Mycorrhiza (2011) 21:569–576
DOI 10.1007/s00572-011-0365-3

ORIGINAL PAPER

# Mycorrhizal fungi associated with *Monotropastrum humile* (Ericaceae) in central Japan

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Received: 8 December 2010 / Accepted: 28 January 2011 / Published online: 19 February 2011


**Abstract** We explored the diversity of mycorrhizal fungi associated with *Monotropastrum humile* in the central part of Japan's main island. We collected 103 *M. humile* individuals from 12 sites with various forest types. We analyzed the DNA sequences of the internal transcribed spacer region from fungal and plant nuclear ribosomal DNAs to assess the genetic diversity of the fungi associated with *M. humile* roots and to position the plant with respect to known Monotropoideae groups, respectively. The plants formed a monophyletic clade with other members of *M. humile* but were separated from *M. humile* var. *glaberrimum* and other monotropes (97% bootstrap support). Of the 50 fungal phylotypes, 49 had best matches with the Russulales, and the other had highest similarity with the Thelephoraceae. Our phylogenetic analysis suggests that *M. humile* roots have a highly specialized association with fungal partners in the Russulaceae. Moreover, a few fungal phylotypes from the *M. humile* roots had positions neighboring those from *Monotropa uniflora* roots. These results indicated that the genetic diversity of mycorrhizal fungi of *M. humile* was highly specific to the Russulaceae, but with high diversity within that family, and that the fungi associated with *M. humile* differ from those associated with *M. uniflora*.

**Keywords** Internal transcribed spacer (ITS) · Monotropoid mycorrhiza · Phylotypes · Russulaceae · Thelephoraceae

**Electronic supplementary material** The online version of this article (doi:10.1007/s00572-011-0365-3) contains supplementary material, which is available to authorized users.

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## Introduction

The Monotropoideae subfamily of the Ericaceae consists of 15 species in 10 genera (Wallace 1975). All species in this subfamily lack or mostly lack chlorophyll and are thus characterized as achlorophyllous or non-photosynthetic plants (Leake 1994). Such plants are believed to obtain fixed carbon from surrounding photosynthetic plants via mycorrhizal mycelia and have thus been referred to as myco-heterotrophic plants (Bidartondo 2005; Björkman 1960; Leake 1994; Leake et al. 2004; Selosse et al. 2006). The roots of plants in this subfamily are now well known to form monotropoid mycorrhizas (Duddridge and Read 1982; Lutz and Sjolund 1973; Massicotte et al. 2005, 2007, 2010; Matsuda and Yamada 2003; Robertson and Robertson 1982). Two genera that contain three species (*Monotropa hypopitys* L., *Monotropa uniflora* L., and *Monotropastrum humile* (D. Don) H. Hara) are naturally distributed in Japan (Kitamura et al. 1975). *Monotropa* spp. are widely distributed throughout the Northern Hemisphere, whereas *M. humile* is a monotypic genus found in limited areas of eastern Asia, from the Himalayas to Japan (Wallace 1975). The mycorrhizal status and fungal symbionts of *M. humile* have recently been determined (Matsuda and Yamada 2003; Yamada et al. 2008).

Mycorrhizal studies of the Monotropoideae have included their histology (Duddridge and Read 1982; Lutz and Sjolund 1973; Massicotte et al. 2005, 2007, 2010; Robertson and Robertson 1982), eco-physiology (Björkman 1960; Bruns and Read 2000; Trudell et al. 2003), and the identification of fungal symbionts (Bidartondo and Bruns 2001, 2002; Cullings et al. 1996; Yang and Pfister 2006; Young et al. 2002). All the species examined so far have specific associations with certain fungal genera that are also known to be ectomycorrhizal: *Gautieria*, *Hydnellum*, *Lactarius*, *Rhizopogon*, *Russula*, and *Tricholoma* (Bidartondo and Bruns 2001, 2002; Cullings et al. 1996; Yang and Pfister 2006).

