Mycorrhiza (2002) 12:75–82
DOI 10.1007/s00572-001-0153-6

ORIGINAL PAPER

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Monotropa uniflora: morphological and molecular assessment of mycorrhizae retrieved from sites in the Sub-Boreal Spruce biogeoclimatic zone in central British Columbia

Accepted: 16 November 2001 / Published online: 20 February 2002

Abstract Plant species in the subfamily Monotropoideae are achlorophyllous and have developed a complex mode of nutrition, receiving photosynthates from neighboring trees via shared fungi. To explore the mycorrhizal associations of *Monotropa uniflora* in central British Columbia (B.C.), plants were sampled from three sites: a *Betula*-dominated site and two sites with a mixture of conifer and hardwood trees. Fifteen *M. uniflora* root-clusters were sampled (five per site) and the mycorrhizal diversity was assessed using morphological and molecular (PCR-RFLP analysis and DNA sequencing) methods. Both methods showed that root-clusters (often comprising several hundred mycorrhizal tips) belonging to the same plant appeared to involve fungus monocultures in the family Russulaceae. All mycorrhizae exhibited typical *Russula* morphology and had mantle cystidia. Two root-clusters, one each from sites 1 and 3, lacked one of the two types of cystidia present on all other root-clusters. PCR-RFLP analysis resulted in three fragment patterns for the 15 root clusters. One molecular fragment pattern included the two root-clusters displaying the single cystidium type plus an additional root-cluster with both cystidia types. DNA sequencing of a portion of the ITS2 region of the ribosomal DNA suggests that the three variants represent different species; two of the variants clustered with the hypogeous fungi *Martellia* and *Gymnomyces*. The study provides increased evidence of low diversity and high specificity in the *Monotropa*-fungus relationship and suggests that *M. uniflora* associates uniquely with fungi in the family Russulaceae in central B.C.

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Keywords Indian-Pipe · Monotropoid · Specificity · PCR-RFLP · DNA sequencing

Introduction

Achlorophyllous angiosperms that obtain nutrients and organic carbon by penetrating the roots of other plants through the development of haustoria are regarded as parasites (Kamienski 1882; Furman and Trappe 1971; Leake 1994). However, among the vascular plants, more than 400 species (in 87 genera) are achlorophyllous and heterotrophic, but do not directly parasitize autotrophic species (Leake 1994). These plants are mycoheterotrophic, depending on carbon compounds obtained via fungus linkages to autotrophic host plants (Furman and Trappe 1971; Molina et al. 1992; Leake 1994). The achlorophyllous mycoheterotrophic condition appears to have evolved independently numerous times within widely disparate taxonomic groups. Nevertheless, these plants exhibit strong convergent evolution with respect to adaptations to a unique ecological niche, often comprising dense moist forests with a surface accumulation of organic litter and limited light for autotrophic growth (Leake 1994). Biological and anatomical attributes of mycoheterotrophic plants, including *Monotropa*, have been described by Leake (1994) and Harley (1969).

Monotropa uniflora L. is classified in a subfamily of the Ericaceae, the Monotropoideae, which consists of 10 genera, comprising 12 species, all of which are achlorophyllous and mycoheterotrophic (Wallace 1975). Placement of the Monotropoideae within the Ericaceae has been debated for many years (Wallace 1975); however, recent molecular studies of partial 28S ribosomal RNA gene sequences support this classification (Cullings and Bruns 1992).

Monotropoid mycorrhizae resemble angiosperm ectomycorrhizae by possessing a thick fungus sheath and a Hartig net that is restricted to the epidermal layer (Duddridge 1985; Molina et al. 1992). They differ from ectomycorrhizae by the development of characteristic

fungus pegs in the epidermal cells (Lutz and Sjolund 1973; Duddridge and Read 1982; Robertson and Robertson 1982). Based on this distinct morphology, these mycorrhizae have been placed in their own class of 'monotropoid' mycorrhizae (Duddridge and Read 1982).

The non-photosynthetic achlorophyllous condition of *Monotropa* has led to numerous investigations that ultimately revealed the fungus associations of *Monotropa* (Kamienski 1882; Trappe and Berch 1985). Elias Fries is generally credited as the first to recognize the fungal nature of the ensheathing outer root layer (Trappe and Berch 1985). Almost 40 years later, Kamienski (1882) produced an elegant paper critically evaluating *Monotropa* mycorrhizae and describing in detail the fungus sheath of *M. hypopitys* L. roots. He suggested that nutrients must pass via the mycelium to the host root cells and that the mycorrhizal fungus could be attached via mycelial bridges to autotrophic trees.

