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Monotropa uniflora plants of eastern Massachusetts form mycorrhizae with a diversity of russulacean fungi

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Abstract: Plant species in the subfamily Monotropoideae are mycoheterotrophs; they obtain fixed carbon from photosynthetic plants via a shared mycorrhizal network. Previous findings show mycoheterotrophic plants exhibit a high level of specificity to their mycorrhizal fungi. In this study we explore the association of mycorrhizal fungi and *Monotropa uniflora* (Monotropoideae: Ericaceae) in eastern North America. We collected *M. uniflora* roots and nearby basidiomycete sporocarps from four sites within a 100 km^2 area in eastern Massachusetts. We analyzed DNA sequences of the internal transcribed spacer region (ITS) from the fungal nuclear ribosomal gene to assess the genetic diversity of fungi associating with *M. uniflora* roots. In this analysis we included 20 ITS sequences from *Russula* sporocarps collected nearby, 44 sequences of *Russula* or *Lactarius* species from GenBank and 12 GenBank sequences of fungi isolated from *M. uniflora* roots in previous studies. We found that all 56 sampled *M. uniflora* mycorrhizal fungi were members of the Russulaceae, confirming previous research. The analysis showed that most of the diversity of mycorrhizal fungi spreads across the genus *Russula*. ITS sequences of the mycorrhizal fungi consisted of 20 different phylotypes: 18 of the genus *Russula* and two of *Lactarius*, based on GenBank searches. Of the sampled plants, 57% associated with only three of the 20 mycorrhizal fungi detected in roots, and of the 25 sporocarp phylotypes collected three, were associated with *M. uniflora*. Furthermore the results indicate that the number of different fungal phylotypes associating with *M. uniflora* of eastern North America is higher than that of western North America but patterns of fungal species abundance might be similar between mycorrhizae from the two locations.

Key words: epiparasite, internal transcribed spacer (ITS), monotropoid, mycoheterotroph, mycorrhiza, symbiosis

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INTRODUCTION

A mycoheterotroph (or epiparasite) is an achlorophyllous, nonphotosynthetic plant that obtains fixed carbon from photosynthetic plants via mycorrhizal fungi (Björkman 1960, Leake 1994). Björkman (1960) showed a connection between *Monotropa hypopithys* and trees when he injected radioactive glucose and phosphorus into the phloem of pine and spruce trees under which the mycoheterotroph grew. He found that the radioactivity passed from tree to *M. hypopithys* and physical separation from trees was severely detrimental to *M. hypopithys* growth. Björkman called this obligate interaction ''epiparasitism'' because the mycoheterotroph indirectly parasitizes trees. Bidartondo and Bruns (2001) suggest this indirect connection might make the mycoheterotroph successful because it can ''cheat'' the photosynthetic symbiont; the photosynthetic host cannot select against the mycoheterotroph without selecting against its own mutualist mycorrhizal fungi.

Among the mycoheterotrophs studied to date, all have specific associations with certain ectomycorrhizal (Cullings et al 1996, Bidartondo and Bruns 2001, Bidartondo and Bruns 2002) or arbuscular mycorrhizal fungi (Bidartondo et al 2002). Results of Bidartondo and Bruns (2002) indicate that evolution of members of the Monotropoideae is tightly coupled to that of their mycorrhizal symbionts.

Studies of *M. uniflora* from various locations in eastern and western North America, Eurasia and Japan have shown that it specifically associates with fungi of the family Russulaceae (Basidiomycota) (Cullings et al 1996, Bidartondo and Bruns 2001, Young et al 2002). Bidartondo and Bruns (2001) sampled a total of 35 *M. uniflora* plants from Nova Scotia, Oregon, Virginia, Vermont and Japan and identified *Russula brevipes, R. paludosa, R. cremoricolor, R. postiana, R. integra*, and *Lactarius theiogalus* as mycorrhizal associates. Young et al (2002) sampled a total of 15 *M. uniflora* plants from three sites in British Columbia and found three mycorrhizal fungi, two sequences of which clustered with the hypogeous genera *Martellia* and *Gymnomyces* (both Russulaceae) in phylogenetic analysis. These previous studies hinted at an intriguing geographic variation in the species diversity of mycorrhizal fungi associated with the mycoheterotroph; diversity was lower in western North American populations than those in eastern North America (Bidartondo and Bruns 2001, Bidartondo 2005, Bidartondo and Bruns 2005).