Members of the Russulaceae were suggested as fungal partners of *Monotropastrum* spp. based on molecular analyses (Bidartondo and Bruns 2001; Yokoyama et al. 2005) and morphological examinations (Imamura and Kurogi 2003; Kasuya et al. 1995; Yamada et al. 2008). Recently, *M. humile* var. *glaberrimum* H. Hara was found to associate with a member of the Thelephoraceae (Yokoyama et al. 2005). These results suggest that there may be widespread but specific fungal associations in the genus *Monotropastrum*. On the other hand, *M. uniflora*, a close relative of *Monotropastrum*, showed a geographic variation in the species diversity of russulacean fungi in North America (Bidartondo and Bruns 2001, 2005; Yang and Pfister 2006; Young et al. 2002). These studies concluded that *M. humile* can also have a geographically specific association of mycorrhizal fungi that are mainly in the Russulaceae. However, few studies have unambiguously analyzed the mycorrhizal fungi associated with *Monotropastrum* spp. Moreover, the identification of the fungal symbionts on myco-heterotrophic plants would be an important first step for the establishment of a clearer picture of common mycelial networks (Selosse et al. 2006) and for understanding co-evolution between two organisms (Selosse and Roy 2009). The degree of species diversity of mycorrhizal fungi associated with the plants would provide a clue for evolutionary processes within the Monotropoideae and parallel evolutionary paths toward myco-heterotrophy in understory Ericaceae and Orchidaceae (Tedersoo et al. 2007).

The aim of our study was to identify the mycorrhizal fungi associated with *M. humile* in central Japan. We hypothesized that the host specificity of the fungi associated with *M. humile* would be high at the family level (i.e., the Russulaceae), irrespective of the sampling sites. To test this hypothesis, we determined the fungal ribosomal DNA of internal transcribed spacer (ITS) regions that could be used as a "DNA barcoding" site for fungal discrimination (Begerow et al. 2010). We also examined the DNA of the sampled plants to position *M. humile* with respect to other known Monotropoideae groups.

## Materials and methods

### Site descriptions and sampling procedures

During the flowering season (from May to August) in 2002, 2005, 2007, and 2009, we visited 12 sites in different types of forest vegetation in central Japan and collected a total of 103 individual flowering *M. humile* (Table 1). The forest types at sites D, E, and H have previously been described (Matsuda and Yamada 2003). Sites A, E, and G were located in secondary forest dominated by *Quercus serrata* Thunb., *Quercus acutissima* Carruthers, or both species. Sites B, C, D, F, and H were naturally regenerated forests; sites B, D, and F were composed of diverse broad-leaved and coniferous tree species in the genera *Abies*, *Carpinus*, *Fagus*, and *Quercus*, whereas sites C and H were on mountain tops and were dominated by *Quercus crispula* Blume. Site I was located at the border between an artificial *Cryptomeria japonica* D. Don forest and naturally regen-

**Table 1** Collection sites for the *Monotropastrum humile* specimens used in this study

| Collection site | Altitude (m) | Latitude, longitude | Numbers of collected plants |
|---|---|---|---|
| A Misato, Tsu, Mie | 140 | 34° 44' N, 136° 23' E | 9 (9) |
| B Mt. Minamimatayama, Taiki, Mie | 982 | 34° 17' N, 136° 18' E | 15 (15) |
| C Mt. Gozaisho, Komono, Mie | 1,212 | 35° 1' N, 136° 25' E | 37 (37) |
| D Misugi, Tsu, Mie | 650 | 34° 27' N, 136° 13' E | 11 (4) |
| E Kiwa, Mie | 600 | 33° 51' N, 135° 51' E | 10 (4) |
| F Mt. Houraiji, Aichi | 684 | 34° 58' N, 137° 35' E | 1 (1) |
| G Makino, Shiga | 300 | 35° 29' N, 136° 1' E | 2 (1) |
| H Mt. Kariyasu, Fukui | 548 | 36° 13' N, 136° 20' E | 8 (1) |
| I Tateyama, Toyama | 240 | 36° 36' N, 137° 20' E | 4 (4) |
| J Minamiminowa, Nagano | 772 | 35° 51' N, 137° 56' E | 4 (4) |
| K Ina, Nagano | 1,500 | 35° 49' N, 137° 51' E | 1 (1) |
| L Hiraya, Nagano | 1,100 | 35° 16' N, 137° 38' E | 1 (1) |

Values in parentheses indicate the number of plants used for DNA sequencing analyses

erated mixed *Quercus* spp. forest. Site J was a mixed forest of *Q. serrata* and *Pinus densiflora* Sieb et. Zucc. on the campus of Shinshu University. Site K was a deciduous needle-leaved *Larix leptolepis* Gordon forest mixed with occasional *P. densiflora*. Site L was a deciduous broad-leaved forest composed of *Fagus crenata* Blume, *Q. crispula*, and *Magnolia obovata* Thunb.