The identity of fungus symbionts, host specificity and habitat requirements for many of the Monotropoideae remain largely unknown (Leake 1994); however, some members appear highly specific in their fungus associations, including two *Monotropa* spp. which may be specialists: *M. hypopitys* associates with suilloid fungi and *M. uniflora* associates with fungi in the Russulaceae (Cullings et al. 1996). Fungus symbionts described in the literature for *M. uniflora* include *Boletus* spp. (Riley 1971), *Armillaria mellea* (Vahl:Fr.) Kumm. (Campbell 1971), the order Helotiales (Ascomycotina) (Riley and Eichenmuller 1970), *Pezizella* spp. (Riley 1971; Riley et al. 1977), *Hymenoscyphus monotropae* Kernan & Finocchio (Helotiales) (Kernan and Finocchio 1983) and the Russulaceae (Martin 1986; Cullings et al. 1993, 1996). Suspected fungus associates for other species within the Monotropoideae include *Elaphomyces* spp. (Reess 1885; Castellano and Trappe 1985), *Boletus* spp. (Francke 1934; Khan 1972), *Tricholoma* spp. (Martin 1986), *Truncocolumella citrina* (Zeller) Singer & Smith (Castellano and Trappe 1985), *Rhizopogon vinicolor* Smith, *Cenococcum geophilum* Fr., species in the Cantharellaceae, and suilloid taxa (*Rhizopogon* and *Suillus* spp.) (Cullings et al. 1993, 1996). Mycorrhizal tree hosts potentially forming linkages with *M. uniflora* and completing the tripartite relationship include the following genera and species: *Pinus* and *Abies* (Björkman 1960), *Tsuga*, *Quercus* and *Acer* (Furman 1966), *Carya*, *Sassafras*, *Tsuga*, *Quercus* and *Betula* (Riley 1971; Riley et al. 1977), *Tsuga*, *Quercus*, *Pinus*, *Fagus* and *Acer* (Campbell 1971), *Acer* (Kernan and Finocchio 1983), *Pseudotsuga menziesii* (Mirb.) Franco, *Calocedrus decurrens*, and *Pinus* spp. (Luoma 1987).

To explore further the identity and host specificity of fungus associates of *M. uniflora* in its northern range, sites were selected near Prince George in the Sub-Boreal Spruce (SBS) biogeoclimatic zone in the central interior of British Columbia, Canada. The diversity of *M. uniflora* mycorrhizae from these sites is described using a combination of morphology (light microscopy) and molecular (PCR-RFLP analysis and DNA sequencing) techniques.

Materials and methods

Site locations and sampling procedures

Three sites (approximately 54° 07′ N latitude and 122° 04′ longitude) containing numerous *M. uniflora* plants were located in the SBS biogeoclimatic zone (Meidinger et al. 1991) in central British Columbia. Two sites were in forested areas within the city limits of Prince George: one was dominated by mature *Betula papyrifera* Marsh., the other by a mixture of conifer and hardwood species. A third site, another mixed conifer/hardwood stand, was located approximately 15 km west of Prince George in a forested area in the town of Miworth. Plants were collected at weekly intervals through the month of July and the first week of August 1997, resulting in a total of 15 root clusters, five from each of the three sites. *Monotropa* flowering stems emerged in early July and this was expected to coincide with mycorrhiza development. Root-clusters were harvested with the surrounding soil and stored at 5°C for up to 1 week until examined. Potential mycorrhizal host trees were identified on each site and fungus sporocarps in the genus *Russula* were sampled on the sites at the same time that root-clusters were harvested.

Mycorrhiza and sporocarp characterization

Immediately prior to mycorrhiza assessment, roots were soaked in cold water to facilitate the removal of soil and organic debris. Initial morphological characterization of mycorrhizae was made using a dissecting microscope. Each large root-cluster (Figs. 1, 2) was divided into smaller sections (4–8) for ease of examination. A minimum of 10 tips per section were examined and described according to the protocols of Ingleby et al. (1990), Agerer (1987–1998), Goodman et al. (1996) and Massicotte et al. (1999). Squash mounts and hand sections (mounted in water or 5% KOH) were examined to determine microscopic features (100–1000× Olympus CH-2 compound microscope). Ten turgid root tips that appeared to have intact meristems were harvested from each root-cluster and frozen at –20°C for subsequent molecular analysis.

Sporocarps were characterized to genus and fresh morphological characters recorded. Approximately 1 ml of fungus tissue was sampled from selected sporocarps and frozen at –20°C for subsequent molecular analysis; dried sporocarp samples were kept as potential voucher specimens in case a match was found between sporocarp and root samples.

PCR-RFLP molecular analysis

DNA was extracted from mycorrhizae and sporocarps using a CTAB protocol (Baldwin and Egger 1996). PCR was used to am-

Figs. 1–6 *Monotropa uniflora* roots colonized by mycorrhizal ▶ fungi

Fig. 1 Typical *Monotropa* cluster of hundreds of mycorrhizal root tips (*arrowheads*) from which several achlorophyllous stems (*) are emerging

Fig. 2 Smaller *M. uniflora* cluster (*) with two emerging stems. Passing through the cluster is a portion of a pine root system (*arrowheads*); *bar* mm

