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Authors:

YAMADA Akiyoshi, KITAMURA Daisei, SETOGUCHI Masanobu, MATSUDA Yosuke, HASHIMOTO Yasushi, MATSUSHITA Norihisa, and FUKUDA Masaki

Title:

Monotropastrum humile var. *humile* is associated with diverse ectomycorrhizal Russulaceae fungi in Japanese forests

Yamada A (Corresponding Author), Kitamura D, Setoguchi M, Fukuda M

Faculty of Agriculture, Shinshu University, 8304 Minami-minowa, Nagano, Japan

E-mail: akiyosh@shinshu-u.ac.jp

Tel: +81-265-77-1631

Fax: +81-265-77-1629

Matsuda Y

Graduate School of Bioresources, Mie University

Hashimoto Y

Obihiro University of Agriculture and Veterinary Medicine

Matsushita N

Graduate School of Agriculture and Life Science, University of Tokyo

Abstract

Monotropastrum humile is nearly lacking in chlorophyll and obtains its nutrients, including carbon sources, from associated mycorrhizal fungi. We analyzed the mycorrhizal fungal affinity and species diversity of *M. humile* var. *humile* mycorrhizae to clarify how the plant population survives in Japanese forest ecosystems. We classified 78 samples of adult *M. humile* var. *humile* individuals from Hokkaido, Honshu, and Kyusyu Islands into 37 root mycorrhizal morphotypes. Of these, we identified 24 types as *Russula* or *Lactarius* fungal taxa in the Russulaceae, Basidiomycetes, but we could not identify the remaining 13 types in their genus in the Basidiomycetes. The number of fungal species on *M. humile* var. *humile* was the highest in the plant subfamily. The diversity of fungal species revealed its increased trends in natural forests at the stand level, fagaceous vegetation, and cool-temperate climate. The most frequently observed fungus colonized mainly samples collected from sub-alpine forests; the second most frequently observed fungus colonized samples collected from sub-alpine to warm-temperate forests. These results suggest that Japanese *M. humile* populations are associated with specific but diverse fungi that are common ectomycorrhizal symbionts of various forest canopy trees, indicating a tripartite mycorrhizal relationship in the forest ecosystem.

Keywords
Ectomycorrhizal symbiosis, fungal species diversity, myco-heterotrophy, non-photosynthetic plants, tripartite relationship

Introduction

Some non-photosynthetic vascular plants have a distinct ecological trait in which they obtain all of their metabolic carbon sources from associated fungi in an organic form. This type of plant nutrition is known as myco-heterotrophy (Leake 1994, Smith and Read 1997). The plant subfamily Monotropoideae (Ericaceae) produces a distinct root anatomy, i.e., monotropoid mycorrhizae, in which fungi form penetration pegs in the root epidermal cells, as well as a fungal sheath and Hartig net mycelium between the root epidermis, respectively (Duddridge and Read 1982, Massicotte et al. 2006, Robertson and Robertson 1982). It is possible that the mycorrhizal fungi supply all of the required plant nutrients, including carbon, nitrogen, and phosphorus. Interestingly, the associated fungi are also ectomycorrhizal symbionts of autotrophic woody plants such as Pinaceae, Fagaceae, Salicaceae, and Betulaceae, which are often dominant in the canopies of various forest ecosystems in the northern hemisphere (Molina et al. 1992). Therefore, carbon sources for monotrope plants are derived originally from the photosynthates of such forest canopies via the connecting mycorrhizal mycelium in soil (Björkman 1960, Bidartondo 2005, Bidartondo and Bruns 2002). The Monotropoideae comprises 15 species in 10 genera that are distributed in a variety of forests in the northern hemisphere, from tropical rain forests to boreal forests (Wallace 1975).

Cullings et al. (1996) reported an extremely specific monotropoid mycorrhization between *Pterospora andromedea* of the Monotropoideae and *Rhizopogon subcaerulescens* in the Basidiomycetes in the Sierra Nevada Mountains of North America, based on fungal DNA fingerprinting of the mycorrhizae, although the fungal identity was later corrected as *R. salebrosus* and *R. arctostaphyli* species groups (Bidartondo and Bruns 2002). They also hypothesized that this monotrope plant evolved rapidly as a specialist and is strictly associated with a single fungal clade (species group). In addition, the fungal group may advantageously obtain more carbohydrate from ectomycorrhizal hosts than do other ectomycorrhizal fungi within a diverse ectomycorrhizal fungal community. In this respect, various levels of mycorrhizal specificity have been clarified recently in the Monotropoideae at the worldwide geographic level, i.e., the association of each plant species with a fungal species group, genus, or family (Bidartondo 2005, Bidartondo and Bruns 2001, Bidartondo et al. 2000, 2002, Kretzer et al. 2000, Yang and Pfister 2006, Young et al. 2002). For example, *Pleuricospora fimbriolata*, *Sarcodes sanguinea*, and *Allotropa virgata* are associated with a single fungal species, i.e. *Gautieria monticola*, *Rhizopogon ellenae*, and *Tricholoma magnivelare*, respectively; *Monotropa hypopithys*, *Pityopus californicus*, and *Hemitomes congestum* are associated with a single fungal genus, i.e. *Tricholoma*, *Tricholoma*, and *Hydnellum*, respectively; and *Monotropa uniflora* is associated with a single fungal family, i.e. Russulaceae. However, further research is required for each monotrope in local populations to clarify their ecological significance in forests. In fact, researchers have analyzed few of the mycorrhizal fungal relationships among Asian populations of the Monotropoideae (Bidartondo and Bruns 2002, Yokoyama et al. 2005).