At each site, we collected *M. humile* plants from different populations separated by at least 1 m. The distances between any two sites ranged from 20 to 330 km. The plants were carefully excavated using a shovel to include the surrounding soil and were taken to the laboratory. All samples were stored at 4°C for less than 2 weeks until the roots could be processed. Root balls were soaked in running tap water to loosen soil particles around the roots. We then excised at least five root tips from each *M. humile* root ball and stored them separately in 1.5-mL tubes at −80°C until DNA extraction. Several roots from each sample were examined microscopically to confirm the formation of monotropoid mycorrhizas (Matsuda and Yamada 2003).

Because the taxonomic identity of *M. humile* is now ambiguous (Tsukaya et al. 2008), we also examined DNA samples from undamaged mature stems of two *M. humile* plants from each of the different populations and three *M. uniflora* plants from one population at site A that were sampled and stored under the same conditions.

## Molecular methods and analyses

We extracted DNA from individual root tips of the *M. humile* individuals following the protocol of Matsuda and Hijii (1999). For three plants collected at site E, we used nine root tips per plant for the extraction to confirm whether fungal colonization of the root systems was by a single species. We used the cetyltrimethylammonium bromide method and amplified the ITS region within the rDNA by means of the polymerase chain reaction (PCR) using the ITS primer pair of ITS1f and ITS4b, which specifically amplifies rDNA from Basidiomycete fungi (Gardes and Bruns 1993). We only PCR-amplified two samples from plants collected at site J, using the primer pair of ITS1f and LR21 (Tedersoo et al. 2006). For samples that failed to amplify the ITS region, a nested PCR using the ITS1 and ITS4 primer pair (White et al. 1990) was also performed. We used a Takara Model TP600 PCR Thermal Cycler Dice (Takara Bio Inc., Otsu, Japan) to run the thermal program designed by Gardes and Bruns (1993).

Prior to the sequence analysis, we digested the PCR products from 31 plants collected at sites D, E, G, and H with three restriction enzymes (*Afa*I, *Hae*III, and *Hin*fI) to discriminate restriction-fragment length polymorphism (RFLP) types. PCR products of the representative RFLP types and direct PCR products were purified using the Jetsorb Gel Extraction Kit (Genomed, Bad Oeynhausen, Germany) following the manufacturer's instructions. The purified DNAs were analyzed using a sequence reaction with the Quick Start Kit (Beckman Coulter, Brea, CA, USA) with either of the forward or reverse primers. The cycle and sequence conditions were the manufacturer's recommended program. The DNAs were then sequenced with a CEQ2000XL DNA sequencer (Beckman Coulter). This process was done at least two times using independent root samples. ITS regions that were successfully sequenced were submitted to the DNA Data Bank of Japan (DDBJ) under the accession numbers AB594932 to AB594977 and AB600187 to AB600190. Sequence similarities were determined using the BLAST sequence-similarity search tool (Altschul et al. 1997) provided by the DDBJ/EMBL/GenBank (http://blast.ddbj.nig.ac.jp/top-j.html) to infer the putative identity of the fungal phylotypes.

For the extraction of plant DNA, we used the DNeasy Plant Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The ITS region of the extracted DNA was amplified using the ITS1 and ITS4 primer pair. Positive PCR products were cleaned using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). DNA sequencing was conducted bidirectionally with one of the primers, after sequence reactions with the DYEnamic ET Dye Terminator sequencing reagents (GE Healthcare), using an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA, USA). The two successfully sequenced *M. humile* samples were deposited in the DDBJ (accessions AB594724 and AB594725). Three *M. uniflora* plants showed identical sequences, which were therefore submitted to DDBJ as a single representative accession (AB600869).

## Sequence and phylogenetic analyses

To construct fungal phylogenetic trees, we used selected ITS sequences from the Russulaceae and Thelephoraceae to assess the positions of our sample phylotypes since our sample was highly similar to those groups (see the "Results" section). In addition, we incorporated some fungal sequences that originated from *M. uniflora* roots that were presented by Bidartondo and Bruns (2001), Young et al. (2002), and Yang and Pfister (2006) into the alignment to position our samples within a wider context. For the DNA sequence of the *Monotropastrum* plants, we incorporated sequences for related taxa from Tsukaya et al. (2008) as well as additional data deposited in the DDBJ to generate phylogenetic trees.

