosum* hosted a different non-AMF community than *T. ceratophorum* and *T. officinale*. Our results suggest that host identity has a stronger effect on rhizosphere fungi than habitat. Furthermore, although host identity influenced both AMF and non-AMF, this effect was stronger for the mutualistic AMF community.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-011-9968-7) contains supplementary material, which is available to authorized users.

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Introduction

Fungi are ubiquitous and play a key role in plant communities as pathogens, saprobes, and symbionts. These organisms can influence plant growth and fitness [1], community structure and composition [2], and ecosystem function [3]. A growing body of evidence indicates that many of these effects depend on fungal identity [4, 5]; however, only a small fraction of the estimated 1.5 million fungal species has been characterized [6]. This is particularly true for soil microbial communities where diversity is thought to be especially high [7, 8], yet technical issues

hamper species isolation and identification. The lack of information regarding soil fungi poses a problem in terms of fully understanding the importance of fungal diversity and predicting how changes in the fungal community will impact ecosystem processes [9].

Partner identity and environmental conditions have been shown to influence the strength of plant–fungal interactions and subsequent feedbacks [4, 10, 11]. Even fungi capable of colonizing numerous host species (e.g., arbuscular mycorrhizal fungi) often exhibit some degree of host specificity [10, 12–14], which can generate species-specific feedbacks that influence both above- and belowground community composition [2]. Since abiotic conditions can alter these plant–fungal feedbacks [11], rhizosphere communities also likely vary along environmental and habitat gradients (e.g., [15]). Characterizing the rhizosphere fungi associated with widespread hosts that co-occur along such gradients may provide insight into the relative importance of host identity and habitat in structuring these fungal communities. Alpine ecosystems are particularly well suited to testing these questions since the heterogeneous nature of alpine soil, frequency of disturbance, and sharp environmental boundaries [16] could generate significant variation in fungal communities across relatively small spatial scales. At the same time, many alpine plant species are distributed across fairly wide environmental gradients at and above treeline (e.g., *Polemonium viscosum* [17]). In this study, we take advantage of significant microhabitat heterogeneity in treeline communities to test the relative importance of habitat and host identity on rhizosphere fungi.

Most fungal phyla and families are functionally diverse, containing pathogens, saprobes, and mutualistic symbionts, which makes it difficult to assign a specific function to a group of even closely related fungi. In contrast, arbuscular mycorrhizal fungi (AMF) comprise the phylum Glomeromycota and are functionally similar to each other. AMF are generally viewed as mutualistic symbionts, providing their host plants with soil resources in exchange for photosynthate [18]. In this study, we focus on AMF as an identifiable functional group within the overall rhizosphere community. Studies employing molecular methods to characterize AMF diversity indicate that a single host species can associate with as many as 38 AMF taxa [14]. However, many AMF appear to have limited geographic distributions [19, 20] and exhibit varying degrees of host specificity [12, 13]. Consequently, both habitat and host identity may be important determinants of AMF diversity and composition.

Over a decade ago, Gardes and Dahlberg [21] highlighted the general lack of information about mycorrhizal associations in arctic and alpine ecosystems. Since that time, relatively few studies have explored or characterized fungal diversity in these ecosystems. Mycorrhizal fungal diversity is predicted to be lower in arctic and alpine regions due to environmental constraints, dispersal barriers, and an increased number of facultative or nonmycorrhizal host species [22, 23]. Support for this hypothesis based on studies conducted across latitudinal and elevational gradients is mixed [15, 20, 24, 25]. Thus, additional surveys spanning a greater number of sites and host species is needed to fully address the question of fungal diversity in arctic and alpine ecosystems. In this study we expand the information available for alpine ecosystems by assessing fungal diversity in three host species and two treeline habitats.

Using a combination of sample-specific DNA tags and direct 454 pyrosequencing we tested whether host identity or habitat affected the richness, diversity, and composition of AMF and non-arbuscular mycorrhizal (non-AMF) fungi associated with *Taraxacum ceratophorum*, *Taraxacum officinale*, and *P. viscosum* plants growing in open meadow and willow understory habitats at treeline on Pennsylvania Mountain, Colorado, USA. Based on the importance of species-specific feedbacks involving mycorrhizal and non-mycorrhizal fungi in other study systems [26, 27] we hypothesized that rhizosphere fungal communities vary among *T. ceratophorum*, *T. officinale*, and *P. viscosum*. Given the patchy distribution of fungi in alpine ecosystems [24, 28, 29], we also hypothesized that unique fungal communities persist in open meadow and willow understory habitats. We evaluated these hypotheses for both AMF and non-AMF to determine if the mutualistic AMF community and functionally diverse non-AMF community respond similarly to host and habitat characteristics.

Methods

Study System and Sampling

Root samples were collected from the treeline region on Pennsylvania Mountain (Park County, CO, USA; 39°15′ N, 106°07′ W, 3,590–3,630 m a.s.l) in early August of 2008. This site is located east of the continental divide, and as such, has a relatively dry climate with an average precipitation of 3.7 cm/month and an average temperature of 12°C during June–August (NOAA National Climate Data Center 2002, http://hurricane.ncdc.noaa.gov/cgi-bin/climatenormals/climatenormals.pl). At this location, treeline is a mosaic of willow shrub and open meadow habitats, and environmental conditions vary across this willow-meadow ecotone. Specifically, temperature, light availability, and wind exposure are higher in the open meadow whereas leaf litter is more abundant in the willow understory [30, 31]. Many plant species occur in both habitats, but at this site overall plant diversity and density is greater in the open

meadow than the willow understory [32]. Likewise, on Pennsylvania Mountain colonization by AMF is higher in the open meadow while ectomycorrhizal fungi are more abundant in the willow understory [33]. Other microbes, including salicylate-mineralizing fungi, have also been shown to associate with alpine willows at other locations in the Rocky Mountains [28]. Sampling across the willow-meadow ecotone allowed us to evaluate whether microhabitat affects the diversity and composition of rhizosphere fungi.