M. uniflora is distributed widely in New England (Seymour 1989), where few of the plants have been sampled for mycorrhizal associates. To further explore the identity and diversity of mycorrhizal fungi associated with *M. uniflora* in the plant's eastern North American range, we selected four sites in eastern Massachusetts, in the eastern mixed forest biome. We used sequencing, GenBank searches and phylogenetic analysis of sequences to study the association of eastern *M. uniflora* plants with members of the Russulaceae.

MATERIALS AND METHODS

Selection and description of field sites.—Four field sites were located in four towns in eastern Massachusetts. The Concord Field Station (Bedford) and Estabrook Woods (Concord) have a range of evergreen and deciduous tree genera, including *Pinus, Tsuga, Acer, Betula, Quercus* and *Fagus*. Whipple Hill (Lexington) is on a large rock outcropping with a mosaic of habitats, including grassland, wetlands, bare rock, low shrub stands and forest, including a *Carya* stand. Flint's Pond woods (Lincoln) surrounds the Lincoln reservoir and has large stands of *Betula* and *Fagus*. The four field sites occur within an area of approximately 10 km × 10 km. Within each site, the minimum and maximum distances between any two samples were approximately 1 m and 1 km, respectively.

Sampling M. uniflora *roots and fungal sporocarps.*—Throughout Jul and Aug 2003, we sampled the highly branched and tightly interwoven roots of *M. uniflora*. We sampled these root balls by locating a flowering *M. uniflora* plant and carefully following the shoot to the roots. We collected soil samples containing a portion of the tight *M. uniflora* root ball.

We stored the soil samples for a maximum of 2 d at 4 C until root harvesting. Root harvesting involved soaking the soil samples in tap water to loosen the soil around the roots, separating and cleaning the roots. We harvested at least four root tips from each *M. uniflora* root ball, storing them separately in 1.5 mL tubes at −80 C until extraction.

As *Russula* and other fungi fruited, we collected sporocarps within a radius approximately 5 m of sampled *M. uniflora* individuals to cursorily survey what fungi were available to *M. uniflora* plants in an area. Immediately upon return from a field site we made spore prints from the collected mushrooms and took notes on mushroom characters. We harvested 1–4 approximately 0.5 cm^3 pieces of sporocarp tissue from each mushroom and stored the pieces separately at −80 C for DNA extraction. We dried the rest of the specimens in a heat-desiccator at least 48 h. Specimens used for DNA sequencing are in the Farlow Herbarium (TABLE I).

Molecular methods and analyses.—We extracted DNA from two root tips per *M. uniflora* individual and from one sample of every sporocarp, following the protocol of Gardes and Bruns (1993), except without β-mercaptoethanol. We amplified the ITS region of root fungus and sporocarp DNA for sequencing. The ITS region, located in the ribosomal RNA gene, is good to use for fungal identification because it has sufficient variation to place unknowns at the species level or to species group (White et al 1990, Horton and Bruns 2001). We used the basidiomycete-specific primer pair ITS1F and ITS4B (Gardes and Bruns 1993) and amplified the ITS region in 25 μL reactions with the following final concentrations as recommended by the manufacturer of Taq polymerase (Invitrogen). We used a GeneAmp PCR System 9600 (Perkin Elmer) to run the program designed by Gardes and Bruns (1993).

We sequenced a total of 56 mycorrhizal fungi and 25 sporocarp samples. We used a QIAquick PCR Purification Kit (QIAGEN) to clean the ITS1F-ITS4B PCR products for cycle sequencing. Each cycle-sequencing reaction contained 1.5 μL cleaned PCR product, 2.0 μL BigDye v. 3 (Applied Biosystems), 1.6 μL 1 μM primer, 2.9 μL purified H_2O. We used a DNA Engine thermal cycler (MJ Research Inc.) for cycle-sequencing reactions with the program recommended by the BigDye manufacturer. We purified cycle-sequencing reactions by ethanol precipitation and performed electrophoresis in an ABI-Prism 3100 automated DNA sequencer (Applied Biosystems). If either or both of the external primers did not provide a good sequence, we used these internal primers: ITS3 (White et al 1990) and 5.8 S (http://www.biology.duke.edu/fungi/mycolab/primers.htm). We searched for any matching sequences from GenBank with the Basic Local Alignment Search Tool (BLAST).