Monotropastrum humile (D. Don) H. Hara of the Monotropoideae is distributed throughout eastern Asia, from the Himalayas to the Islands of Japan. The population of *M. humile* consists of two varieties, i.e. *M. humile* var. *humile* (D. Don) H. Hara (=*M. humile* var. *tripetalum* (Mikino) H. Hara) and *M. humile* var. *glaberrimum* Hara, and only former variety distributes in Japan (Hara 1965, Yokoyama et al. 2005). Recently, taxonomic implication between the two varieties of *M. humile* has been revised based on the distinct difference of their symbiotic mycorrhizal fungal clade (Yokoyama et al. 2005). In Japan, *M. humile* var. *humile*, along with two other monotropes (*Monotropa hypopithys* and *Monotropa uniflora*), is commonly distributed under various types of forest vegetation such as oak, beech, and pine. The flowering phenology of *M. humile* var. *humile* has been studied extensively because of the plant's distinct appearance (Tsukaya 1998). We recently reported that *M. humile* var. *humile*, like other monotropes, exclusively forms typical monotropoid mycorrhizae under various types

of forest vegetation ranging from warm-temperate to sub-alpine climatic zones in Japan (Matsuda and Yamada 2003). We observed monotropoid mycorrhizae on adult plants throughout the flowering and seed maturation stages. Although we did not analyze the fungal identity, we observed distinct variations in the external characteristics of the mycorrhizal fungal sheath, i.e., color, luster, and texture, indicating differences in the associated fungal species.

Therefore, we attempted to distinguish the morphotypes of *M. humile* var. *humile* mycorrhizae to clarify the diversity of the plant's associated fungal species. Our findings of the mycorrhizal fungal diversity in *M. humile* should reveal that the plant's reproduction and nutritional ecology is obligately dependent on mycorrhizal fungal ecophysiology and the related ectomycorrhizal symbiotic system in forests.

Materials and Methods

Collection of samples

We collected flowering individuals of *Monotropastrum humile* var. *humile* from several different types of forest vegetation on Hokkaido, Honshu, and Kyushu Islands, Japan. Most collection sites had been surveyed previously during the flowering season of *M. humile*, following the method set out by Tsukaya (1998). In general, we collected one or two plant individuals from each forest, but selected three or five individuals from relatively large *M. humile* populations. For each sample, we recorded the dominant tree species of the forest canopy. The mycorrhizal root system, i.e., root ball, of each individual plant was dug out from the soil humus layer using a shovel. Each root sample was placed in a polyethylene bag, transported as quickly as possible to the laboratory, and stored in a refrigerator for no longer than one week until processing. If necessary, samples were stored in a cool box until they were brought to the laboratory.

Microscopic observation of monotropoid mycorrhizae

We washed the root system of each *M. humile* var. *humile* sample using tap water and removed any remaining soil particles using fine forceps. We placed at least 10 mycorrhizal root tips per plant on slide glasses, mounted them using lactic acid, and covered them with cover glasses; we then sealed the preparations using nail polish. We set each preparation on a differential interference contrast Nomarski microscope and observed it under high magnification (Yamada et al. 2001). We recorded microscopic characteristics of mycorrhizal external structures such as the fungal sheath, extraradical hyphae, and rhizomorphs to distinguish mycorrhizal morphotypes at the fungal genus level based on the methods set out by Ingleby et al. (1990) and Agerer (1987–2002). We expected that these microscopic observations would ensure the identification of each fungal morphotype because monotropoid mycorrhizae of *M. humile* var. *humile* form a developed fungal sheath (Kasuya et al. 1995, Matsuda and Yamada 2003). Because *M. humile* var. *humile* is reportedly to associate with a *Russula* species (Bidartondo and Bruns 2001, Yokoyama et al. 2005), we also conducted fluorescent microscopic observations or a sulfovanillin staining procedure (Largent et al. 1977) to check for the presence of laticiferous hyphae, which is a characteristic of *Lactarius* in the Russulaceae. To confirm the validity of the mycorrhizal morphotyping at the fungal species level, we checked the fungal ribosomal DNA (rDNA) using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis (Gardes and Bruns 1993; Yamada et al. 2001) for each mycorrhizal sample collected in 2001. We extracted fungal DNA from several fresh mycorrhizal tips in each sample based the CTAB method and PCR amplified the ITS region within the rDNA using the ITS primer pair ITS IF/4B, which specifically amplifies Basidiomycete fungi. We used an aliquot of the PCR product for a second step PCR (nested PCR) using another ITS primer pair, ITS 1/4 that is eukaryote-specific, because some samples

produced insufficient product during the first PCR step to allow RFLP analysis. For the RFLP analysis, the products obtained during the second PCR were digested using endonuclease, i.e., *Hif* I, *Hae* III, or *Taq* I.

Data analysis

We analyzed the plant–fungus relationships using either a plant-centered (i.e., phytocentric) or fungus-centered (i.e., mycocentric) framework (Southworth et al. 2005). We used a phytocentric perspective to clarify how environmental and plant factors are related to mycorrhizal fungal distribution and species diversity and used a mycocentric perspective to clarify how mycorrhizal fungi specialize on *M. humile* var. *humile* within the plant subfamily. To estimate fungal species diversity, we used the Shannon-Weaver diversity index (*H'*) and the related index of equitability (*E'*). If necessary, we statistically analyzed numerical data using one-way ANOVA (KaleidaGraph ver. 3.6J, HULINKS Inc., Tokyo, Japan).

Results

Sample collection

A total of 78 individual *Monotropastrum humile* samples were collected under a variety of forest vegetation ranging from sub-alpine to warm-temperate zones (see Appendix 1). The flowering season of *M. humile* varied from April to October; this finding generally supports the results of Tsukaya (1998), with the exception of one sample. Sample 01T-1, which was collected in October, revealed a complication with *Monotropa uniflora*, so we observed its fruit anatomy (i.e., sap fruit and parietal placenta) to confirm its taxonomic validity as the genus *Monotropastrum*.