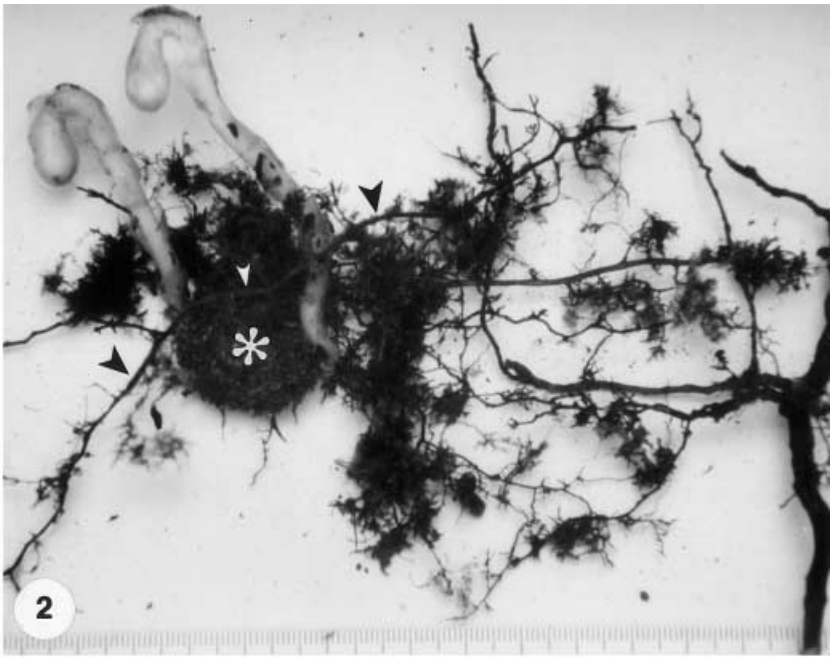
Fig. 3 Mycorrhizal tip showing bristle morphology of the outer mantle due to abundant cystidia (*arrowheads*)

Fig. 4 Surface view of mycorrhizal mantle showing elongated (*arrowheads*) and fusiform/flask-shaped (*arrows*) cystidia

Fig. 5 Light micrograph showing fusiform/flask-shaped cystidia (*) with small apical knobs (*arrowheads*). The basal septate portion of an elongated cystidium (*arrow*) is also present

Fig. 6 Light micrograph showing the elongated or awl-shaped cystidia with abundant surface crystals (*arrowheads*)


1
10 mm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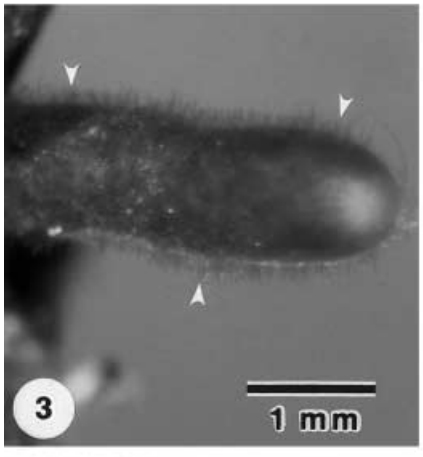
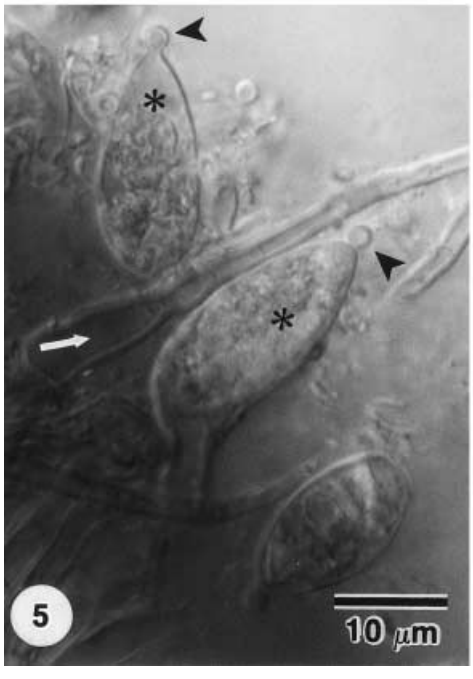
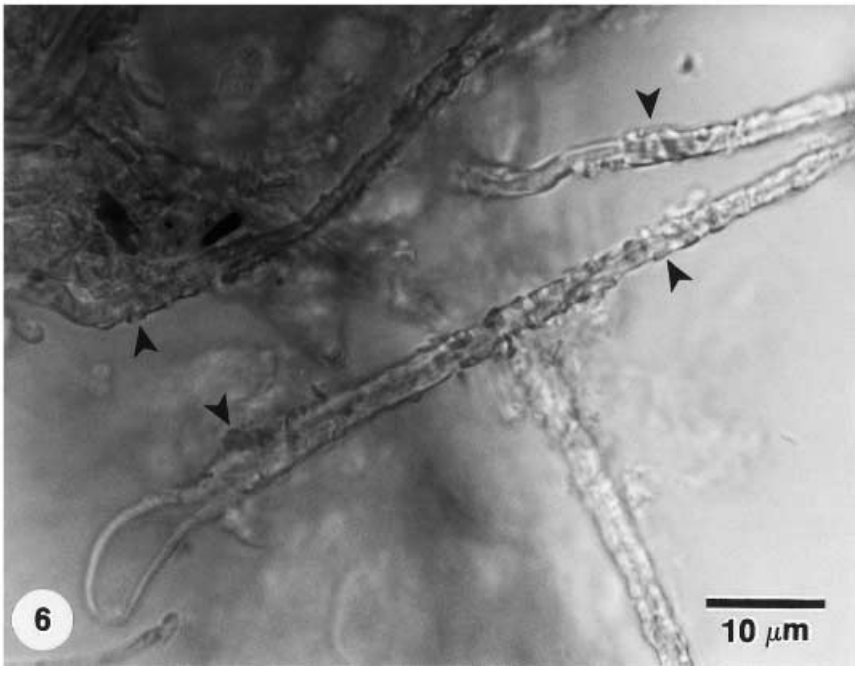