Sequence and phylogenetic analysis. To view the placement of sample mycorrhizal fungus phylotypes within a larger phylogeny of *Russula* and *Lactarius* species, we aligned the 56 sample root isolate ITS sequences with 44 *Russula* and *Lactarius* GenBank ITS sequences. We also aligned 12 *M. uniflora* root fungal sequences from GenBank, generated by Bidartondo and Bruns (2001) and Young et al (2002) (TABLE I). We added to the alignment 20 ITS sequences of sample nearby *Russula* sporocarps to assess the range of fungi available to *M. uniflora* plants. We manually aligned the sequences with Sequence Alignment Editor v. 2.0 (Rambaut 1996). A single phylotype (labeled A-KK) represents identical sample sequences. We submitted the sample ITS sequences to GenBank (TABLE I) and the alignment to TreeBase (http://www.treebase.org, accession number S1646).

We performed phylogenetic analyses with PAUP*4.0b10 (Swofford 2002). We used *Gloeocystidiellum aculeatum* as outgroup because it is a member of the corticioid clade, which has been shown to form a sister group with members of the Russulaceae (Hibbett and Thorn 2001). The parsimony analysis was a heuristic search including all characters with 1000 replicates, starting trees obtained via stepwise addition, random sequence addition, tree-bisection-reconnection (TBR) branch-swapping, and an unlimited number of trees to be saved. We performed bootstrap analysis with 500 replicates with a heuristic search with 100 replicates, random sequence addition, MAXTREES set to 100 not to be increased, starting trees obtained via stepwise addition, and TBR branch-swapping.

TABLE I. GenBank accession numbers of sequences generated in this study (A–KK), and Farlow Herbarium (FH) collection numbers for sporocarps sequenced, followed by GenBank accession numbers of sequences from other studies included in our analysis

Phylotype/ Taxon	GenBank	FH
A	DQ777969	
B	DQ777970	
C	DQ777971	C.LN16.F1.SY
D	DQ778000	
E	DQ777999	
F	DQ777972	
G	DQ777973	
H	DQ777996	H.ES04.F1.SY
		H.ES18.F1.SY
		H.LN13.F1.SY
I	DQ777994	I.ES25.F1.SY
J	DQ777974	
K	DQ777975	K.LN04.07.F1.SY
L	DQ777976	
M	DQ777977	
N	DQ777989	N.ES26.F1.SY
O	DQ777983	O.LN14.F3.SY
P	DQ777984	
Q	DQ777995	Q.WH25.26.F1.SY
R	DQ777985	
S	DQ777978	
T	DQ778003	T.ES19.F1.SY
		T.ES19.F2.SY
		T.WH23.F1.SY
U	DQ777979	
V	DQ778004	V.WH18.F1.SY
		V.WH18.F2.SY
W	DQ777980	
X	DQ777986	
Y	DQ777988	Y.LN20.F1.SY
Z	DQ777992	Z.ES22.F1.SY
AA	DQ777987	AA.ES24.F1.SY
BB	DQ777993	BB.ES04.06.F1.SY
CC	DQ778002	CC.ES19.F4.SY
DD	DQ778005	DD.ESTA.F2.SY
EE	DQ887001	EE.LN09.F3.SY
FF	DQ777997	FF.LN14.F1.SY
GG	DQ777998	GG.LN18.F1.SY
HH	DQ777982	HH.WH19.F1.SY
II	DQ777981	II.LN19.F1.SY
JJ	DQ777990	
KK	DQ777991	
Gloeocystidiellum aculeatum	AY061739	
Lactarius quietus	AJ272247	
L. subsericatus	AF140255	
L. theiogalus	AF349716	
Russula adusta	AY061652	
R. amoenipes	AY061656	
R. aquosa	AY061657	
R. archaea	AY061737	
R. atropurpurea	AY061654	
R. aurata	AY061659	
R. betularum	AY061729	
R. brevipes	AF349714	
R. caerulea	AY061661	
R. camarophylla	AY061662	
R. chloroides	AY061663	
R. claroflava	AY061665	
R. cremoricolor	AJ277910	
R. cuprea	AY061667	
R. cyanoxantha	AY061669	
R. decolorans	AY061670	
R. delica	AY061671	
R. drimeia	AY061672	
R. emetica	AY061673	
R. heterophylla	AY061681	
R. integra	AF230896	
R. lepida	AY061686	
R. mustelina	AY061693	
R. nauseosa	AY061733	
R. nitida	AY061696	
R. ochroleuca	AY061697	
R. odorata	AY061698	
R. pallidospora	AY061701	
R. paludosa	AY061703	
R. parazurea	AY061704	
R. postiana	AF230898	
R. puellula	AY061710	
R. pulverulenta	AY061736	
R. raoultii	AY061712	
R. rubra	AY061717	
R. sphagnophila	AY061719	
R. velenovskyi	AY061721	
R. versicolor	AY061722	
R. vinosa	AY061724	
R. violacea	AY061725	
R. xerampelina	AY061734	
2344	AF349715	
2371	AF349709	
MG15	AF349708	
NC2172	AF349710	
NS2087	AF349712	
S123	AF311975	
S132	AF311976	
S144	AF311977	
S323	AF311978	
VT2364	AF349711	
VT2407	AF349717	
VT2408	AF349713	