Mycorrhizal distinction

All collected plant individuals developed the distinct mycorrhizal root system, i.e., root ball, and all of the mycorrhizal tips had a well-developed fungal sheath, as reported previously (Matsuda and Yamada 2003). Mycorrhizal tips within each mycorrhizal sample exhibited the same morphological characteristics, i.e., each mycorrhizal sample contained a single mycorrhizal morphotype. We classified these 78 mycorrhizal samples into 37 morphotypes using microscopic observations (see Appendix 2). Within these morphotypes, we classified 15 and 9 types as *Russula* and *Lactarius* species, respectively, both of which are in the Russulaceae, Basidiomycetes (Fig 1). Although we were unable to identify the causal fungal genera of the other 13 types, the morphology of their fungal sheaths indicated similarity to *Russula*- or *Lactarius*-associated morphotypes, i.e. well organized regular synenchymatous or irregular synemchymatous structure, and lacking in a clamp connection on a hyphal septum. No samples had pigmented hyphal wall that infers the affinity to Thelephoraceae. PCR-RFLP analysis showed that all tested mycorrhizal morphotypes had different restriction patterns (data not shown). Even the mycorrhizal samples that we were unable to identify the genus through microscopic observations exhibited rDNA amplification by PCR, indicating that these mycorrhizal fungi also belonged to the Basidiomycetes.

Plant–fungus association from a phytocentric perspective

Natural or semi-natural forests exhibited higher fungal diversity than did man-made forests, although this difference was not significant because of the limited sample size (Table 1). Three independent plant individuals collected from the sampling site of Ina, Nagano (adjacent to a large cropland), were associated with the same mycorrhizal fungus. In contrast, four distinct fungi were observed at the sampling site of Azumi, Nagano (natural sub-alpine forest), including both *Russula* and *Lactarius*.

The fungal diversity was similar among the collection sites in Hokkaido, Ibaraki, and Nagano, which had climatic zones ranging from sub-alpine to warm-temperate (Table 2). All samples collected from the Shigakougen-Asama area exhibited different morphotypes; samples from this area had the highest *E'* values. The largest number of *M. humile* samples was recorded in Fagaceae forests, whereas the smallest number was recorded in mixed Fagaceae-Pinaceae forests. Interestingly, although Betulaceae trees were commonly distributed in many forest sites, we found no *M. humile* in pure Betulaceae stands.

With regard to climate, the largest number of *M. humile* samples was recorded in cool-temperate areas, and these areas had the highest *H'* values (Table 2). Although the smallest *H'* value was recorded in a warm-temperate area because of the small sample size, this area produced the highest *E'* values among the three different climates.

Plant–fungus association from a mycocentric perspective

Of the 37 mycorrhizal morphotypes that we distinguished, only six morphotypes appeared in different regions, as well as in a large number of samples (Table 3). Of these, morphotype 26, a *Russula*-related species, was collected from both Fagaceae- and Pinaceae-dominated forests. Another five morphotypes were mostly collected from Fagaceae or Fagaceae-related mixed forests. With regard to climate, morphotype 26 was mainly collected from sub-alpine forests; its collection sites in cool-temperate forests were limited to higher altitude areas and locations near sub-alpine forests. Although morphotype 20 was collected from a wide range of climatic zones, the other four morphotypes were mainly collected from cool-temperate zone.

We identified the causal fungal genera of 24 morphotypes (i.e., *Russula* or *Lactarius*) and compared their frequency of occurrence (Table 4). Both genera were observed in greater numbers in Fagaceae forests, and fewer were observed in mixed forests. With regard to climate, both genera were observed in greater numbers in cool-temperate forests, and fewer were observed in warm-temperate forests. No *Lactarius* was found in warm-temperate forests, even though we collected eight samples from this climate.

Discussion

The Japanese *Monotropastrum humile* var. *humile* population is associated with diverse and specific fungi belonging to *Russula*, *Lactarius* and several unidentified species. Previously, this monotrope species had a confirmed association only with *Russula* sp. (Bidartondo and Bruns 2001, Yokoyama et al. 2005). However, we reported the plant–fungus specificity here for the first time and suggest that *M. humile* var. *humile* may have a specific mycorrhizal association with a single fungal family, i.e., the Russulaceae, Basidiomycetes. To our knowledge, *M. humile* var. *humile* has greater fungal species diversity (see Appendix 2; more than 30 species) than does any other previously reported monotrope species (Bidartondo and Bruns 2001, 2002, Yang and Pfister 2006). *Monotropa hypopithys* is distributed widely in the northern hemisphere, and eight *Tricholoma* species have been identified from among the 44 samples collected in Eurasia and North America (Bidartondo and Bruns 2001). Researchers have collected *Monotropa uniflora* mycorrhiza samples from North America and have identified seven, three and twenty Russulaceae species from among 33,15, and 56 samples, respectively (Bidartondo and Bruns 2001, Yang and Pfister 2006, Young et al. 2002). The fungal diversity of *M. humile* var. *humile* mycorrhizae probably reflects the high species diversity of ectomycorrhizal fungi, including Russulaceae, in Asian forest ecosystems (Matsuda and Hijii 1998, Yamada and Katsuya 2001). *Monotropa humile* might have evolved to expand its association with fungal species under such circumstances. If this is the case, the genetic diversity of a *M. humile* var. *humile* population in a given local area should be linked to the level of ectomycorrhizal fungal

diversity.