To characterize the rhizosphere fungal community in alpine willow and meadow habitats, we sampled roots from three widely distributed herbaceous plant species. *T. ceratophorum* (Asteraceae) and *P. viscosum* (Polemoniaceae) are native alpine plants, whereas *T. officinale* (Asteraceae) is an exotic species in North America. All three plant species co-occur in open meadow and willow understory habitats on Pennsylvania Mountain. Previous research shows that *T. ceratophorum* is more heavily colonized by AMF than either *T. officinale* or *P. viscosum* [33] and more responsive to colonization than *T. officinale* [32]. Sampling these three host species allowed us to evaluate whether AMF and non-AMF exhibit host specificity.

We sampled roots from ten plants per species per habitat for a total of 60 individuals distributed across a 0.7-km distance (sampling area=3,350 m^2). Plants sampled from the open meadow were at least 3 m from the nearest willow. Replicate plants in either habitat were at least 3 m from each other. From each plant, we collected multiple fine root fragments from the upper 10 cm of soil, totaling approximately 15 cm of root length per plant. The roots were rinsed with water to remove soil particles, surface sterilized for 15 min with 10% bleach, and preserved in 2× CTAB buffer until DNA extraction.

DNA Extraction and Sequencing

Total DNA was extracted from each root sample and eluted in 100 μl of the EB elution buffer using Qiagen Plant DNeasy kits (Qiagen, Valencia, CA, USA). We quantified the resulting DNA concentrations using a ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted each template to 2.0 ng μl^{-1}. Fungal small subunit (SSU) ribosomal DNA sequences were amplified using primer constructs that combined the A and B primers for 454 pyrosequencing with the fungal-specific SSU primers nu-SSU-0817-5′ and nu-SSU-1536-3′ [34]. A 5-base pair (bp) DNA tag was inserted between the A and nu-SSU-1536-3′ primers for post-sequencing sample identification. These SSU primers amplify DNA from fungi in all the major taxonomic groups [34]. The resulting 762 bp amplicon includes the V4 (partial), V5, V7, and V8 (partial) variable regions. SSU genes have been shown to be more informative than ITS genes for AMF [35]; however, these genes may lack resolution for some non-AMF taxa [36, 37]. Each template was PCR-amplified in 20 μl reactions containing 10 ng of the DNA template, 200 μM of each dNTP, 200 nM of each primer, 2.5 mM of $MgCl_2$, 1 U of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), and 4 μl of the supplied PCR buffer. The PCR reactions were run under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by final extension at 72°C for 8 min. Each sample was amplified in three separate reactions; 10 μl of each amplicon was pooled, resulting in a total of 30 μl/sample. The pooled amplicons were purified using Agencourt AMpure PCR purification kits (AgenCourt Bioscience Corporation, Beverly, MA, USA). Equal amounts of the purified amplicons were combined to create three sequencing pools consisting of 20 randomly selected samples per pool. All six combinations of habitat and host species were represented in each pool. The sequencing pools were adjusted to a concentration of 10 ng μl^{-1} using a RapidVap vacuum evaporation system, and then sequenced from the A-primer construct in a one sixteenth region of a 454 reaction using a GS FLX sequencer (454 Life Sciences, Branford, CT, USA) at the University of Florida's Interdisciplinary Center for Biotechnology Research (Gainesville, LF, USA). Raw 454 pyrosequencing files are deposited in the Sequence Read Archive (accession number, SRA023882.1).

Bioinformatics and OTU Designation

Sequences without a correct DNA tag or primer sequence, shorter than 195 bp, or with more than one ambiguous base were omitted from the dataset. DNA tags on the remaining sequences were replaced with a root sample designation, aligned using CAP3 [38], and assigned to operational taxonomic units (OTUs) at 90%, 95%, 97%, and 99% sequence similarity using a minimum overlap of 100 bp. To facilitate comparisons with other studies, we focus on the results for 97% sequence similarity; patterns in fungal richness, diversity, and evenness were similar at all four levels of sequence similarity unless otherwise noted. Other CAP3 parameters (e.g., matches, mismatches, and gap penalties) were left at the default settings. Root samples that did not meet a minimum sequencing depth of 50 sequences were omitted from the analysis, which reduced the total sample size to 36 individual root samples.

Sequencing errors (e.g., insertion and deletion errors, chimeric sequences, and low-quality reads) may inflate richness and diversity estimates based on 454 pyrosequencing data [39, 40]. Some authors suggest that the majority of singletons, sequences that occur only once in the dataset

and can comprise >60% of taxa detected through pyrosequencing, are the result of such errors [37, 41]. Consequently, we removed all singletons from the dataset to reduce the likelihood that sequencing errors might inflate fungal richness and diversity estimates.

One randomly selected sequence per OTU was assigned to a phylogenetic group (AMF or non-AMF) using two methods. First, we conducted a BLASTN search to identify the reference taxon to which the query had the highest sequence similarity, which we assume corresponds to taxonomic relationships and phylogenetic distance (Table S1 in the Electronic Supplementary Materials (ESM)). Second, we used ClustalX (version 2.0) to perform a "full/slow" multiple sequence alignment of OTU and reference sequences from the BLASTN search followed by neighbor-joining tree construction. Reference sequences from GenBank were retained in their full length to assist in supporting the backbone of the tree, although this increased the overall length of the alignment by including additional portions of the ribosomal region. Nucleotide positions containing gaps were included in the analysis. Overall, the phylogenetic analysis enabled us to verify phylogenetic assignments and account for potential issues with the BLASTN search due to lack of coverage and misidentified sequences in GenBank. We removed two OTUs from the dataset because ambiguous BLASTN and phylogenetic distance results prevented definitive assignment to a phylogenetic group. Sequences of each OTU used in the BLASTN and phylogenetic analyses are deposited in GenBank (accession numbers are listed in Table S1 in the ESM; alignment and tree files are available in Appendix A in the ESM).