3
1 mm


4
20 µm


5
10 µm


6
10 µm

plify an approximate 1,100-bp fragment of the nuclear-encoded ribosomal RNA (rDNA) gene region. The targeted region contains the 3′-end of the 18S rRNA gene, the internal transcribed spacers (ITS1 and ITS2), the 5.8S rRNA gene, and approximately 350 bp at the 5′-end of the 28S rRNA gene. This region exhibits sufficient variability to enable detection of species-specific RFLP patterns with a minimal number of enzymes (Egger 1995). The fungus-specific primer used to amplify the ITS region was NL6Bmun (CAAGCGTTTCCCTTTCAACA) (Egger 1995), in conjunction with the universal primer ITS1 (TCCGTAGGTGAACCTCGCG) (White et al. 1990).

PCR reactions were run in 30-µl volumes containing 1–2 units of DNA polymerase (Ultra Therm, BioCan Scientific), one-tenth volume of 10× reaction buffer (provided by the manufacturer), 2 mM $MgCl_2$, 50 mM each of dATP, dCTP, dTTP's and dGTP (Pharmacia Biotech), and 0.8 mM of each oligonucleotide primer. A drop of sterile mineral oil overlaid the PCR reaction to prevent evaporation. PCR was carried out on a Perkin-Elmer model 480 thermocycler for 35 cycles of denaturation at 94°C for 45 s, annealing at 48°C for 45 s, and extension at 72°C for 130 s. The cycle included a 55 s ramp time between annealing and extension, and the extension time was increased by 1 s each cycle. To ensure effective PCR conditions, samples were heated to 94°C for 1 min before the first cycle, and a 5 min final extension at 72°C was added at the end of the last cycle.

The resulting PCR product was assayed by RFLP analysis. Three restriction endonucleases were used: *Alu*I (AGCT), *Hin*fI (GANTC), and *Rsa*I (GTAC) (Pharmacia Biotech). These enzymes cleave frequently and contain no overlap in their recognition sequences, thus all detected mutations are independent (Egger 1995). Digestion products were subjected to electrophoresis at 90 V for approximately 4 h in a 2.5% agarose gel composed of 1% regular agarose and 1.5% low-melting point (NuSieve, FMC Bioproducts) agarose containing 0.73 µg/ml of ethidium bromide.

RFLP fragment patterns were photographed and digitized using the Biophotonics 2000I imaging system (BioCan Scientific). Patterns were subsequently analyzed using the program RFLPscan Plus, Version 3.0, Scanalytics) using 1 kb ladder (Life Technologies) as a molecular standard. Two tolerance levels were used to determine the percent similarity between fragments from different samples. A tolerance level of 2% was set for comparison of fragments within each gel; a tolerance level of 6% was used to compare samples between gels to compensate for changes in gel density and fluctuations in buffer concentrations which might alter DNA migration.

DNA sequencing

To determine phylogenetic affinities of the fungus symbionts, four mycorrhizal samples were sequenced in both forward and reverse directions. The samples comprised the three RFLP variants: S123 and S132 represented two of the variants, S144 and S323 represented the third variant but were obtained from sites separated by a distance of 15 km. A 250-bp fragment spanning the ITS2 region of the ribosomal RNA gene repeat was sequenced using the primers ITS3 (GCATCGATGAAGAACGCAGC) (White et al. 1990) and ITS8mun (CTTCACTCGCCGTTACTA) (Egger 1995). PCR reactions were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR product was quantified using a GeneQuant II RNA/DNA Calculator (Pharmacia Biotech) before sequencing using a Thermo Sequenase Cycle Sequencing Core Kit (Amersham Life Science).