Some researchers have theorized that *Pterospora andromedea* evolved to be specifically associated with certain *Rhizopogon* species groups, all of which specifically associate with conifer trees and obtain more carbon from their hosts than do other ectomycorrhizal fungi in the forest ecosystem (Cullings et al. 1996). However, Young et al. (2002) published results for *Monotropa uniflora*–Russulaceae associations that do not support this theory because of differences in ecological traits among the latter fungal group, which is associated with diverse coniferous and deciduous tree species. Because the Russulaceae is often the most frequent ectomycorrhizal taxon to fruit in oak, pine, and fir forests (Richardson 1970, Murakami 1987; Kernaghan et al. 1997; Matsuda and Hijii 1998, Yamada and Katsuya 2001), to survive *M. humile* var. *humile* might have adapted to the considerable fungal biomass of Russulaceae in ectomycorrhizal forests. Indeed, *Russula* species can be the dominant mycorrhizal biomass in soil within both conifer and deciduous broad-leaf forests (Gardes and Bruns 1996). In addition, because the Russulaceae is one of the largest ectomycorrhizal fungal families, with more than 1200 species (Kirk et al. 2001), it would often be favorable for *M. humile* var. *humile* to adapt to such fungi to find partners in forests (Bidartondo 2005). The absence of *Lactarius*-related mycorrhizae in the *M. humile* samples collected from warm-temperate forests (Table 3) suggests the relative importance of *Russula* species in the ectomycorrhizal fungal community in these areas. *Lactarius* species are common in warm-temperate forests, but are relatively rare in tropical areas, whereas *Russula* species are sometimes dominant even in tropical rain forests (Lee et al. 1997, Watling and Lee 1998, Adhikari 2000). In contrast, in boreal and even sub-arctic climatic zones, both *Lactarius* and *Russula* are common ectomycorrhizal taxa (Bills et al. 1986, Brunner et al. 1992, Gardes and Dahlberg 1996). These general distribution patterns of Russulaceae fungi and higher species diversification of *Russula* can help to explain our findings.

It is not clear why no *M. humile* var. *humile* individuals were observed in pure Betulaceae stands. *Betula* species compose forest canopies along with *Quercus*, *Pinus*, and *Abies* species in cool-temperate and sub-alpine climate areas in Japan, and these trees can share ectomycorrhizal fungi, including *Russula*. A *Betula–M. humile* var. *humile* combination might be quite rare, in which case we might have merely missed it, or the combination might be prohibited by a certain interaction between plants such as a chemical communication known as allelopathy (Rice 1984) that has been observed in birch (Santamour and Lundgren 1997, Keinänen et al. 1999). The sister species *M. uniflora* is known to occur in *Betula*-dominated forests and to be associated with Russulaceae fungus (Young et al. 2002). This implies habitat segregation between these two monotropes that share the mycorrhizal fungal taxa at the family level; may be considered to have a sympatric relationship in that they both inhabit forest vegetation. Because we did not conduct a small-scale analysis of root systems among the plant species, such analyses will be necessary to clarify the presence/absence of the *Betula–M. humile* var. *humile* combination in mixed forests that contain *Betula*.

The Japanese forests contained three monotrope species: *M. hypopithys*, *M. uniflora*, and *M. humile* var. *humile*. *Monotropa hypopithys* and *M. uniflora* are associated specifically with the *Tricholoma* and Russulaceae, respectively (Bidartondo and Bruns 2001, 2002, Yang and Pfister 2006, Young et al. 2002). If these three monotropes co-exist within the same forest site, *M. uniflora* and *M. humile* var. *humile* may antagonize each other by seeking the same fungal taxa. In fact, in Nagano prefecture, these three monotrope species or the latter two species are often observed within the same forest (unpublished data). Phylogenetic data for these monotropes (Bidartondo and Bruns 2002) indicate that *M. humile* and *M. uniflora* occur within a clade that is beyond the taxonomy of the genera *Monotropastrum* and *Monotropa*, similar to the *Pterospora andromedea–Sarcodes sanguinea* relationship. In addition, the sympatric *P. andromedea* and *S. sanguinea* share the same fungal sister species group in the

genus *Rhizopogon* sect. *Amylopogon*, but never overlap in their mycorrhizal fungi at the species level. This suggests that *M. humile* and *M. uniflora* also share mycorrhizal fungi at the family level, but do not share the same fungal species, thus avoiding the scramble for nutrient acquisition; DNA fingerprinting for fungal identification would clarify whether this is the case. The fact that every plant sample that we analyzed exhibited a single mycorrhizal morphotype suggests that each *M. humile* var. *humile* individual is associated with a single fungal species after it germinates from seed and sustains the same fungus throughout its life span; this has been suggested for some other monotropes based on in-situ germination experiments (Bidartondo and Bruns 2005, Leake et al. 2005). *Monotropa humile* var. *humile* may be an ideal study species to allow researchers to determine how plants can use only a single fungal host in the presence of diverse potential candidates at seed germination and subsequent developmental stages.

The fundamental issue of whether the monotropoid mycorrhiza is a mutualistic association is difficult to explain at present (Bidartondo 2005). The association has a basically tripartite relationship that comprises a symbiotic ectomycorrhizal fungus, a plant, and an additional myco-heterotrophic monotrope. The fungus–monotrope association via monotropoid mycorrhizae is shortsightedly a host–parasite relationship in which the intracellular contents (carbon, nitrogen, phosphorus, etc.) of the fungal mycelium are merely taken up by the plant roots. However, the carbon source appears to originate from the ectomycorrhizal host tree, and the ectomycorrhizae that associate with both seedling and adult monotropes increase in biomass significantly (Bidartondo et al. 2000; Kretzer et al. 2000, Bidartondo and Bruns 2005, Leake et al. 2005). Therefore, it is possible that the ectomycorrhiza–monotrope relationship can be categorized as mutualistic. The mutualistic aspect of the symbiosis will be enforced if the associated fungus increases mushroom production in relation to increased mycorrhizal biomass and then acquires increased fitness, although an opposite phenomenon is reported that mushroom productions of *Tricholoma terreum* and *T. magnivelare* decrease by the association with *M. hypopithys* and *Allotropa virgata*, respectively (Martin 1985, Bidartondo 2005). Although we lack such quantitative data for the associated ectomycorrhizae, it is plausible that *M. humile* var. *humile* has a mutualistic association with the host fungi, based on the evolutionary linage of the monotropes. In addition, if the presence of *M. humile* var. *humile* increases the neighboring ectomycorrhizal biomass, any advantages of autotrophic hosts such as growth promotion by increased Russulaceae ectomycorrhizae through the monotropoid mycorrhizal association should reveal a tripartite mutualistic association within the forest ecosystem.