RESULTS

We commonly found *Russula, Lactarius, Boletus, Amanita, Cortinarius*, and *Paxillus* sporocarps in the vicinity of the sampled *M. uniflora* individuals.

Sequencing mycorrhizal fungi obtained from roots of 56 *M. uniflora* individuals resulted in 20 different

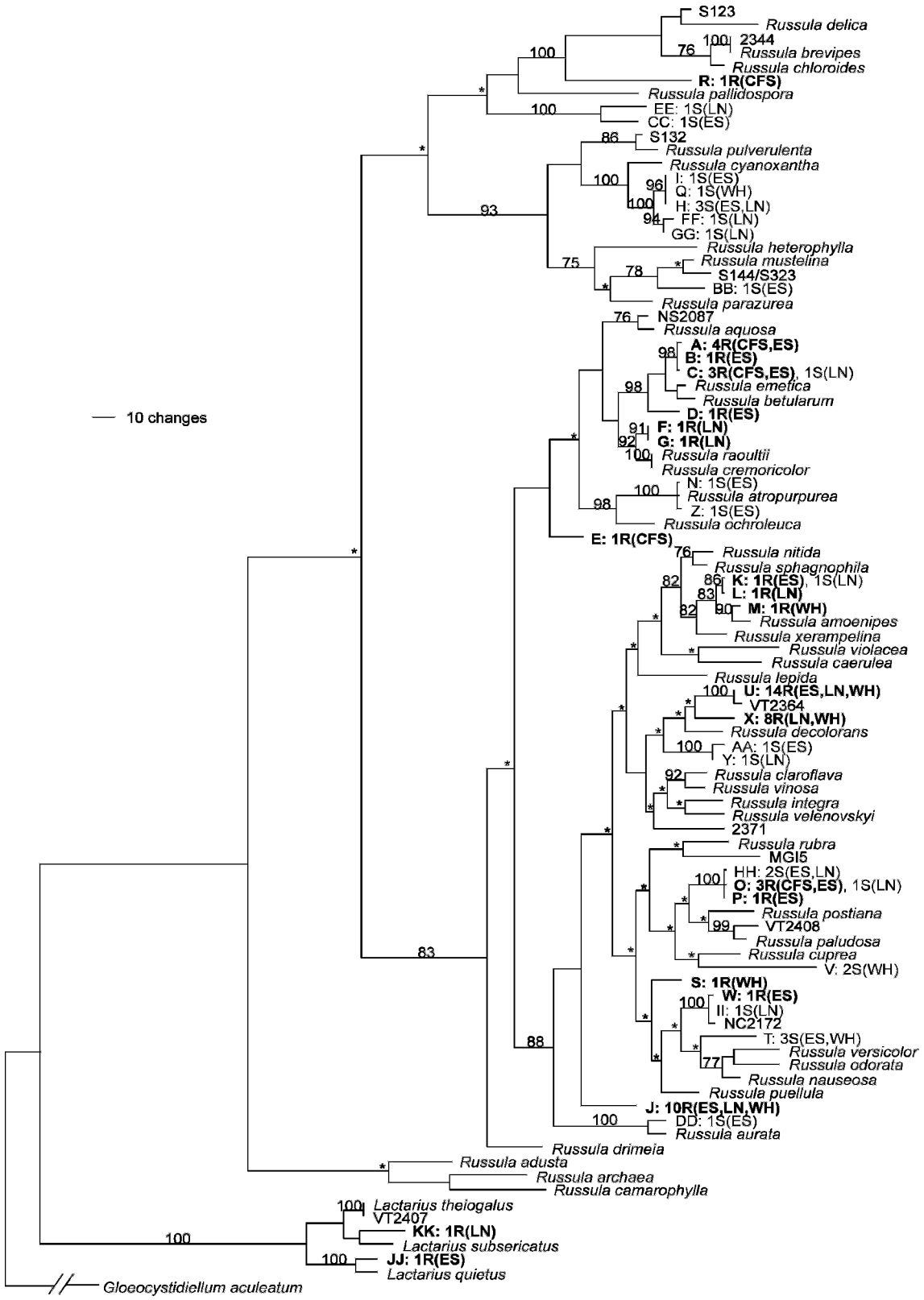

FIG. 1. This example maximum parsimony tree (of 5420 trees) shows the 37 sample fungal sequences from root tips and sporocarps (labeled A–KK) and 56 GenBank sequences. Of the 56 sampled root fungi, 20 different phylotypes were found. Boldface emphasizes root fungal sequences. Lightface denotes root fungal sequences from previous studies (i.e. S123, NC2172). Gray print denotes sporocarp sequences generated in this study and nonroot GenBank sequences. Numbers above

phylotypes, and sequencing 25 sporocarps resulted in 20 phylotypes. The sequences of mycorrhizal fungi and sporocarps together represent 37 phylotypes total (labeled A–KK, FIG. 1). Most frequent phylotypes among mycorrhizal fungus isolates were phylotypes U (25% of 56 mycorrhizal fungi isolated), J (18%), X (14%), A–C (14%), and O–P (7%). The rest (22%) of the mycorrhizal fungus samples represented unique phylotypes.

All phylotypes clustered with *Russula* species, except two, which grouped with *Lactarius* species (FIG. 1). GenBank searches of the root fungal sequences gave the same results as closest matches (54 *Russula*, two *Lactarius*), although none of the phylotypes matched 100% with any GenBank sequences. Only