Representative sequences were aligned using version 6 of MAFFT (Katoh and Toh 2008; http://mafft.cbrc.jp/alignment/server/index.html), using the L-INS-i (slow;

iterative refinement) method and adjusting the scoring matrix as "1PAM/$\kappa$=2". No further manual corrections were required to obtain reproducible results. Phylogenetic analyses were performed using version 4 of MEGA (Tamura et al. 2007). The maximum-parsimony tree was obtained using the close-neighbor-interchange algorithm (Nei and Kumar 2000) with search level 7, in which the initial trees were obtained by means of the random addition of sequences (ten replicates). The replicate trees, in which more than 65% the associated taxa were clustered together in the bootstrap test (1,000 replicates), were shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset using the "complete deletion" option.

## Results

The ITS matrix of plants for 23 accessions contained 169 aligned positions, of which 52 were parsimony-informative. Figure 1 shows one of the 2,103 most-parsimonious trees in 149 lengths. The consistency index was 0.679, and the retention index was 0.817. The ITS region obtained from the *Monotropastrum* samples we examined were placed into the same clade as other *M. humile* plants that originated from either Taiwan or Japan with a 97% bootstrap value. In addition, the *M. humile* clade was separated from the closest group of *M. humile* var. *glaberrimum* and *M. uniflora*, indicating that the *Monotropastrum* plants we examined were highly likely to be phylogenetically part of the *M. humile* clade.

We confirmed that the representative roots of all *M. humile* individuals that formed monotropoid mycorrhizas possessed a fungal mantle, a Hartig net, and fungal pegs (Matsuda and Yamada 2003). For three *M. humile* plants collected at site E, the ITS-RFLP analyses succeeded for eight, eight, and nine roots of the nine roots we examined, and we obtained an identical RFLP pattern both within and between these individuals. We grouped 31 *M. humile* results into 12 RFLP types using the three restriction enzymes (data not shown). The representative RFLP types and the rest of the individuals were sequenced, and 50 sequences were successfully obtained. The data from each individual root and the representative RFLP types were treated as different phylotypes. Of the 50 phylotypes, 49 had the closest match with members of the Russulaceae, and the last phylotype showed higher similarity with the Thelephoraceae (Table S1).

Figure 2 shows one of the 1,044 equally most-parsimonious trees (length=549). There were a total of 223 positions in the final dataset, of which 104 were parsimony-informative. The consistency index was 0.385, and the retention index was 0.843. Of the 50 phylotypes, 49 were clustered into a Russulales clade supported by a 67% bootstrap value (Fig. 2). Within the clade, 24 phylotypes were placed in a *Russula* clade with a 66% bootstrap value. An additional ten phylotypes were positioned in a *Lactarius* and hypogeous *Arcangeliella* clade, with a 91% bootstrap value. The remaining 15 phylotypes were found in smaller groups of phylotypes, most of which neighbored known fungal taxa and were supported by high bootstrap values. The 50th phylotype, E3, was designated into a *Thelephora–Tomentella* clade with a 99% bootstrap value.

Multiple phylotypes were detected within each site and tended to be located within similar clades (e.g., at sites A, C, and J). However, some phylotypes from different sites were placed in a single or similar group within the phylogenetic tree (e.g., sites B and C in the *Lactarius* clade and sites A, F, and J in the *Russula* clade; Fig. 2). Although fungal phylotypes from the *M. uniflora* roots were present in the phylogenetic tree, most *M. humile* phylotypes that were supported with higher bootstrap values were nested with other *M. humile* samples or with known russulacean phylotypes. A few phylotypes (e.g., E3) were nested with