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Legend of Figure

Fig 1. Microscopic characteristics of mycorrhizal fungal sheath indicating *Russula* (**A-D**) and *Lactarius* (**E-H**). **A**: Obclavate cystidia of morphotype 5 (sample 98A-1), **B**: acicular cystidia of morphotype 5 (sample 98A-1), **C**: intracellular crystal of morphotype 26 (sample 00B-1), **D**: thick-walled extraradical hyphae of morphotype 28 (sample 97B-1), **E**: laticiferous hyphae of morphotype 12 (sample 00A-1), **F**: autofluoresence of a latex-containing laticiferous hyphae of morphotype 12 under UV irradiation (sample 00A-1), **G**: interlocking irregular synenchymatous tissue of morphotype 12 (sample 00A-1), **H**: oblong cells consisting of irregular synenchymatous tissue of morphotype 10 (sample 98B-1). Bars: 10µm.

Table 1. Fungal species diversity in the sampled forest sites.

Forest category	Collection site (vegetation*)	Number of:		Diversity Index***	
		Samples collected	Morphotypes observed	*H'*	*E'*
Man-made or occasionally managed forest	Minami-minowa, Nagano (Pd, Qs)	5	2	0.500	0.721
	Ina, Nagano (Qs, Cc)	3	1		
	Komagane, Nagano (Qs, Qat)	3	2	0.637	0.919
	Naka, Ibaraki	3	2	0.637	0.919
	(mean with SE in parentheses; n = 3)			0.591 (0.046)	0.853 (0.013)
Natural or semi-natural secondary forest	Azumi, Nagano (Av, Td, Bp, Bm)	5	4	1.332	0.961
	Matsunoyama, Niigata**	3	2	0.637	0.919
	Shinhotaka, Gifu	3	2	0.637	0.919
	Iijima, Nagano	3	3	1.099	1.000
	(mean with SE in parentheses; n = 4)			0.926 (0.174)	0.949 (0.019)

*Please see Appendix 1 for the abbreviation.

**Samples from two collection sites were pooled because they were neighboring stands with the same vegetation.

***Means of both *H'* and *E'* between forest categories were not statistically different, i.e. P = 0.170 and 0.167, respectively.

Table 2. Fungal species diversity in relation to geographic and environmental parameters.

Parameter	Category	Number of:		Diversity index	
		Samples collected	Morphotypes observed	*H'*	*E'*
Local area*	Shihoro (4)	4	3	1.039	0.946
	Ibaraki (2)	4	3	1.039	0.946
	Myoukou - Togakushi Mts. (4)	5	4	1.332	0.961
	Shigakougen - Asama Mts. (5)	5	5	1.609	1.000
	Norikura - Hotaka Mts. (5)	11	6	1.540	0.860
	Utsukushigahara - Yatsugatake Mts. (6)	7	5	1.475	0.917
	Foot of the Kiso Mountains (14)	23	8	1.623	0.781
Vegetation **	Fagaceous	41	22	2.994	0.969
	Conifer	23	16	2.483	0.896
	Mixed	14	7	1.362	0.700
Climatic zone**	Sub-alpine	18	11	2.057	0.858
	Cool-temperate	52	23	2.862	0.913
	Warm-temperate	8	6	1.733	0.967

*Collection sites in each categorized local area are listed in Appendix 1. Shihoro (4): Kamikawa, Shihoro, Kamishihoro; Ibaraki (2): Hitachi-ohmiya, Naka; Myoukou - Togakushi Mts. (4): Myoukoukougen, Shinano, Togakushi; Shigakougen - Asama Mts. (5): Kijimadaira, Yamanouchi, Suzakia, Tobu; Norikura - Hotaka (5): Azumi, Shinhotaka; Utsukushigahara - Yatsugatake Mts. (6): Shiojiri, Shimosuwa, Yachiho, Chino, Minamimaki; Foot of the Kiso Mountains (14): Minowa, Minami-minowa, Ina, Komagane, Iijima, Narakawa, Iida.

**Vegetation and the related climatic zone of each collection site are shown in Appendix 1.

Table 3. Comparison of the distribution patterns of frequently sampled mycorrhizal types.

Mycorrhizal			Numbers of samples in the following categories*							
			Vegetation			Climatic zone			Forest condition	
Type	Genus	Number of samples collected	Fag	Con	Mix	Sa	Ct	Wt	Artificial	Natural
26	*Russula*	11	4	7	0	7	4	0	0	11
1	*Russula*	10	4	1	5	0	9	1	8	2
2	*Russula*	7	4	0	3	0	7	0	7	0
20	unknown	5	3	1	1	1	2	1	3	2
11	*Lactarius*	3	2	0	1	0	3	0	0	2
14	*Lactarius*	3	3	0	0	0	3	0	0	3

*Abbreviations: Fag: fagaceous; Con: coniferous; Sa: sub-alpine; Ct: cool-temperate; Wt: warm-temperate.

Table 4. Comparison of distribution patterns between *Russula* and *Lactarius* mycorrhizae.

Mycorrhizal Genus	Sample number	Frequency (%) of samples in the following category*					
		Vegetation			Climatic zone		
		Fag	Con	Mix	Sa	Ct	Wt
Russula	45	46.7	33.3	20.0	28.9	62.2	8.9
Lactarius	15	60.0	26.7	13.3	26.7	73.3	0.0

*See Table 3 for abbreviations.

Appendix 1. List of *Monotropastrum humile* var. *humile* samples.