We conducted a more thorough neighbor-joining analysis of the AMF taxa to evaluate confidence in our phylogenetic group assignments and to identify families represented in the dataset. All non-AMF sequences were removed from the alignment. To improve our ability to separate clades of AMF, we added high-quality reference sequences comprising 69 unrepresented Glomeromycota taxa and six basidiomycete and ascomycete outgroup taxa [42]. Next, we conducted 1,000 neighbor-joining bootstrap replicates to assess the confidence of tree topology. Finally, we trimmed the edges of the matrix, realigned the sequences using MUSCLE [43], and recalculated the neighbor-joining tree with 1,000 bootstrap replicates (alignment and tree files are available in Appendix B in the ESM).

Richness and Diversity Indices

OTU frequencies were determined for each root sample, and these data were used to calculate OTU richness and diversity (Table S2 in the ESM). Overall OTU richness (S) was calculated by summing the number of OTUs within each root sample. Shannon's diversity index ($H' = -\sum p_i(\log_e(p_i))$), where p_i is the relative abundance of each OTU, was calculated for each root sample using ESTIMATES (version 8.2.0; [44]). OTU evenness was calculated as the ratio of Shannon's diversity index and OTU richness ($H'/\ln S$). The above richness and diversity indices were calculated separately for AMF and non-AMF datasets at all four levels of sequence similarity.

Statistical Analyses

Each root sample represented an experimental unit, which allowed us to statistically evaluate differences in OTU richness, diversity, and evenness due to host species and habitat. For each response variable (richness, diversity, and evenness), we evaluated the effects of host identity and habitat on the rhizosphere fungal community using analysis of variance (ANOVA). To characterize differences in the response of AMF and non-AMF communities to host species and habitat we conducted separate ANOVAs for each fungal community. The relationship between AMF and non-AMF richness and diversity per root sample was evaluated using linear regression. The above statistical analyses were conducted using the statistical program JMP (version 8.0.2; SAS Institute, Inc.). Overall sampling effort per host species was assessed using rarefaction analysis in ESTIMATES.

Differences in fungal community composition were analyzed using PC-ORD (version 4.1, [45]). The relative abundance of AMF and non-AMF OTUs based on 97% sequence similarity were used to calculate community distance estimates using the Sorensen (Bray–Curtis) index; these estimates were visualized using nonmetric multidimensional scaling [46]. The optimal number of dimensions (k) was selected using a Monte Carlo test of significance at each level of dimensionality (k=1–6) by comparing 100 runs with empirical data to 100 randomized runs with a random seed starting value. The three-dimensional solution produced stress values significantly smaller than those in the randomized runs for both fungal communities. Differences in community composition based on this three-dimensional ordination were analyzed using multi-response permutation procedure (MRPP) [46]. Since MRPP cannot account for interactions between factors, we analyzed differences due to host identity and habitat separately. For this analysis, we report the P value and the chance-corrected within-group agreement (A), which represents within-group homogeneity compared with the random expectation.

Results

Sequencing Analysis and Bioinformatics

After controlling for sequencing depth per sample and sequence quality (presence of the DNA tag and primer, sequence length, and ambiguous bases), we retained a total of 3,921 sequences from 36 root samples collected from three host species and two habitats (average length=250 bp and median length=245 bp). The total number of sequences acquired per root sample (average=109 and standard deviation=49) did not differ significantly among host species (F=0.71, df=2, P=0.50, and 4.2% variance), habitat (F=0.55, df=1, P=0.46, and 1.6% variance), or host species within habitat (F=0.69, df=2, P=0.51, and 4.1% variance) indicating no bias in average sequencing depth among treatments. However, there was substantial variation in sequencing depth among individual root samples (Table S2 in the ESM).

The aligned matrix of OTUs and reference taxa included 9,148 nucleotide positions, of which 68.8% (6,291 nucleotide positions) was variable. Based on the BLASTN search and neighbor-joining analysis, 13.8% of the sequences represented AMF (44 OTUs at 97% sequence similarity). The remaining 86.2% of the sequences represented various non-AMF taxa (186 OTUs at 97% sequence similarity). While it was difficult to reliably assign non-AMF sequences to a specific taxonomic group, the phylogeny and BLASTN results generally agreed as to which phylum the remaining sequences belong (non-AMF OTUs were 58% Ascomycota, 26% Basidiomycota, 14% Zygomycota, and 2% Chytridiomycota). Rarefaction analysis indicates that we approached but did not achieve OTU saturation for each host species (Fig. 1a).

To further characterize the distribution of OTUs within the phylum Glomeromycota, we conducted a more thorough neighbor-joining analysis using the AMF taxa only. After trimming the ends of the matrix, the alignment was 2,424 nucleotide positions in length and 56.6% (1,369 nucleotide positions) variable. The neighbor-joining phylogram of AMF OTUs and reference sequences (Fig. 2; full tree with labeled tips is available in Appendix B in the ESM) recovered a topology generally congruent to the published hypothesis of phylogenetic relationships within Glomeromycota [42]. OTUs were not equally distributed taxonomically or phylogenetically within Glomeromycota (Fig. 2). Most AMF richness occurred in Glomeraceae, with *Glomus* group A containing 28 OTUs and *Glomus* group B containing ten, together representing over 80% of the total number of AMF OTUs and 92% of AMF sequences. OTUs represented the longest branches in the tree and tended to cluster in clades without reference sequences, which suggests that these OTUs represent divergent taxa largely