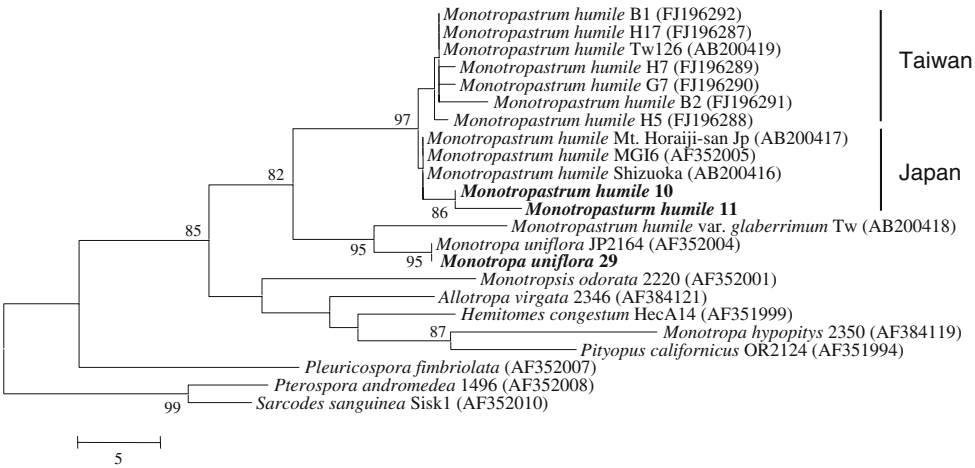


**Fig. 1** One of the 2,103 maximum-parsimony phylogenetic trees constructed from the ITS sequences of *Monotropastrum humile* collected in central Japan and from other allied species. The tree is rooted with *Sarcodes sanguinea* and *Pterospora andromedea*. Data from the present study are *boldfaced*. Bootstrap values higher than 65% are indicated at the nodes