Site name	Latitude (N), Longitude (E)	Alt. (m)	Forest canopy*	Y/M of sampling	N	Sample ID
Kamikawa, Hokkaido	43˚ 40', 143˚ 06'	1000	Pj, As, Pg	03/Jul	1	03T-1
Shihoro, Hokkaido	43˚ 31', 143˚ 09'	700	As, Pg	02/Aug	1	02Q-1
Kamishihoro, Hokkaido	43˚ 22', 143˚ 12'	600	As, Bp, Alm	03/Jul	1	03R-1
Kamishihoro, Hokkaido	43˚ 20', 143˚ 10'	1000	As, Pj	03/Jul	1	03S-1
Toubetsu, Hokkaido	43˚ 25', 141˚ 39'	300	Lk, As, Bp	03/Jun	1	03I-1
Kuromatsunai, Hokkaido	42˚ 39', 140˚ 20'	100	Fc	02/Jun	1	03B-1
Miyako, Iware	39˚ 40', 141˚ 58'	50	Pt	97/Jun	1	97B-1
Asahi, Yamagata	38˚ 14', 139˚ 59'	600	Fc	98/Jul	1	98B-1
Hitachiohmiya, Ibaraki	36˚ 41', 140˚ 24'	250	Qs	98/Jun	1	98A-1
Naka, Ibaraki	36˚ 29', 140˚ 30'	40	Qs, Qat	97/Jun, 01/Jun	3	97A-1, 01F-1, 01F-2
Matsunoyama, Niigata	37˚ 05', 138˚ 38'	250	Fc, Qs, Qc	03/Jun	2	03C-1, 03C-2,
Matsunoyama, Niigata	37˚ 05', 138˚ 37'	250	Fc, Qs, Qc	03/Jun	1	03Q-1
Myoukoukougen, Niigata	36˚ 54', 138˚ 08'	1400	Fc	03/Jun	2	03F-1, 03F-2
Shinano, Nagano	36˚ 49', 138˚ 10'	900	Qc	02/Jun	1	02F-1
Shinano, Nagano	36˚ 47', 138˚ 10'	900	Qc, Bp	03/Jul	1	03N-1
Togakushi, Nagano	36˚ 43', 138˚ 07'	1100	Qc, Bp, Pd	02/Jul	1	02J-1
Kijimadaira, Nagano	36˚ 50', 138˚ 30'	1500	Fc	02/Jul	1	02O-1
Yamanouchi, Nagano	36˚ 42', 138˚ 30'	1700	Td, Bp, Am	02/Jul	1	02L-1
Yamanouchi, Nagano	36˚ 43', 138˚ 30'	1600	Bm, Bp, Qc	02/Jul	1	02P-1
Suzaka, Nagano	36˚ 33', 138˚ 20'	1400	Qc, Bp	02/Jul	1	02N-1
Toubu, Nagano	36˚ 25', 138˚ 26'	2100	Lk, Av, Bm	03/Jul	1	03P-1
Hakuba, Nagano	36˚ 44', 137˚ 49'	1000	Fc, Qc	02/Jul	1	02I-1
Azumi, Nagano	36˚ 07', 137˚ 36'	1800	Av, Td, Bp, Bm	00/Jul, 01/Aug	5	00A-1, 00A-2, 01O-1, 01O-2, 01Q-1
Azumi, Nagano	36˚ 06', 137˚ 37'	1600	Av	01/Aug	1	01P-1
Shiojiri, Nagano	36˚ 09', 138˚ 00'	800	Qs	02/May	1	02A-1
Shimosuwa, Nagano	36˚ 08', 138˚ 08'	1400	Qc	02/Jul	1	02M-1
Yachiho, Nagano	36˚ 04', 138˚ 22'	1800	Qc, Bp	01/Jul	2	01N-1, 01N-2
Chino, Nagano	36˚ 03', 138˚ 20'	2100	Av, Td	01/Jul	1	01M-1
Chino, Nagano	36˚ 03', 138˚ 20'	1800	Av, Td	00/Aug	1	00B-1
Minamimaki, Nagano	35˚ 59', 138˚ 25'	1700	Av, Td, Bp, Bm	03/Jul	1	03O-1

Ohshika, Nagano	35˚ 33', 138˚ 06'	1600	Ah, Td, Qc	03/Jul	2	03J-1, 03J-2
Minowa, Nagano	35˚ 55', 138˚ 00'	800	Qs, Pd	02/Jun	1	02C-1

Appendix 1. (continued)

Site name	Latitude (N), Longitude (E)	Alt. (m)	Forest canopy*	Y/M of sampling	N	Sample ID
Minami-minowa, Nagano	35˚ 54', 137˚ 56'	900	Qs, Cc	01/Jun	1	01D-1
Minami-minowa, Nagano	35˚ 52', 137˚ 56'	780	Pd, Qs	01/May	5	01A-1, 01A-2, 01A-3, 01B-1, 01B-2
Ina, Nagano	35˚ 51', 137˚ 56'	700	Qs, Cc	01/Jun	3	01C-1, 01C-2, 01C-3
Ina, Nagano	35˚ 48', 137˚ 53'	800	Pd	02/Jun	1	02E-1
Komagane, Nagano	35˚ 44', 137˚ 56'	900	Qs, Qat	01/Jun	3	01E-1, 01E-2, 01E-3
Komagane, Nagano	35˚ 44', 137˚ 53'	900	Qs, Pd	01/Jul	1	01J-1
Iijima, Nagano	35˚ 41', 137˚ 53'	900	Ts, Pd	02/Jun	1	02B-1
Iijima, Nagano	35˚ 40', 137˚ 50'	1300	Lk	03/Jul	1	03K-1
Iijima, Nagano	35˚ 41', 137˚ 51'	1250	Fc, Qc	03/Jul	1	03L-1
Iijima, Nagano	35˚ 41', 137˚ 50'	1400	Pk, Td	03/Jul	1	03M-1
Iida, Nagano	35˚ 33', 137˚ 49'	700	Qat	02/Jun	1	02D-1
Iida, Nagano	35˚ 27', 137˚ 55'	950	Qs, Pd, Pk, Ts	01/Oct, 03/Jun	2	01T-1, 03D-1
Narakawa, Nagano	35˚ 52', 137˚ 51'	1400	Ah, Td, Qc, Bg	02/Jun	1	02H-1
Ootaki, Nagano	35˚ 51', 137˚ 32'	1300	Cc, Qc	02/Jun	1	02G-1
Shinhotaka, Gifu	36˚ 16', 137˚ 36'	2100	Pjh	01/Jul	1	01K-1
Shinhotaka, Gifu	36˚ 17', 137˚ 35'	1300	Qc, Bp	01/Jul	3	01L-1, 01L-2, 01L-3
Shinhotaka, Gifu	36˚ 17', 137˚ 35'	1300	Qc, Fc, Ah	02/Jul	1	02K-1
Asahi, Toyama	36˚ 58', 137˚ 35'	50	Cs, Qa	03/May	1	03A-1
Shiramine, Ishikawa	36˚ 09', 136˚ 37'	700	Fc, Qc	03/Jun	2	03G-1, 03H-1
Ohama, Fukui	35˚ 24', 135˚ 38'	800	Fc	03/Jun	2	03E-1, 03E-2
Higashiyama, Kyoto	nd		nd	01/Apr	1	01R-1
Miike, Miyazaki	31˚ 57', 131˚ 02'	1300	Cs	01/Jun	1	01G-1
Tairoike, Kagoshima	31˚ 55', 131˚ 02'	1400	Pd, Af	01/Jun	1	01H-1