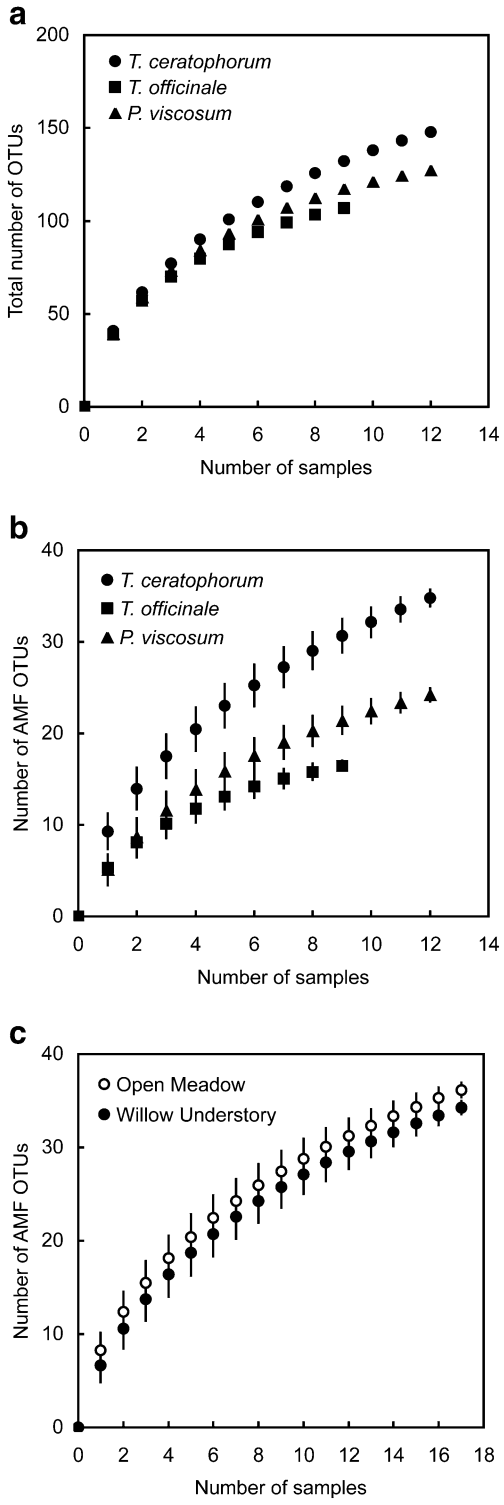

Figure 1 Species accumulation curves showing the number of fungal OTUs verses the number of root samples analyzed. **a** Total number of fungal OTUs detected in root samples from *T. ceratophorum* (*circles*), *T. officinale* (*squares*), and *P. viscosum* (*triangles*); **b** number of AMF OTUs detected in root samples from each host species (*symbols* as described above); **c** number of AMF OTUs detected in roots samples from open meadow (*open circles*) and willow understory (*solid circles*) habitats. OTUs were designated based on 97% sequence similarity