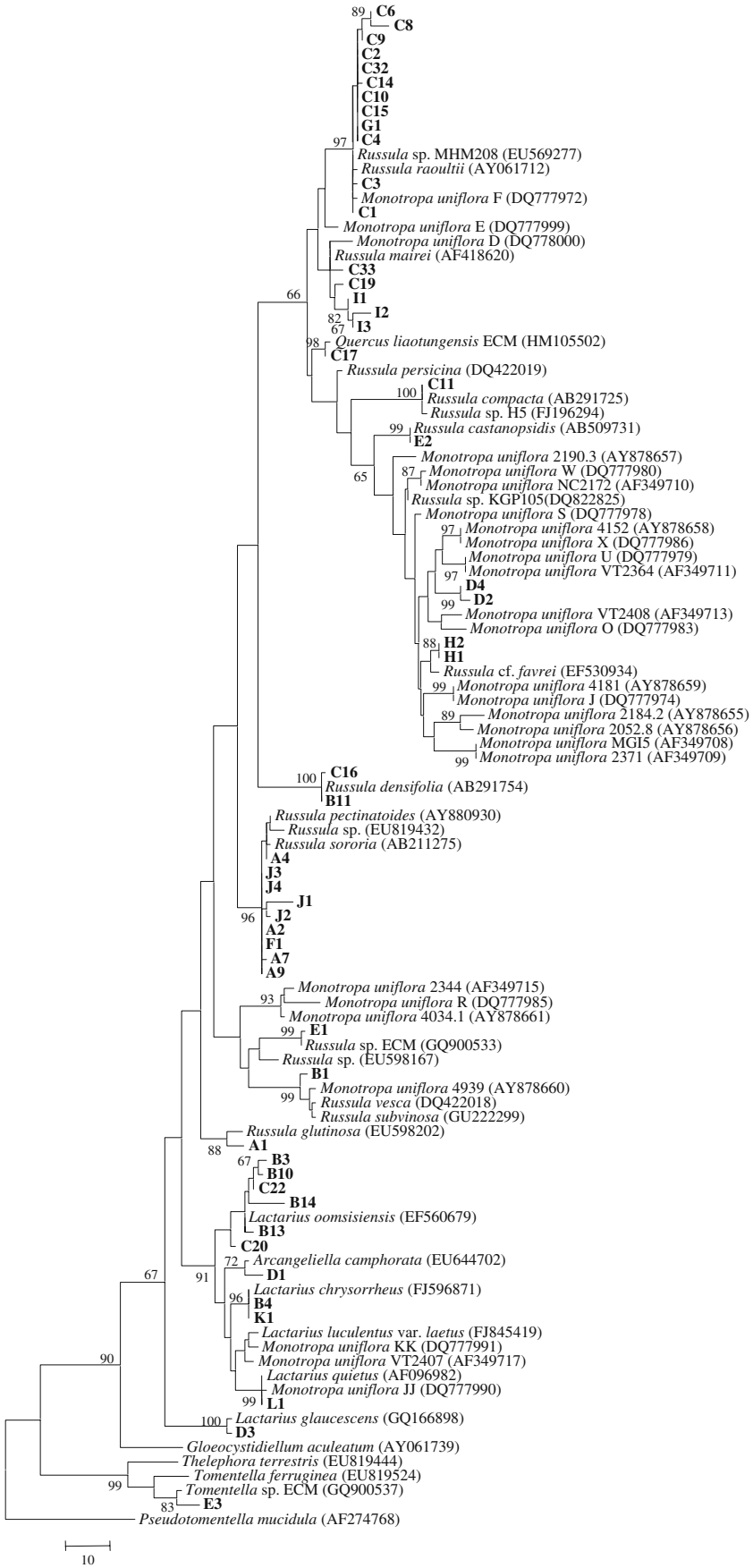


**Fig. 2** One of the 1,044 maximum-parsimony phylogenetic trees constructed from the ITS sequences of fungi associated with *Monotropastrum humile* roots collected in central Japan. The tree is rooted with *Pseudotomentella mucidula*. Data from the present study are *boldfaced*. Bootstrap values higher than 65% are indicated at the nodes

phylotypes originating from fungi that are known to form ectomycorrhizas.

## Discussion

Our investigation of the diversity of *M. humile* mycorrhizas suggested that most of the fungi we detected were members of the Russulaceae (Fig. 2). Because the plants were collected at 12 sites with large variation in geographical locations and vegetation types, this result suggests that russulacean fungi are the main mycobionts of this species. This finding is supported by previous results for the same genus (Yamada et al. 2008; Yokoyama et al. 2005) and for other monotrope plants such as *M. uniflora* (Bidartondo and Bruns 2001; Yang and Pfister 2006; Young et al. 2002). *Russula* spp. and *Lactarius* spp. are well-known ectomycorrhizal fungal groups that are dominant ectomycorrhizas in forest ecosystems (Matsuda and Hijii 1998, 2004; Yamada and Katsuya 2001); this dominance in forest ecosystems may facilitate their association with *Monotropastrum* plants.

We determined that the RFLP pattern of multiple root tips from the same plants was identical. Because an RFLP pattern characterized by two or more enzymes can indicate the number of fungal species (Horton 2002), each *M. humile* plant appeared to be associated with a single fungal phylotype, as has been suggested for other monotrope plants (Bidartondo and Bruns 2001, 2005; Cullings et al. 1996; Yang and Pfister 2006; Young et al. 2002). Moreover, the overall number of detected phylotypes was high, as previously reported for *M. humile* (Yamada et al. 2008) and for *M. uniflora* (Yang and Pfister 2006). However, Bidartondo and Bruns (2001) indicated that *Russula brevipes* Peck was the only fungus that colonized *M. uniflora* plants sampled over a 9,400-km$^2$ area in Oregon. Young et al. (2002) suggested that only three mycorrhizal fungi were associated with 15 *M. uniflora* plants sampled at three sites in central of British Columbia, Canada. These results indicate that at the fine scale of individual root systems, monotrope plants would typically have high fidelity for a limited number of compatible fungal species. However, from the wider perspective of a forest ecosystem, *M. humile* showed a certain level of fungal specificity at the genus or family level toward russulacean fungi (Yamada et al. 2008). In ectomycorrhizal fungal communities, the larger the number of host tree species in a forest ecosystem, the more diverse the fungi associated with the ecosystem (Ishida et al. 2007). Thus, differences in the diversity of tree species may be responsible for differences among studies in the number of fungi associated with monotropes. Even if this is the case, we found some phylotypes (sites B and C and sites A, F, and J) that comprised a single clade at multiple sites (Fig. 2; Yang and Pfister 2006). This suggests that *M. humile* has a wide range of fungal associations, though directed toward russulacean fungi, and that there might be some unknown recognition process between the plant and fungal partners rather than random associations with available members of the Russulaceae in situ (Bidartondo and Bruns 2005).

Although most of the fungal phylotypes of *M. humile* were placed within the Russulaceae, a few were clustered with known *M. uniflora* fungal symbionts. Similarly, fungi from *M. uniflora* roots clustered with the same fungi, even though their phylogenetic relationships with fruiting bodies of neighboring *Russula* species were examined and found not to nest (Yang and Pfister 2006). Given the large number of known *Russula* species around the world (Kirk et al. 2008), insufficient sequence data have been accumulated, and an adequate taxonomic resolution of this genus therefore remains to be developed (Ryberg et al. 2009). Although it is too early to express a definite conclusion, the unique clades revealed by our root samples indicate that the associated fungi might be common groups for myco-heterotrophic plants and may indicate the existence of potential cryptic species.