The sequencing master mix contained 3 units of Thermo Sequenase, 0.34 pmol/µl of Cy5.5 labeled oligonucleotide primer (ITS3 or ITS8), one-tenth volume of 10× Sequencing Buffer (Visible Genetics Inc.), 15% dimethylsulfoxide, and 700 ng of PCR template, with the volume adjusted to 22 µl with sterile distilled water. Aliquots (5 µl) of master mix were added to four tubes containing 3.0 µl of either A-, G-, T-, or C- Termination Mix (750 µM each dNTP and 2.5 µm ddNTP in 0.1 mM, pH 8. PCR reactions were carried out on a PTC 100 thermocycler (MJ Research, Inc.) for 35 cycles of denaturation at 94°C for 40 s, annealing at 52°C for 20 s, and extension at 72°C for 1 min. The cycle included a 55 s ramp time between annealing and extension. To ensure effective PCR conditions, samples were heated to 94°C for 2 min before the first cycle, and a 2 min final extension at 72°C was added to the last cycle. After PCR, approximately 6 µl of Stop Loading Dye (Amersham Life Science) was added to each reaction tube. Tubes were stored at 4°C until used.

The OpenGene automated DNA system (Visible Genetics Inc.) was used for sequencing. Samples were heated to 75°C for 2 min, then placed on ice, prior to loading onto a 6% polyacrylamide gel. Approximately 2 µl of sample/stop dye solution was loaded into each well. The sequencing gel was maintained at 50°C with an approximate voltage of 1,400 mV for 40 min. The OpenGene software (Visible Genetics Inc.) was used to edit sequences and to correct errors in base calling. Sequences were imported into MacVector version 6.5 (Oxford Molecular) and aligned using the default parameters of the Clustal algorithm (Higgins et al. 1992) as implemented in MacVector. An unambiguous portion of the alignment was used to calculate differences between sequences.

To infer phylogenetic affinities of the mycorrhizal samples, they were aligned with ITS sequences of Russulaceae species obtained from GenBank, then subjected to phylogenetic analysis using PAUP. Samples included in the analysis were: *Gymnomyces ammophilus* Vidal & Calonge (AF230890), *Lactarius acerrimus* Britz. (AJ278139), *L. controversus* (Fr.) Fr. (AJ272245), *L. deliciosus* (L: Fr.) Gray (U80999), *L. insulsus* Fr. (AJ272243), *L. quietus* (Fr.) Fr. (AJ272247), *Martellia pila* (Pat.) Vidal (AF230893), *Russula foetens* (Pers.: Fr.) Fr. (AF230895), *R. fragilis* (Pers.: Fr.) Fr. (AF230897), *R. integra* (L.) Fr. (AF230896), *R. mairei* Peck (AF230899), *R. postiana* Romell (AF230898), *Zelleromyces giennensis* Calonge, Moreno-Arroyo & Gomez (AF230900), and *Z. hispanicus* Calonge & Pegler (AF231913). *Amanita tenuifolia* Murrill (AF085492) and *Amylostereum laevigatum* (Fr.) Boidin (AF218396) were included as outgroup taxa.

The data matrix was analyzed using the maximum-likelihood procedure in PAUP* 4.0b4a (PPC) (Swofford 1999). A maximum-likelihood tree was constructed using the heuristic search algorithm with 10 cycles of random addition and the transition/transversion ratio estimated by optimizing the maximum likelihood parameter. Empirical base frequencies were used, with the Hasegawa-Kishino-Yano (HKY) two parameter model for unequal base frequencies. An equal rate of substitution was assumed for all sites. Starting branch lengths were obtained using the Rogers-Swofford method and the starting trees for branch swapping were obtained by stepwise addition. The tree bisection-reconnection (TBR) branch swapping algorithm was used, with the "Multrees" option in effect. Branch support was determined by bootstrap analysis (Felsenstein 1985) calculated using 500 repetitions. Bootstrapping was performed using the neighbor-joining algorithm as implemented in PAUP. Distance values were estimated by maximum-likelihood with parameters as indicated above, except that the starting trees were obtained by neighbor-joining.

Results

Morphological characterization of *M. uniflora* mycorrhizae

Microscopic examination revealed that all root tips within root-clusters were colonized by mycorrhizal fungi and all mycorrhizae in each cluster appeared to be a monoculture derived from one fungus species. Little morphotype variation existed between mycorrhizae from different root-clusters. All mycorrhizae exhibited a monopodial pyramidal branching pattern. Most were straight, up to 4.2 mm in length, and 0.6–1.4 mm in width. The mantle (20–35 µm thick) had a short spiny

Table 1 Approximate restriction fragment band sizes of the amplified fungal rDNA ITS region from *Monotropa uniflora* mycorrhizae after digestion with the restriction enzymes *Alu*I, *Hin*fI and *Rsa*I. The primers used were ITS1 and NL6Bmun. All mycorrhizae were described morphologically as belonging to the family Russulaceae