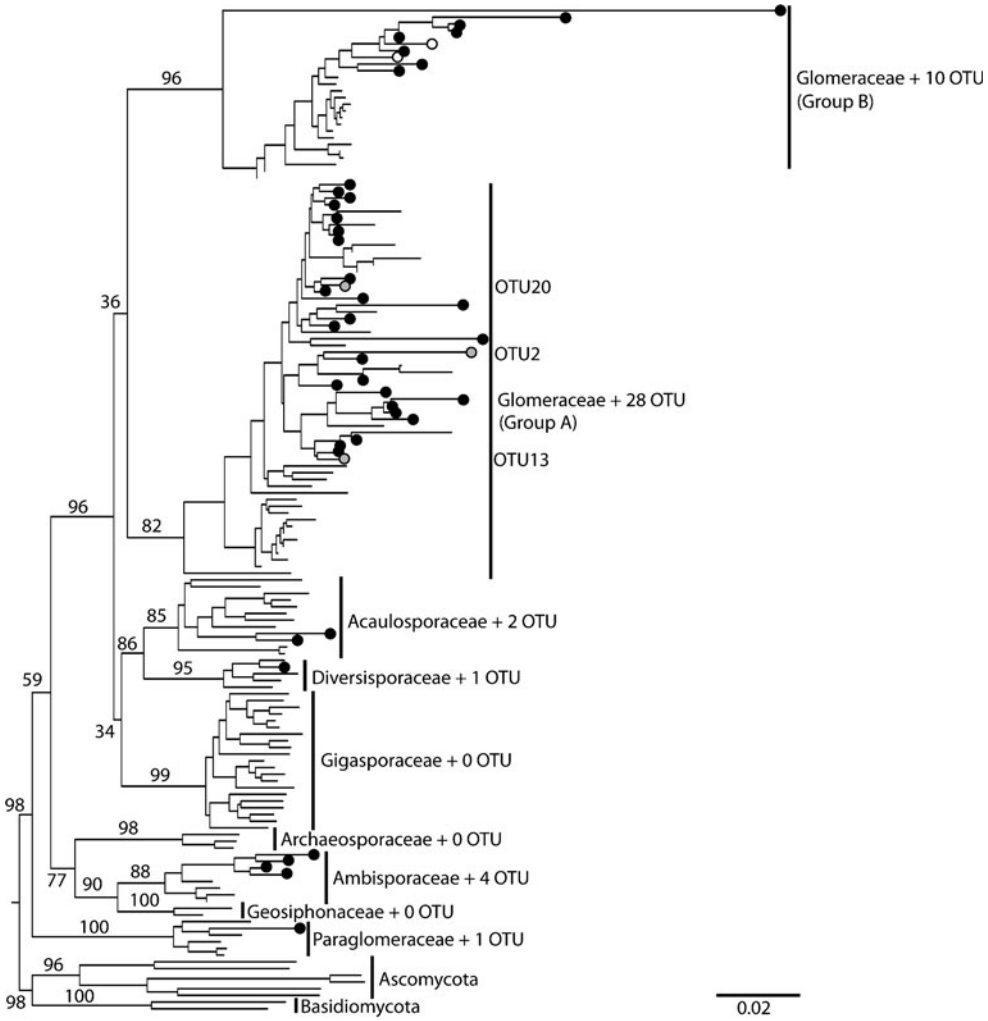

Figure 2 Neighbor-joining phylogram of AMF OTUs, top BLASTN reference sequences, additional AMF reference sequences from unrepresented Glomeromycota lineages, and outgroup taxa from Basidiomycota and Ascomycota inferred from small subunit ribosomal DNA sequences (alignment length=2,424 nucleotide positions). *Black circles* represent tips belonging to OTU sequences, *gray circles* indicate the most abundant AMF OTU in each host species (OTU 20=*T. ceratophorum*, OTU 13=*T. officinale*, and OTU 2=*P. viscosum*), and *open circles* indicate two AMF OTUs removed from the study because of insufficient sampling depth. OTUs were designated based on 97% sequence similarity. Numbers by nodes along the tree backbone represent 1,000 neighbor-joining bootstrap replicates. Clades are labeled according to the family assignments of reference sequences included in the lineage. The number of AMF OTUs found in each clade is noted after the *plus sign*. Glomeraceae groups A and B follow the categories assigned by Schwarzott et al. [59]

uncharacterized by current fungal systematics. Additionally, our assembly parameters required a relatively high number of nucleotide changes (>7 bp in a 245 bp sequence) to identify as sequence as a separate OTU; thus, it is unlikely that sequencing artifacts produced this level of divergence.

Richness and Diversity Indices

The total number of AMF taxa based on 97% sequence similarity was similar in open meadow (37 OTUs) and willow understory (35 OTUs) habitats, and 28 of the OTUs were present in both habitats. In contrast, the total number of AMF taxa varied among *T. ceratophorum* (36 OTUs), *T. officinale* (17 OTUs), and *P. viscosum* (25 OTUs). Eight AMF OTUs were present in all three host species, while 18 were present in only one host species. Individual root samples were colonized by 0–12 AMF OTUs (Table S2 in the ESM). AMF richness per root sample was significantly higher in the open meadow than the willow understory (Table 1; ANOVA, habitat effect, P=0.05). On average, there were 6.3 OTUs per root sample from the open meadow and 5.6 OTUs per root sample from the willow understory. The difference between habitats was only significant when OTUs were designated based on 97% sequence similarity (Fig. S1a in the ESM). AMF richness and diversity per root sample were significantly higher in *T. ceratophorum* compared with *T. officinale* and *P. viscosum* (ANOVA, host effect, P=0.003 and 0.01, respectively; Table 1 and Fig. 3a, b). The observed host effect on AMF richness and diversity was significant regardless of what level of sequence similarity was used to designate OTUs (Fig. S1d, e in the ESM). AMF evenness per root sample did not differ among host species or between habitats (Table 1 and Fig. 3c). Host identity explained more than 30% of the variation in AMF richness and diversity (Table 1). In contrast, habitat explained only 11.5% of the variation in AMF richness and 6.9% of the variation in AMF diversity (Table 1). Rarefaction analysis further supports the greater effect of host species compared with habitat on AMF richness (Fig. 1b, c).

The total number of non-AMF taxa based on 97% sequence similarity was similar in open meadow (135 OTUs)

Table 1 Analysis of variance on the effects of host identity and habitat on rhizosphere AMF and non-AMF communities

Response	Host identity				Habitat				Host identity × habitat			
	F	*df*	*P*	Variance (%)	*F*	*df*	*P*	Variance (%)	*F*	*df*	*P*	Variance (%)
AMF												
OTU richness (*S*)	7.88	2	0.003	39.3	4.59	1	0.05	11.5	1.21	2	0.32	6.0
Shannon's Index (*H*′)	5.67	2	0.01	32.1	2.42	1	0.14	6.9	1.48	2	0.25	8.4
Evenness (*H*′/lnS)	0.31	2	0.74	2.7	1.52	1	0.23	6.7	0.83	2	0.45	7.4
Non-AMF												
OTU richness (*S*)	0.09	2	0.91	0.5	2.13	1	0.15	6.0	1.74	2	0.19	9.9
Shannon's Index (*H*′)	0.86	2	0.43	3.9	2.00	1	0.17	4.5	5.80	2	0.007	25.9
Evenness (*H*′/lnS)	1.35	2	0.27	6.1	0.19	1	0.67	0.4	5.08	2	0.01	23.1

Richness and diversity estimates were calculated for OTUs designated based on 97% sequence similarity

and willow understory (134 OTUs) habitats, and 83 of the OTUs were present in both habitats. In contrast, the total number of non-AMF OTUs varied among *T. ceratophorum* (115 OTUs), *T. officinale* (93 OTUs), and *P. viscosum* (105 OTUs). Thirty-seven of these OTUs were present in all three host species while 95 non-AMF OTUs were present in only one host species. Individual root samples were colonized by 7–31 non-AMF OTUs (Table S2 in the ESM), and average non-AMF richness per root sample was similar across treatments (Table 1; ANOVA, $P>0.1$). Average non-AMF diversity and evenness per root sample varied among host species, but only in the willow understory where non-AMF