A member of the Thelephoraceae was suggested to be a mycorrhizal fungus of *M. humile* var. *glaberrimum* (Yokoyama et al. 2005). Although the taxonomic identity of *Monotropastrum* plants remains controversial (Tsukaya et al. 2008), our phylogenetic analysis nested the examined plants with other *M. humile* and separated them clearly from *M. humile* var. *glaberrimum*. Thus, our study is the first report that *M. humile* associates with a member of the Thelephoraceae. However, it is not clear why *Monotropastrum* spp. associate with the Thelephoraceae as well as the Russulaceae. In addition, although we collected four *M. humile* plants at the same location, only one was associated with a member of the Thelephoraceae; the others were associated with members of the Russulaceae. Thus, further studies are needed to clarify the fungal species diversity associated with *M. humile* by focusing on more intensive sampling of each site and sampling of sites in more diverse geographic areas. In addition, the degree of genetic diversity of *M. humile* populations might explain the degree of fungal specificity, particularly if associations with the Russulaceae and Thelephoraceae arose in different genetic lineages. Studying the relationship between host and fungal genetic diversity would provide important clues to how fungal specificity evolved within the Monotropoideae.

**Acknowledgments** We thank Naoto Oonishi (Taiki town office, Mie Prefecture) and Naoya Yamano (The Japan Serow Center in Mie Prefecture) for providing information on the plant materials, the members of the Laboratory of Forest Pathology and Mycology, Mie University, for their assistance in the field, and two anonymous reviewers for their insightful suggestions on this manuscript. This study was supported in part by Grants-in-Aid for Scientific Research from the Japan Ministry of Education, Science, Sports and Culture to YM (20651061 and 22688011).

## References


Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Begerow D, Nilsson H, Unterseher M, Maier W (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol 87:99–108

Bidartondo MI (2005) The evolutionary ecology of myco-heterotrophy. New Phytol 167:335–352

Bidartondo MI, Bruns TD (2001) Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure. Mol Ecol 10:2285–2295

Bidartondo MI, Bruns TD (2002) Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. Mol Ecol 11:557–569

Bidartondo MI, Bruns TD (2005) On the origins of extreme mycorrhizal specificity in the Monotropoideae (Ericaceae): performance trade-offs during seed germination and seedling development. Mol Ecol 14:1549–1560

Björkman E (1960) *Monotropa hypopitys* L.—an epiparasite on tree roots. Physiol Plant 13:308–327

Bruns TD, Read DJ (2000) In vitro germination of non-photosynthetic, myco-heterotrophic plants stimulated by fungi isolated from the adult plants. New Phytol 148:335–342

Cullings KW, Szaro T, Bruns TD (1996) Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites. Nature 379:63–66

Duddridge JA, Read DJ (1982) An ultrastructural analysis of the development of mycorrhizas in *Monotropa hypopitys* L. New Phytol 92:203–214

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evol Int J Org Evol 39:783–791

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rust. Mol Ecol 2:113–118

Horton TR (2002) Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. Plant Soil 244:29–39

Imamura A, Kurogi S (2003) Difference in monotropoid mycorrhiza formation between *Monotropastrum globosum* and its forma *roseum*. Mycoscience 44:63–65

Ishida TA, Nara K, Hogetsu T (2007) Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. New Phytol 174:430–440

Kasuya MCM, Masaka K, Igarashi T (1995) Mycorrhizae of *Monotropastrum globosum* growing in a *Fagus crenata* forest. Mycoscience 36:461–464

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9:286–298

Kirk PM, Cannon PF, Minter DW, Stalpers JA (eds) (2008) Dictionary of the fungi, 10th edn. CAB International, Wallingford

Kitamura S, Mutata G, Hori M (1975) Coloured illustrations of herbaceous plants of Japan (Sympetalae). Hoikusha, Osaka

Leake JR (1994) The biology of myco-heterotrophic ("saprophytic") plants. New Phytol 127:171–216

Leake JR, McKendrick SL, Bidartondo M, Read DJ (2004) Symbiotic germination and development of the myco-heterotroph *Monotropa hypopitys* in nature and its requirement for locally distributed *Tricholoma* spp. New Phytol 163:405–423

Lutz RW, Sjolund RD (1973) *Monotropa uniflora*: ultrastructural details of its mycorrhizal habit. Am J Bot 60:339–345