*Abbreviations: Af: *Abies firma*; Ah: *Abies homolepis*; Am: *Abies mariesii*; As: *Abies sachalinensis*; Av: *Abies veitchii*; Alm: *Alnus maximowiczii*; Bg: *Betula grossa*; Bm: *Betula maximowicziana*; Bp: *Betula platyphylla* var. *japonica*; Cc: *Castanea crenata*; Cs: *Castanopsis sieboldii*; Fc: *Fagus crenata*; Lk: *Larix kaempferi*; Pg: *Picea glehnii*; Pj: *Picea jezoensis*; Pjh: *Picea jezoensis* var. *hondoensis*; Pd: *Pinus densiflora*; Pk: *Pinus koraiensis*; Pt: *Pinus thunbergii*; Qa: *Quercus acuta*; Qat: *Quercus acutissima*; Qc: *Quercus crispula*; Qs: *Quercus serrata*; Td: *Tsuga diversifolia*; Ts: *Tsuga sieboldii*.

Appendix 2. Characteristics of the mycorrhizal morphotypes of *Monotropastrum humile* var. *humile*

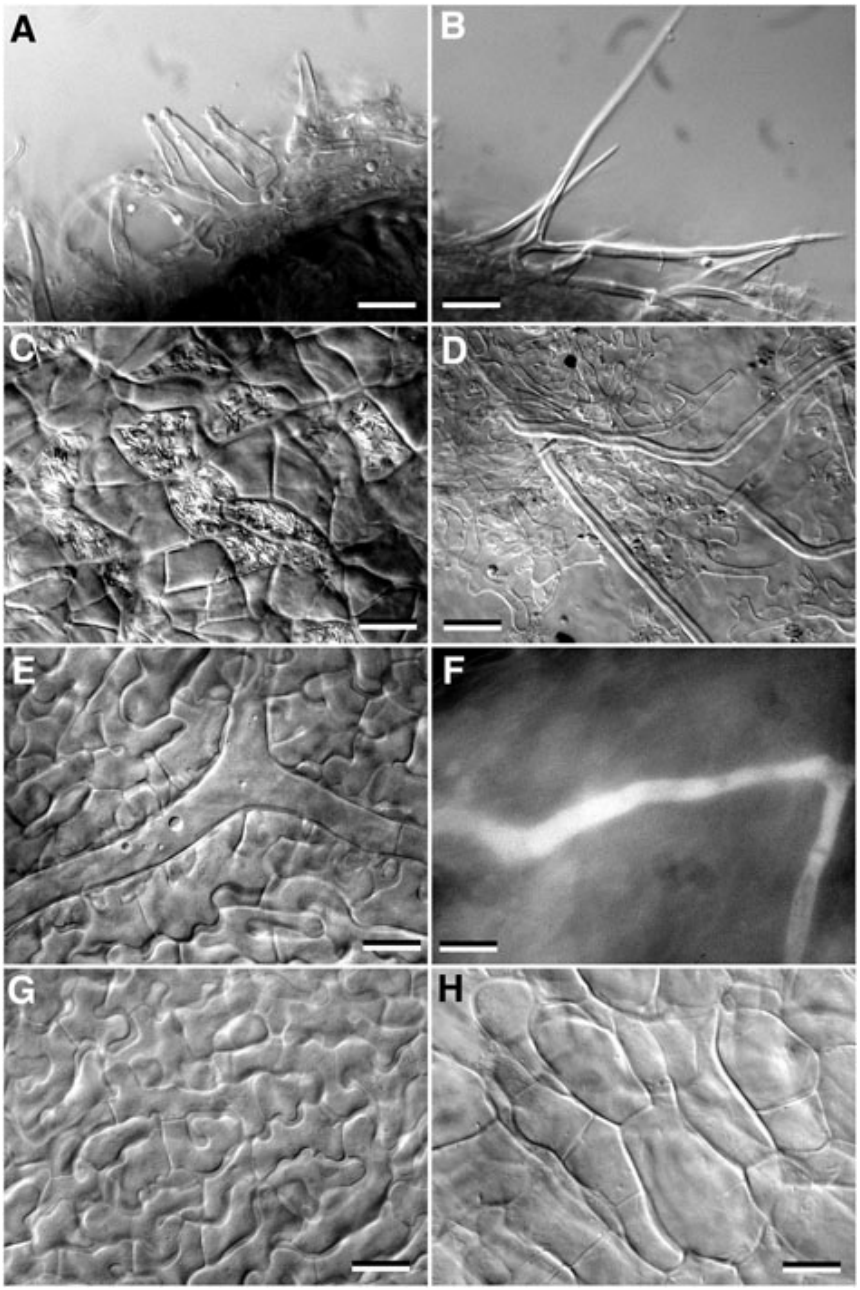
Morphotype	Fungal sheath			Diameter (µm) of the extraradical hyphae	Putative fungal genus	Related samples
	Surface layer*		Cystidium** and other specialized hyphae			
	Texture	Size (µm) and other characteristics of cells				
1	NSS/NS		Frequent, obclavate with knob at the apex, 15–35 µm in length	2.0–2.5	*Russula*	01A-1, 01A-2, 01A-3, 01B-1, 01E-2, 01E-3, 02A-1, 02D-1, 02H-1. 03K-1
2	NSS		Sparse, obclavate, 12–20 µm in length	2.0–2.5	*Russula*	01B-2, 01C-1, 01C-2, 01C-3, 01E-1, 01J-1, 02C-1
3	NS		Frequent, obclavate, 15–30 µm in length		*Russula*	01R-1
4	FP/NP		Obclavate to subulate, 40–80 µm in length	3.0–5.0	*Russula*	02P-1
5	NSS		Obclabate with knob at the apex, 15–35 µm; acicular with dichotomous branching, 30–60 µm		*Russula*	98A-1
6	NSS		Obclavate to sublate, thick-walled, 30–45 µm in length	2.0–2.5	*Russula*	02Q-1, 03T-1
7	NSS		Obformis to more slender, 20–35 µm in length	2.0–2.5	*Russula*	03I-1
8	ISI/ISN	5–10 x 10–15	Laticiferous hyphae present	2.0–2.5	*Lactarius*	01M-1
9	ISI/ISN	10–15 x 10–15	Laticiferous hyphae present	2.0–2.5	*Lactarius*	02J-1
10	ISN	7–20 x 15–35	Laticiferous hyphae present	2.0–2.5	*Lactarius*	98B-1, 02F-1
11	ISN	15–25 x 20–35	Laticiferous hyphae present	2.0–2.5	*Lactarius*	02K-1, 03E-1, 03E-2
12	ISI	4–8 x 12–20, thick-walled	Laticiferous hyphae present		*Lactarius*	00A-1
13	ISN	15–20 x 25–35	Laticiferous hyphae present	2.0–3.0	*Lactarius*	01L-1, 01L-3
14	ISI	7–10 x 10–15, thick-walled	Laticiferous hyphae present	2.5–3.5	*Lactarius*	03H-1, 03L-1, 03N-1
15	ISI	5–10 x 7–15	Laticiferous hyphae present	2.0–2.5	*Lactarius*	03P-1
16	ISI/NS	5–10 x 15–25	Laticiferous hyphae present	2.0–2.5	*Lactarius*	03O-1
17	ISI	5–10 x 15–20		1.5–2.0	unknown	01D-1, 03B-1
18	ISI	2–5 x 7–12		2.5–3.5	unknown	01G-1
19	ISI	4–8 x 10–15, thick walled		1.5–2.5	unknown	03D-1, 03M-1
20	ISI/ISN	5–10 x 10–20, thick-walled		2.0–2.5	unknown	01F-1, 01F-2, 01O-2, 01T-1, 02N-1