RFLP type	Approximate fragment sizes (bp)			Site:Plant	Site:No. of tips amplified (*n*=75)
	*Alu*I	*Hin*fI	*Rsa*I		
A	470 233 190 134	314 259 170 116 110	477 415	1:1, 1:4, 1:5; 2:1, 2:2, 2:3, 2:4, 2:5; 3:2, 3:3, 3:4	1:15; 2:25; 3:15
B	466 188 159 141 128	380 315 160 147	834 131	1:2; 3:1, 3:4	1:5; 3:10
C	275 254 188 131	365 325 165 153	991	1:3	1:5

texture with a matte lustre (Fig. 3). The outer mantle had a net prosenchyma arrangement progressing to a net synenchyma inner mantle. Young tips were a light tawny apricot color becoming darker with age; many root apices had a creamy white translucent color with a faint tinge of purple. No rhizomorphs or emanating hyphae were observed, but there were two distinct forms of cystidia (Figs. 4, 5, 6). Type 1 cystidia were fusiform to flask-shaped and often possessed one or two small apical knobs (Figs. 4, 5). They ranged from 20–40 µm in length and 6–10 µm in width, and had a basal septum (width 2–4 µm). Type 1 cystidia did not appear to be ornamented but often contained cytoplasmic deposits. These cystidia were found on mycorrhizae from all root-clusters from all three sites. Type 2 cystidia were bristle-like or awl-shaped with abundant crystal-like ornamentation (Figs. 4, 5, 6). These cystidia ranged from 50–140 µm in length and from 2–4 µm in width, often tapering to approximately 1 µm at the tip. They appeared to have thicker cell walls than type 1 cystidia, numerous septa, and many had an enlarged basal cell (Figs. 5, 6). No clamps were observed on either cystidium type. Type 2 cystidia were found on mycorrhizae from all root-clusters except one root-cluster each from sites 1 and 3. In five of the root-clusters, poorly developed *Cenococcum geophilum* was observed colonizing one or two root tips near the root apices. No other morphotypes were observed on these root systems.

Site-associated sporocarps and potential host trees

Sporocarps from several *Russula* species were commonly retrieved on the sites. Two species were given tentative identities (*R. cf. emetica* and *R. cf. rosacea*) but other *Russula* sporocarps could not be identified with confidence. All sites contained a variety of mycorrhizal host trees. Sites 1 and 3 contained the greatest diversity of trees with seven species common to both sites: *Populus tremuloides* Michx., *Betula papyrifera*, *Abies lasiocarpa* (Hook.) Nutt., *Pinus contorta var. latifolia* Engelm., *Alnus viridis* (Chaix) DC., *Picea engelmannii* Parry ex Engelm. × *glauca* (Moench) Voss and *Salix* sp. Site 2 was dominated by mature *B. papyrifera* and had only a minor component of *P. tremuloides* and *A. lasiocarpa*.

PCR-RFLP analysis and DNA sequencing

Fragment patterns from PCR-RFLP analysis indicated that each root-cluster was a monoculture formed by one mycorrhizal fungus. In total, three distinct fragment patterns were identified from all root-clusters from the three sites (Table 1). One fragment pattern appeared to dominate the mycorrhizal associations of *M. uniflora*. It represented 11 of the 15 root-clusters: three of five root-clusters on site 1, all root-clusters on site 2, and three of five clusters on site 3 (Table 1). A second fragment pattern was identified for one root-cluster from site 1 and two clusters from site 3. The third pattern was only identified from one cluster from site 1. This resulted in three different fragment patterns on site 1, one on site 2, and two on site 3 (Table 1). No exact matches were found between fragment patterns of sporocarps and mycorrhizae and, therefore, no sporocarps were deposited as voucher specimens.

DNA sequencing resulted in an unambiguous alignment for 246 bp. Four sequences were obtained for the three different fragment patterns; these have been deposited in Genbank under the accession numbers: S123 (AF311975), S132 (AF311976), S144 (AF311977) and S323 (AF311978). An analysis of base substitutions and gaps for the four sequenced samples indicated that the two samples (S144 and S323) which represented the same fragment pattern from two separate sites were identical. These two differed from S123 at 57 nucleotide positions (23.2%) and from S132 at 52 positions (21.1%). S123 and S132 differed from each other at 59 positions (24.0%).

Phylogenetic analysis of the four samples indicated that they all nested within the Russulaceae (Fig. 7). Three of the sequenced mycorrhizae (S132, S144 and S323) clustered with the hypogeous fungi *Gymnomyces* and *Martellia*, with the sequence for S132 being very close to that of *Martellia pila*. S144/S323 represented 11 of the plants sampled while S132 represented only one plant. S123 (representing the remaining three plants) also nested within the Russulaceae but occupied an ambiguous position (Fig. 7). Bootstrap support for the branches was generally low, reflecting the highly variable nature of the ITS region, although the grouping of S132 with *M. pila* was strongly supported at 93%.

