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ORIGINAL ARTICLE

Localization of helotialean fungi on ectomycorrhizae of *Castanopsis cuspidata* visualized by in situ hybridization

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Abstract Non-ectomycorrhizal fungi that associate with typical ectomycorrhizae often remain hidden, and their localization inside ectomycorrhizal (ECM) roots has remained uncharacterized. In this study, the fungal community associated with the ectomycorrhizae of *Castanopsis cuspidata* was investigated using a culture-dependent isolation technique. Additionally, the species composition and localization were determined using molecular techniques. The results of the isolation and identification of fungal species revealed the predominance of a few species belonging to the order Helotiales. Furthermore, the fungal community structures were significantly different depending on the taxa of the ectomycorrhiza-forming fungi. A taxon-specific probe was developed to analyze the localization of one dominant Hyaloscyphaceae (Helotiales) species in ECM tissues by in situ hybridization. Hybridization signals were detected on the surface of the fungal mantle and around the ECM fungal cells within the mantle. Hyphal penetration into ECM hyphal cells of fungal mantles was also observed. Signals were not detected in the Hartig net or plant tissues inside the mantle in healthy ectomycorrhizae. These findings suggest that the analyzed species interact not only with host plant as root endophyte but also directly with the ECM fungi.

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Introduction

Within an ectomycorrhizal (ECM) community, non-ECM fungi have often been detected during field surveys (Kernaghan et al. 2003; Rosling et al. 2003; Urban et al. 2008; Leski et al. 2010). Such non-ECM fungi seem to ubiquitously colonize ECM roots as they have been frequently detected with molecular-based or culture-dependent approaches (Kernaghan and Patriquin 2011; Vohník et al. 2013). The non-ECM fungi commonly belong to the order Helotiales (phylum: Ascomycota), which represents the largest group in the class Leotiomycetes with 13 families and 395 genera (Wang et al. 2006). These species cover a broad range of niches and have been described as saprobes, plant pathogens, endophytes, and mycorrhizal fungi. Dark septate root endophytes, such as the *Phialocephala fortinii* s.l.–*Acephala applanata* species complex (Grünig et al. 2008) or the ericoid mycorrhizal *Hymenoscyphus ericae* aggregate (Vrålstad et al. 2000), have been extensively investigated as the major colonizers of the ectomycorrhiza-forming roots of diverse host plants, including conifers and angiosperms. However, in some cases, different types of ectomycorrhiza-associated fungi with non-melanized and unidentified hyphae are the predominant species (Kernaghan and Patriquin 2011, 2015). Members of the helotialean group have recently been identified as the dominant species in the roots of Fagaceae trees in the temperate and subtropical forests of Japan (Toju et al. 2013a, b, 2014). These results may illustrate the complex colonization patterns and variability of fungal root endophyte species in different hosts and environmental conditions.

Despite the increasing number of studies on ectomycorrhiza-associated helotialean species, their ecological role remains unclear. Considering ECM fungi are important for the growth and regeneration of host trees, ectomycorrhiza-associated fungi likely also affect forest ecosystems. Some ectomycorrhiza-associated helotialean species have been described as ECM fungi (Vrålstad et al. 2002a; Villarreal-Ruiz et al. 2004; Tedersoo et al. 2008; Wang et al. 2011; Huang et al. 2014), while others are regarded as putative endophytes, mycoparasites, plant pathogens, or entomopathogens (Tedersoo et al. 2009). The behaviors of ectomycorrhiza-associated fungi have been analyzed on ectomycorrhizae-forming trees under experimental conditions. The fungal isolates were often endophytic (Hashimoto and Hyakumachi 2001; Vrålstad et al. 2002a; Bergero et al. 2003; Grelet et al. 2009; Vohník et al. 2013). The endophytic fungi on ECM roots appear to coexist with ECM fungi. However, it is unclear how root-associated helotialean fungi interact with ECM fungi in the field. There is currently limited information regarding the localization of ectomycorrhiza-associated fungi in ECM roots because of the technical difficulties associated with targeting the fungi.