three of the 25 sequenced sporocarps had sequences that matched those of mycorrhizal fungi (phylotypes C, K and O) (FIG. 1). Where sporocarps did match mycorrhizal fungi, they were from different locations. Mycorrhizal fungi isolated from *M. uniflora* individuals near the sporocarps with phylotypes C, K and O formed mycorrhizae, not with these types but rather with other *Russula* species. For example for phylotype O mycorrhizal fungus sequences came from Estabrook and the Concord Field Station and the sporocarp with a matching sequence was from the Lincoln site (LN). The fungus from a *M. uniflora* near the sporocarp of phylotype O at LN was phylotype G.

Furthermore all phylotypes representing more than one root sample consisted of samples from 2–3 sites. For example phylotype U, which represented 14 root samples, included mycorrhizal fungus sequences from Estabrook, Lincoln and Whipple Hill (FIG. 1). Also, for all plants with two root tips sampled each (three plants), the fungal sequences from the same plant were identical.

Two fungi from roots grouped with root fungi from previous studies, phylotype U with VT2364 and phylotype W with NC2172, both with 100% bootstrap values (FIG. 1). No roots collected in this study had fungi that clustered with the *R. pulverulenta-R. parazurea* clade (93% boostrap), although two root fungal sequences, S132 and S144/S323, from British Columbia did and from this study six sporocarp phylotypes, I, Q, H, FF, GG, BB (FIG. 1), also grouped there.