Massicotte HB, Melville LH, Peterson RL (2005) Structural features of mycorrhizal associations in two members of the Monotropoideae, *Monotropa uniflora* and *Pterospora andromedea*. Mycorrhiza 15:101–110

Massicotte HB, Melville LH, Tackaberry LE, Peterson RL (2007) *Pityopus californicus*: structural characteristics of seed and seedling development in a myco-heterotrophic species. Mycorrhiza 17:647–653

Massicotte HB, Melville LH, Peterson RL, Tackaberry LE, Luoma DL (2010) Structural characteristics of root-fungus associations in two mycoheterotrophic species, *Allotropa virgata* and *Pleuricospora fimbriata* (Monotropoideae) from southwest Oregon, USA. Mycorrhiza 20:391–397

Matsuda Y, Hijii N (1998) Spatiotemporal distribution of fruit bodies of ectomycorrhizal fungi in an *Abies firma* forest. Mycorrhiza 8:131–138

Matsuda Y, Hijii N (1999) Characterization and identification of *Strobilomyces confusus* ectomycorrhizas on Momi fir by RFLP analysis of the PCR-amplified ITS region of the rDNA. J For Res 4:145–150

Matsuda Y, Hijii N (2004) Ectomycorrhizal fungal communities in an *Abies firma* forest, with special reference to ectomycorrhizal associations between seedlings and mature trees. Can J Bot 82:822–829

Matsuda Y, Yamada A (2003) Mycorrhizal morphology of *Monotropastrum humile* collected from six different forests in central Japan. Mycologia 95:993–997

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, New York, 333 pp

Robertson DC, Robertson JA (1982) Ultrastructure of *Pterospora andromedea* Nuttall and *Sarcodes sanguinea* Torrey mycorrhizas. New Phytol 92:539–551

Ryberg M, Kristiansson E, Sjökvist E, Nilsson RH (2009) An outlook on the fungal internal transcribed spacer sequences in GenBank and the introduction of a Web-based tool for the exploration of fungal diversity. New Phytol 181:471–477

Selosse MA, Roy M (2009) Green plants that feed on fungi: facts and questions about mixotrophy. Trends Plant Sci 14:64–70

Selosse MA, Richard F, He X, Simard SW (2006) Mycorhizal networks: des liaisons dangereuses? Trends Ecol Evol 21:621–628

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tedersoo L, Suvi T, Larsson E, Kõljalg U (2006) Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. Mycol Res 110:734–748

Tedersoo L, Pellet P, Kõljalg U, Selosse MA (2007) Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. Oecologia 151:206–217

Trudell SA, Rygiewicz PT, Edmonds RL (2003) Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. New Phytol 160:391–401

Tsukaya H, Yokoyama J, Imaichi R, Ohba H (2008) Taxonomic status of *Monotropastrum humile*, with special reference to *M. humile* var. *glaberrimum* (Ericaceae, Monotropoideae). J Plant Res 121:271–278

Wallace GD (1975) Studies of Monotropoideae (Ericaceae): taxonomy and distribution. Wasmann J Biol 33:1–88

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols—a guide to methods and applications. Academic, New York, pp 315–322

Yamada A, Katsuya K (2001) The disparity between the number of ectomycorrhizal fungi and those producing fruit bodies in a *Pinus densiflora* stand. Mycol Res 105:957–965

Yamada A, Kitamura D, Setoguchi S, Hashimoto Y, Matsuda Y, Matsushita N, Fukuda M (2008) *Monotropastrum humile* var. *humile* is associated with diverse ectomycorrhizal Russulaceae fungi in Japanese forests. Ecol Res 23:983–993

Yang S, Pfister DH (2006) *Monotropa uniflora* plants of eastern Massachusetts form mycorrhizae with a diversity of russulacean fungi. Mycologia 98:535–540

Yokoyama J, Fukuda T, Tsukaya H (2005) Molecular identification of the mycorrhizal fungi of the epiparasitic plant *Monotropastrum humile* var. *glaberrimum* (Ericaceae). J Plant Res 118:53–56

Young BW, Massicotte HB, Tackaberry LE, Baldwin QF, Egger KN (2002) *Monotropa uniflora*: morphological and molecular assessment of mycorrhizae retrieved from sites in the Sub-Boreal Spruce biogeoclimatic zone in central British Columbia. Mycorrhiza 12:75–82