Appendix 2. (continued)

Morphotype	Fungal sheath			Diameter (µm) of the extraradical hyphae	Putative fungal genus	Related samples
	Surface layer*		Cystidium** and other specialized hyphae			
	Texture	Size (µm) and other characteristics of cells				
21	ISI	3–8 x 10–20		2.0–2.5	unknown	02B-1
22	ISI/ISN	5–10 x 10–20, thick-walled		2.0–2.5	unknown	03A-1
23	ISI	2–4 x 5–10		1.5–2.5	unknown	03J-1, 03J-2
24	NP	subsurface layer: ISI/NS, 2–4 x 5–10		2.0–2.5	unknown	02G-1
25	NSS	subsurface layer: ISN	Thick-walled extraradical hyphae present	1.0–1.5	*Russula*	01H-1
26	ISN/RS	4–12 x 10–20, intracellular crystal, sparsely exfoliated	Thick-walled extraradical hyphae present	2.0–2.5	*Russula*	00B-1, 01K-1, 01L-2, 01N-1, 01N-2, 01O-1, 01P-1, 01Q-1, 02I-1, 02L-1, 03S-1
27	ISI	4–12 x 10–15	Thick-walled extraradical hyphae present	2.0–3.0	*Russula*	03C-1, 03C-2
28	ISI	4–8 x 8–15, intracellular crystal	Thick-walled extraradical hyphae present		*Russula*	97B-1
29	ISN	10–20 x 20–30		2.5–3.0	unknown	03F-1, 03F-2
30	ISI/ISN	4–8 x 8–12		2.0–2.5	unknown	02M-1
31	ISN/RS	10–15 x 15–25, intracellular oily droplet		2.0–2.5	unknown	03Q-1
32	ISN	7–15 x 12–25, thick-walled, intracellular oily droplet	Thick-walled extraradical hyphae present	2.5–3.0	*Russula*	03R-1
33	ISN/RS	8–15 x 15–30, intracellular granules		2.0–2.5	*Russula*	02E-1
34	ISN/RS	5–10 x 7–15	Thick-walled extraradical hyphae present	2.0–2.5	*Russula*	02O-1
35	RS/ISN	4–12 x 10–20			unknown	00A-2
36	RS	3–15 x 6–20, intracellular oily droplet		2.5–3.0	unknown	03G-1
37	RS	8–15 x 10–20, intracellular crystal		nd	*Russula*	97A-1

*Abbreviations: FP: Felt prosenchyma; ISI: Irregular synenchyma, interlocking; ISN: Irregular synenchyma, not interlocking; NP: Net prosenchyma; NS: Net synenchyma; NSS: Net synenchyma, straightly arrayed; RS: Regular synenchyma (Ingleby et al. 1990).

**Terminology is based on Kirk et al. (2001).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