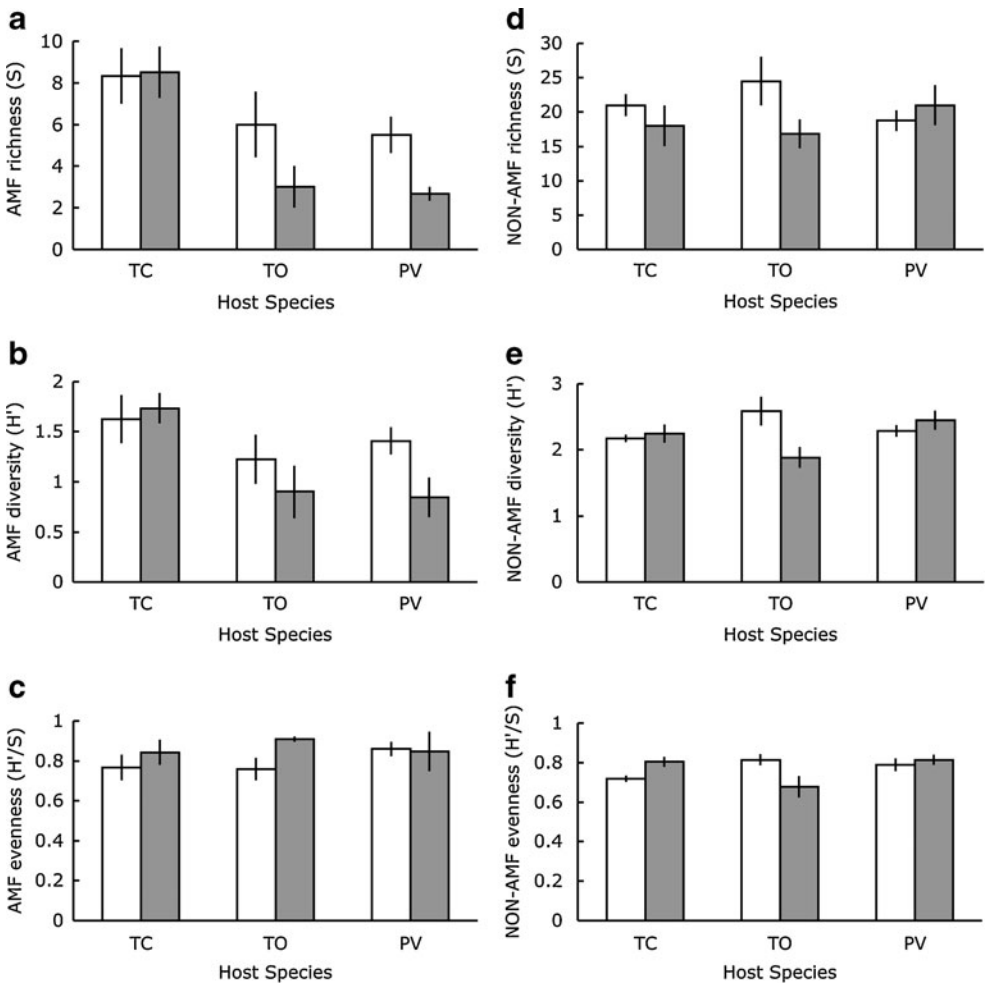

Figure 3 AMF and non-AMF communities colonizing the roots of *T. ceratophorum* (*TC*), *T. officinale* (*TO*), and *P. viscosum* (*PV*) plants sampled from open meadow (*open bars*) and willow understory (*solid bars*) habitats on Pennsylvania Mountain. **a**, **d** Operational taxonomic unit (*OTU*) richness (*S*); **b**, **e** Shannon's diversity index (*H*′); **c**, **f** OTU evenness (*H*′/lnS). Means (±SE) were calculated using OTUs designated based on 97% sequence similarity. AMF richness and diversity were significantly higher in the open meadow than in the willow understory, and in *T. ceratophorum* compared with *T. officinale* and *P. viscosum*. Non-AMF diversity and evenness were significantly reduced in *T. officinale* in the willow understory

diversity and evenness were reduced in *T. officinale* compared with *T. ceratophorum* and *P. viscosum* (Table 1; ANOVA, habitat×host interaction, P=0.007 and 0.01; Fig. 3e, f). The observed habitat by host interaction was not significant at 99% sequence similarity (Fig. S2b, c in the in the ESM). Neither host identity nor habitat explained more than 6% of the variation in non-AMF richness; however, the interaction between host identity and habitat explained over 20% of the variation in non-AMF diversity and evenness (Table 1). Interestingly, non-AMF richness and diversity per root sample were not significantly correlated with AMF richness and diversity (linear regression, P=0.12 and 0.50, respectively).

Community Composition

The most abundant AMF OTU represented 26–46% of the total number of AMF sequences for a given host species, and the identity of this OTU differed among the three host species. OTU 20 (HQ256922, *Glomus* group A, unable to identify a close reference species in the neighbor-joining tree), OTU 13 (HQ256915, *Glomus* group A, *Glomus aureum*), and OTU 2 (HQ256904, *Glomus* group A, *Glomus hoi*) were the most abundant OTUs associated with *T. ceratophorum*, *T. officinale*, and *P. viscosum*, respectively (Fig. 2). The AMF community was characterized using a three-dimensional ordination solution representing 72% of the variation in AMF composition per root sample (Fig. 4a). MRPP analyses indicate that the AMF community differed among host species (A=0.12 and P=0.002) but not between habitats (A=0.006 and P=0.33). Pair-wise species comparisons indicate that the community colonizing *T. officinale* was significantly different from the community colonizing *T. ceratophorum* (A=0.16 and P=0.0008) and *P. viscosum* (A=0.14 and P=0.003). AMF composition was not significantly different between *T. ceratophorum* and *P. viscosum* (A=−0.02 and P=0.74).

A single OTU (OTU 8, HQ256910, Pezizomycotina, unable to identify a close reference species in the neighbor-joining tree) was the most abundant non-AMF OTU in all three host species, representing 21% of the total number of non-AMF sequences. The non-AMF community was characterized using a three-dimensional ordination solution representing 74% of the variation in non-AMF composition per root sample (Fig. 4b). MRPP analyses indicate that the non-AMF community differed among host species (A=0.05 and P=0.01) but not between habitats (A=−0.004 and P=0.26). Interestingly, plants that hosted a more similar AMF community did not also host a more similar non-AMF community. Instead, pair-wise species comparisons indicate that the non-AMF community colonizing *P. viscosum* was significantly different from the community colonizing *T. ceratophorum* (A=0.03 and P=0.04) and *T. officinale* (A=0.08 and P=0.002). Non-AMF composition was not significantly different between *T. ceratophorum* and *T. officinale* (A=0.01 and P=0.23).