DISCUSSION

In our investigation of *M. uniflora* mycorrhizal fungus diversity, all root fungi were members of the Russulaceae, despite the availability of other mycorrhizal fungi (FIG. 1), and each *M. uniflora* plant appears to be colonized by a single fungal phylotype, confirming other studies (Cullings et al 1996, Bidartondo and Bruns 2001, Young et al 2002, Bidartondo and Bruns 2005). The genus *Russula* includes more than 750 species (Kirk et al 2001), an exceptionally large number. To place our isolates we used the sequences used in a previous phylogenetic study of the genus, which broadly sampled classical taxonomic groups (Miller and Buyck 2002). The distribution of sample root fungi and GenBank root fungi in the *Russula* phylogeny shows that compatible fungi are spread widely across the entire genus, at least so far as it has been sampled currently.

Our results confirm findings that the number of mycorrhizal species from eastern North American *M. uniflora* populations is greater than that found from western populations (Bidartondo and Bruns 2001, Bidartondo 2005, Bidartondo and Bruns 2005). Similar to the 20 different phylotypes found in this study of eastern plants, Bidartondo and Bruns (2001) found four different root fungi from plants of a single population in Vermont. In contrast they found *Russula brevipes* to be the only mycorrhizal fungus with *M. uniflora* plants sampled in a 9400 km^2 area in Oregon and Young et al (2002) found only three different mycorrhizal fungi associating with 15 plants sampled from British Columbia. Furthermore we found the most frequently identified associates in multiple sites (FIG. 1), suggesting the genetic diversity of the fungi was not site-specific.

Despite the difference in overall mycorrhizal fungus species-number between eastern and western *M. uniflora* populations, similar species-abundance patterns are evident between western plants and the eastern plants sampled in this study. First, we found the number of root samples per phylotype was not distributed evenly; 57% of the sampled plants associated with only three of the 20 mycorrhizal fungi. Similarly, in western North America, Young et al (2002) found one of three mycorrhizal fungi dominated. Second, we found no species of the *R. pulverulenta-R. parazurea* clade, even though mem-

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branches indicate bootstrap values. An asterisk (*) indicates branches that are collapsed in the strict consensus tree. Our samples have been indicated as: number of sequences retrieved, source of the sample, roots ''R'' or sporocarps ''S,'' and the locations, Concord Field Station (CFS), Estabrook Woods (ES), Flint's Pond Woods (LN) and Whipple Hill (WH).)

bers of the clade formed mycorrhizae with western plants (S144/S323, S123), and members of this clade were present among sporocarps collected in the vicinity of *M. uniflora* plants (phylotypes I, Q, H, FF, GG) (FIG. 1). Third, no root fungi were identical to nearby sporocarps, even when a nearby fungus had been found to form mycorrhizae with *M. uniflora* roots in other locations (phylotypes C, K, O) (FIG. 1). We conclude that only a few fungal species dominated as *M. uniflora* mycorrhizae despite the availability of fungal species that are compatible with *M. uniflora* from other locations. This suggests that specificity might be more complex than random partnering with the available members of the Russulaceae.

Bidartondo and Bruns (2005) suggest a mechanism for the origin of specificity patterns in mycoheterotrophs. They showed in germination experiments that seedlings of *M. uniflora* and other plants from the Monotropoideae developed best when associated with the fungus species found with the maternal plant. They suggest that cues for *M. uniflora* germination are heritable and that fungal performance trade-offs might explain the narrow specificity of individual mycoheterotrophic plants to their mycorrhizal fungi.

Population studies of the genetic diversity of *M. uniflora* might help to explain if specificity to the Russulaceae arose differently in different lineages (Bidartondo and Bruns 2005). *M. uniflora* plants in North America appear to represent a group genetically distinct from those in Japan (Bidartondo and Bruns 2001, Neyland and Hennigan 2004), and *M. uniflora* populations exhibit extensive morphological variation (Wallace 1975). Further sampling of the genetic diversity of *M. uniflora* and its associated fungi across its range might add information to the genetic diversity of the partnership.

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