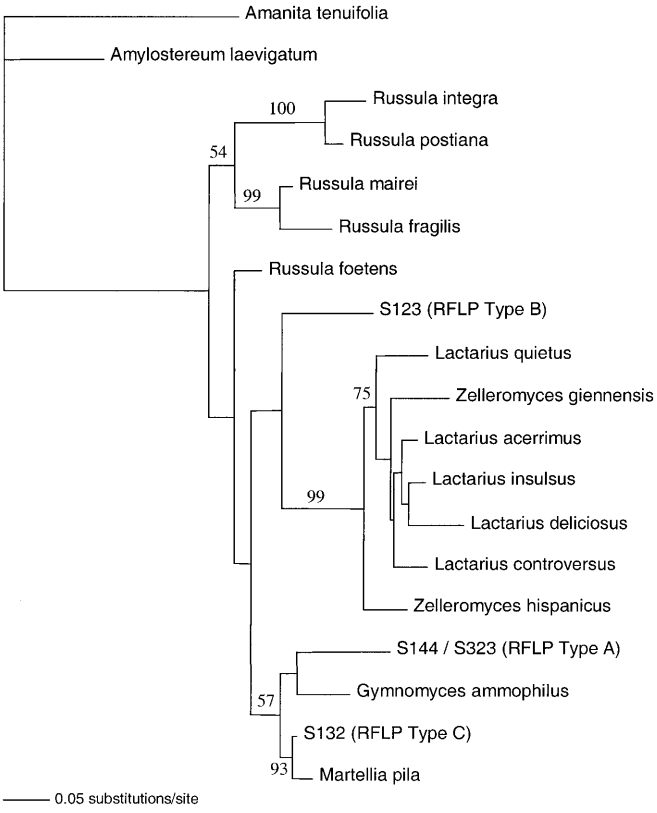


Fig. 7 Maximum-likelihood tree constructed using the heuristic search algorithm in PAUP*. Numbers adjacent to branches indicate bootstrap support (branches without numbers have bootstrap support lower than 50%). RFLP types A, B and C correspond to Table 1

Discussion

Based on both morphological and molecular assessments, the fungus symbionts identified in all root-clusters from all sites belonged in the family Russulaceae. Morphotype features are similar to published descriptions for *Russula* mycorrhizae by Martin (1986), Agerer (1987–1998), Taylor and Alexander (1992) and Kernaghan et al. (1997). Martin (1986) described at least eight symbiotic associations of *M. uniflora*, some of which appeared to belong in the Russulaceae and that exhibited characteristic cystidia. In an ultrastructure study of *M. uniflora*, Lutz and Sjolund (1973) described cystidia similar to our type 1 *Russula*-like cystidia but mention these structures only briefly as hyphal projections. Snetselaar and Whitney (1990), investigating crystal formation by *M. uniflora* mycorrhizae, reported two types of cystidia similar to those described in the present study.

Numerous *Russula* species have been reported to be common ectomycorrhizal symbionts of both conifer and hardwood species (Trappe 1962; Bills et al. 1986; Molina et al. 1992). Taylor and Alexander (1992) described mycorrhizae synthesized between *R. aeruginea* Lindblad:Fr. and *Picea sitchensis* (Bong.) Carr. Kernaghan et al. (1997) described Russulaceae mycorrhizae of *A. lasiocarpa* and *P. engelmannii* Parry ex Engelm. and documented the morphology of *R. brevipes* Pk. mycorrhizae showing cystidia similar to type 1 cystidia in our study. Harley and Smith (1983) noted that mycorrhizae synthesized with various species of *Russula* also developed these characteristic cystidia. In fact, the presence of cystidia on the surface of mycorrhizae has been suggested to be a distinctive feature that may facilitate placement of these morphotypes within the genus *Russula* (Godbout and Fortin 1985). However, other descriptions of *Russula* mycorrhizae suggest variations in mantle features. Taylor and Alexander (1992) synthesized *R. aeruginea–P. sitchensis* mycorrhizae in vitro and reported typical cystidium development after 20 weeks. In contrast, Giltrap (1979) combined the same fungus with *Betula* spp. and found the mycorrhizae lacked cystidia. Taylor and Alexander (1992) suggested that the lack of cystidia reflects an immature stage in development, but went on to emphasize that cystidia have not always been documented for some *Russula* spp. and that other fungus species can produce cystidia. A developmental difference could explain the lack of type 2 cystidia on two root-clusters in our study, but this is not certain.

Cullings et al. (1996) examined the fungus symbionts of several geographically dispersed mycorrhizae of *M. uniflora* at sites that varied from mixed conifer/hardwood forests to sand dune ecosystems. They determined that mycorrhizal fungi grouped in tight terminal clusters with several members in the Russulaceae, specifically, *Lactarius piperatus* (Scop.) Fr., *R. rosacea* (Pers.) Fr. and *R. laurocerasi* Melzer. Our findings support their observation that members of the Russulaceae are major fungus symbionts of *M. uniflora*, and further suggest that mycorrhizae in individual root-clusters are formed by only one fungus species. Although epigeous sporocarps belonging to several *Russula* spp. were collected from our sites, no exact matches were found between fragment patterns of sporocarps and mycorrhizae. DNA sequencing revealed that S132 clustered close to *M. pila* in the phylogenetic analysis and that S144/S323 clustered with a member of the genus *Gymnomyces*, suggesting that they may be more closely related to hypogeous members of the Russulaceae. *Martellia* and *Gymnomyces* sporocarps have been described from conifer and deciduous forests in western North America (Smith et al. 1981; Arora 1986; Miller 1988; Castellano et al. 1989). A paper by Trappe and Castellano (1986) describes the ectomycorrhizae formed in vitro between *Martellia medlockii* and lodgepole pine as well as a weak association between *M. medlockii* and western hemlock. Pine mycorrhizae were described as having a finely pubescent surface with abundant erect hyphae that gave rise to hyphal strands. The authors did not mention cystidia and photo documentation was inconclusive. Hyphal strands were not seen in our study samples. To our knowledge, no other mycorrhizal descriptions exist in the literature for either of these two fungus genera.