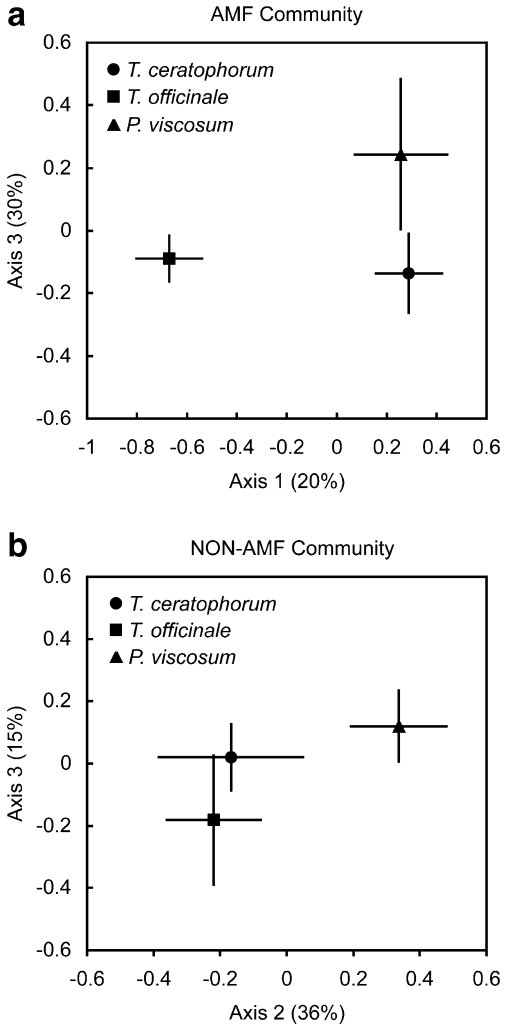

Figure 4 Nonparametric multidimensional scaling (*NMS*) ordination of **a** AMF and **b** non-AMF communities colonizing *T. ceratophorum* (*circles*), *T. officinale* (*squares*), and *P. viscosum* (*triangles*). Plotted are the mean scores (±SE) for each host species along the two axes that represent the greatest amount of variation among host species. *T. officinale* hosted a significantly different AMF community compared with *T. ceratophorum* and *P. viscosum*. *P. viscosum* hosted a significantly different non-AMF community compared with *T. ceratophorum* and *T. officinale*. OTUs were designated based on 97% sequence similarity

Discussion

Fungal communities, particularly those colonizing plant roots, are understudied in many biomes despite their importance to community structure and ecosystem function. In this study, we combined DNA tagging and direct pyrosequencing to characterize the rhizosphere fungi

associated with three herbaceous alpine plant species. Tagging individual root samples with sample-specific DNA tags maximized the number of root samples analyzed, but at a cost in terms of overall sequencing depth per sample. Ultimately, this approach enabled us to statistically compare the effects of host identity and habitat on fungal richness, diversity, and composition. This study expands the available information regarding plant–fungal interactions in alpine ecosystems. Our results indicate that in this system host identity has a greater impact on rhizosphere fungal communities than habitat, and that the effect of host identity is stronger for the mutualistic AMF community.

AMF Diversity in Alpine Hosts

We focused on the AMF community because these fungi form a mutualistic relationship with their hosts and because of the large number of unanswered questions about AMF in alpine ecosystems. Our results do not support the prediction that AMF communities are less diverse in alpine habitats compared with other temperate ecosystems; however, this study is limited to only three host species at a single site. A more expansive survey is needed to fully address the question of AMF diversity at high elevations. Overall, we identified 44 AMF taxa; previously, the highest reported AMF richness in a single study was 48 taxa in a boreonemoral forest [14]. Furthermore, average AMF richness per host species (26) and per individual root sample (6) are comparable to values reported in other studies [19, 47]. Even though overall AMF richness at our study site appears to be relatively high, OTU richness per root sample suggests that only a small portion of the fungal community colonizes an individual host. Similar patterns in sample-specific richness have been noted for leaf endophytes, ectomycorrhizal fungi, and AMF in other systems [47–50], highlighting the patchy distribution of fungi in nature.

Host Identity Impacted Fungal Communities

Sampling multiple independent hosts allowed us to statistically evaluate whether host identity or habitat influenced the rhizosphere fungal community. Our results indicate that host identity has a greater impact than habitat on the richness and diversity of rhizosphere fungi in this system, and that AMF and non-AMF may respond differently to host and habitat characteristics. Variation in AMF richness and diversity among host species mirrored pervious observations of differences in colonization and mycorrhizal responsiveness. More specifically, total AMF richness and AMF richness per root sample was highest in *T. ceratophorum*, a host species that is generally more responsive to mycorrhizal fungi and more heavily colonized than *T. officinale* and *P. viscosum* [32, 33]. In contrast, there was little variation in non-AMF richness and diversity among host species except in the willow understory where fungal diversity per root sample was reduced in the exotic host *T. officinale*. The non-AMF community includes putative fungal pathogens, saprobes, dark septate fungi, and other asymptomatic endophytes. Some research suggests that exotic plants are less vulnerable to pathogens in their introduced range [4]; thus, susceptibility to fungal pathogens may account for some of the difference in non-AMF diversity per root sample in *T. officinale*. Interestingly, there was no correlation between AMF and non-AMF richness or diversity per root sample, suggesting that these two fungal communities respond differently to their environment.

Compared with the observed host effects, habitat had a relatively small impact on the richness and diversity of rhizosphere fungi. In fact, total AMF and non-AMF richness were similar between habitats. Likewise, 64% of AMF OTUs and 45% of non-AMF OTUs were isolated from both open meadow and willow understory hosts, suggesting that many of the rhizosphere fungi at this site are distributed across the willow-meadow ecotone. In contrast, habitat did affect non-AMF diversity per root sample, but only for one of the three host species. In particular, non-AMF diversity was reduced in *T. officinale* plants from the willow understory compared with plants from the nearby open meadow. AMF richness per root sample was also generally lower in understory plants, although in this case habitat only explained 11.5% of the variation in AMF richness (compared with 39% for host identity). The relatively small effect of habitat on AMF richness per root sample may reflect habitat-specific differences in colonization. Previous work shows that AMF colonization is generally reduced in understory plants at this study site [33]. Given the relatively small root sample analyzed per plant, lower AMF colonization may have affected the likelihood of detecting AMF in root samples from the willow understory, resulting in reduced AMF richness per root sample. Ultimately, our results suggest that microhabitat plays a relatively minor role in structuring rhizosphere fungal communities in this system.

AMF and non-AMF community composition also varied among host species but not between habitats, providing further evidence that rhizosphere fungi respond more strongly to host identity in this system. Host identity has been shown to impact the composition of both above- and belowground fungal communities [4, 10, 51]; however, the degree of host specificity can be highly variable both within and among different types of plant–fungal interactions ([52, 53 and references therein). The small number of host species surveyed in this study limits our ability to identify host characteristics that influence rhizosphere fungal community assembly. In some systems, the host's ecological niche influences fungal specialization and

community composition [14]. Other research suggests that phylogenetic relationships among host species can impact fungal specialization such that closely related plants are more likely to share specialized enemies and partners [54]. Sampling additional plant species at our study site could shed light on whether the observed differences in rhizosphere fungal composition are due to the host's ecological niche, phylogenetic relatedness, or some other host characteristic.

Interestingly, in this study the effect of host identity differed for AMF and non-AMF communities. In particular, host identity explained over 30% of the variation in AMF richness and diversity per root sample, but less than 5% of the variation in non-AMF richness and diversity per root sample. Moreover, plant species that hosted a more similar AMF community did not also host a more similar non-AMF community. Taken together, these results suggest that AMF and non-AMF respond differently to their host environment, and that that different plant characteristics influence host specificity by AMF and non-AMF.

Primer Selection and Bias

Primer selection can impact phylogenetic analysis and diversity estimates in studies such as this one. The SSU gene region is generally less informative for Ascomycetes and Basidiomycetes than other ribosomal gene regions [36, 37], which likely reduced our ability to assign non-AMF OTUs to more specific phylogenetic groups. Since OTUs were determined based on sequence similarity, reduced variability in the SSU gene region for Ascomycetes and Basidiomycetes may have also impacted the number of non-AMF taxa detected and overall patterns in non-AMF richness and diversity. In contrast, the SSU gene region is generally more informative than the ITS gene region for Glomeromycetes, and various SSU primers have been used extensively to distinguish phylogentic relationships among AMF taxa ([35, 42] and references therein). In this study, AMF OTUs were assigned with high confidence to families within Glomeromycota; however, assigning OTUs to genera or species was more problematic given the short sequence length. Furthermore, clades containing only OTUs may correspond to taxa currently unrepresented in GenBank. Thus, although 454 pyrosequencing is a powerful tool for studying fungal communities, we found that short 454 sequences of highly conserved gene regions may be insufficient to resolve some phylogenetic relationships without further characterization of taxa present in environmental samples.

Future Directions and Conclusions

The observed variation in AMF and non-AMF communities raises intriguing questions about the implications of fungal diversity and composition for plant fitness. Mycorrhizal fungi differ in their ability to access resources, protect their host against pathogens, and alleviate stress [5, 10, 55], yet it is unclear whether increased fungal diversity within individual plants is always beneficial. For example, Jansa et al. [56] found little evidence that colonization by multiple *Glomus* species enhanced benefits to the host plant, suggesting that increased partner diversity may not translate into increased plant fitness. However, other research suggests that functional specialization occurs at the family level within Glomeromycota, and that colonization by multiple species from different AMF families may increase plant fitness by providing complementary benefits [5]. In contrast, the non-AMF rhizosphere community contains a functionally diverse array of pathogens, saprobes, dark septate fungi, and other root endophytes. Pathogens can vary greatly in terms of virulence and host specificity [4, 57]; however, there is a high degree of functional redundancy among saprobes and other root endophytes [58]. Variation in the diversity and composition of these non-AMF groups may have very different implications for plant fitness compared with variation in mutualistic AMF communities. Likewise, variation in aboveground fungal communities, which also contain a functionally diverse array of species, may impact host plants differently than variation in belowground communities and mycorrhizal fungi, in particular.

Overall, this study demonstrates that host identity has a stronger effect than habitat on fungal community assembly in this system, and that the relative importance of these selection pressures likely differs for mutualistic AMF and other rhizophere fungi. This study also provides novel insights into fungal community variability both within and among alpine host species. While these results further our understanding of alpine fungal ecology, the functional consequences of the observed differences in fungal richness, diversity, and composition remain open for further investigation.

Acknowledgments A NSF doctoral dissertation improvement grant (DEB-0808000) and the University of Missouri funded this study. Lori Eggert at the University of Missouri and the Division of Biology and Ecological Genomics Institute at Kansas State University provided access to their facilities. Ernesto Almira at the Interdisciplinary Center for Biotechnology Research at the University of Florida provided the 454 pyrosequencing service. Gavin Conant assisted with post-sequencing analysis. Candace Galen, Lori Eggert, Marc Brock, Maarja Opik, and three anonymous reviewers commented on the manuscript.

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