The restricted fungus diversity and high degree of specialization of *M. uniflora* found in our study is in

contrast to the high degree of diversity exhibited by most ectomycorrhizal hosts (Molina et al. 1992; Cullings et al. 1996). Achlorophyllous plants depend on mycorrhizal associations for photosynthate acquisition and therefore might benefit by being broadly receptive to many fungi (Molina et al. 1992). Why some members of the Monotropoideae have apparently become highly specialized in their fungus-host relationships raises interesting questions. The *Monotropa*-fungus relationship must have at least two components. The fungus must provide a good supply of fixed carbon and the plant must be able to access this resource. D.P. Janos (personal communication) suggests that if an achlorophyllous plant species can increase its acquisition of photosynthate by specializing with one or a few fungus species, then selection should favor specialization. Through a process of exclusion by the plant of less "generous" fungi, colonization by the "more generous" fungus might be maximized. A high degree of fungus-host specificity with respect to autotrophs might also increase the fitness of the mycobiont if specialization improves resource acquisition. For this to occur, root colonization by the fungus must increase sufficiently to compensate for decreased association with other hosts (Janos 1985). It is possible that a fungus gains its carbon by being a specialist on an abundant autotrophic host, or by being a generalist on numerous less abundant host species. This may be independent of whether or not the fungus is mycorrhizal with an achlorophyllous plant (D.P. Janos, personal communication). Cullings et al. (1996) suggest that suilloid fungi, many of which are specialized on the Pinaceae, have perhaps become very efficient in obtaining photosynthate from their autotrophic hosts. In turn, some members of the Monotropoideae (e.g. *M. hypopitys*, *Pterospora andromedea*) may have increased their fitness advantage by utilizing the suilloid fungi as an efficient source of fixed carbon. Whether this hypothesis can be applied to fungi in the Russulaceae that appear to be associating with *M. uniflora*, is uncertain. Many species within the Russulaceae, in contrast to the suilloid fungi, are generally believed to be broadly mycorrhizal on hosts that include both conifer and deciduous species (Arora 1986; Molina et al. 1992; Taylor and Alezander 1992). However, other members, including *Russula* and *Lactarius* spp. as well as several hypogeous fungi belonging to the genera *Martellia* and *Zelleromyces*, are believed to have a narrow to intermediate host range (Molina et al. 1992). Although many possible fungus symbionts must have existed on our sites, DNA sequencing identified only three fungus species in total for *M. uniflora*. The two fungus symbionts that shared the same PCR-RFLP fragment pattern were conspecific, having identical DNA sequences through a 246-bp region of ITS2. The level of variation between the three distinct fragment patterns (21–24%) was in a range that would be expected for highly divergent species or congeneric taxa. This was supported by the phylogenetic analysis which nested the mycorrhiza samples in divergent clades within the Russulaceae.

In summary, the mycoheterotrophic plant *M. uniflora*, growing on sites in the Sub-Boreal Spruce biogeoclimatic zone in central British Columbia, was highly selective in its association with symbiotic fungi, whether in mixed conifer/hardwood or *Betula*-dominated forests. Each individual root-cluster appeared to consist of mycorrhizae formed by a single fungus species in the family Russulaceae. Morphologically, differences between fungus symbionts were limited to the presence of either one or two cystidial types. PCR-RFLP analysis and DNA sequencing suggested that three species in the Russulaceae (one possibly being *Martellia pila* or a closely related species) were involved in these symbioses. One species representing 11 of 15 plants dominated all three sites. Both morphology and molecular assessments suggest low diversity and high specificity in the *Monotropa*-fungus associations in this region. With respect to ecosystem function, a high level of specialization between symbionts may mean that these relationships, while potentially being efficient conduits between partners, may also be more susceptible to the impacts of disturbance and to reductions of ecosystem biodiversity.

Acknowledgements This project was partially funded by a Natural Sciences and Engineering Research Council of Canada operating grant to H.B.M as well as a British Columbia Student Summer Works grant and a Summer Career Placement grant (Human Resources Development Canada) to H.B.M. The authors wish to thank Rob Countess and Keith Williams, who graciously assisted with sporocarp identification, Aniko Varga, who provided constructive suggestions during the project, and Audrey Young and Sara Fletcher, who patiently assisted with typing the manuscript.

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