The objective of this study was to clarify the colonization patterns of helotialean fungi on ECM tissues in *Castanopsis cuspidata*-dominated forests. *Castanopsis cuspidata* (family: Fagaceae) is an evergreen tree and it is one of the major canopy trees in southwestern Japanese secondary forest (Tagawa 1995). To reveal the potential interactions between ectomycorrhiza-associated fungi and ECM fungi and/or plant hosts, we characterized a community of ectomycorrhiza-associated fungi by isolating and identifying species. We also visualized their localization patterns in ECM roots using a species-specific in situ hybridization technique with a newly developed probe.

Materials and methods

Sampling method

Rhizosphere soil was sampled from the 100-m^2 study site in the Kodaiji-san Mountain National Forest (N 34° 59′ 49″, E 135° 47′ 8″; 150 m above sea level) in Kyoto, Japan. The experimental stands supported *Castanopsis cuspidata* as sole ectomycorrhizae-forming trees with ground vegetation consisting of *Camellia japonica*, *Damnacanthus indicus*, *Pieris japonica*, and *C. cuspidata* seedlings. The study site was divided into 25 4-m^2 subplots. Approximately 100-ml soil cores (5 cm diameter × 5.1 cm depth), including the organic layer, were collected from the center of 12 subplots, which were alternately positioned at the sampling site. If a sample lacked or contained only a few ectomycorrhizae, we arbitrarily selected another point within the same subplot that contained a sufficient number of ectomycorrhizae. Samples for the fungal isolation and localization tests were collected in June 2013 and June to July 2017, respectively.

Fungal isolation from ectomycorrhizae samples

All soil samples were transported to the laboratory and stored at 4 °C until used. Fungi were isolated within 1 week of sampling. All ectomycorrhizae in soil cores were dissected and classified based on color, surface texture, and density of extraradical mycelium for each of individual soil cores. The ectomycorrhizae were transferred to Petri dishes for random selections. They were cleaned by removing debris and then stirring for 30 min in flasks with 100 ml deionized water containing three drops of Tween 80. Representative samples of each ECM morphotype were stored in 2× cetyltrimethylammonium bromide (CTAB) buffer [2% (*w*/*v*) CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris HCl (pH 8.0), and 1% (*w*/*v*) polyvinylpyrrolidone] at − 20 °C for the molecular identification of ectomycorrhiza-forming fungi. All cleaned ectomycorrhizae were surface-sterilized in 5% (*w*/*v*) $Ca(ClO)_2$ for 2 min and then rinsed three times in sterilized deionized water. The ECM root tips were cut into two pieces, and one piece was aseptically placed onto Modified Norkran's C medium (Yamada and Katsuya 1995) supplemented with 100 mg/l chloramphenicol and incubated at 25 °C for 1 month. The obtained isolates were used for molecular identifications. Fast-growing isolates were not identified as they were considered contaminants. However, morphotypes repeatedly isolated across several subplots were used for molecular identifications even if the isolates were fast-growing and potentially contaminants.

Fungal DNA extraction for molecular identifications

We extracted DNA from fungal cultures isolated from ectomycorrhizae as described by Izumitsu et al. (2012). A small amount of mycelia was collected from each isolated colony using a sterilized toothpick and suspended in 100 μl 10-fold diluted Tris-EDTA (TE) buffer in a microtube. Samples were microwaved (100 V, 600 W) twice for 1 min each and then centrifuged at 10,000 rpm for 5 min. The supernatants were used as templates for polymerase chain reaction (PCR) analyses.

A CTAB method was used to extract DNA from ECM root tips. Briefly, ECM root tips in 2× CTAB buffer were frozen in liquid nitrogen and heated three times at 65 °C in a block incubator. The root tips were crushed with a homogenizer pestle and then incubated at 65 °C for 30 min. Samples were purified with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol solutions. The DNA was precipitated with isopropyl alcohol and rinsed with 70% ethanol. Pellets were dried with a CC-105 centrifugal concentrator

(TOMY Digital Biology, Tokyo, Japan) and dissolved in 50 μl 10-fold diluted TE buffer. The extracts were stored at − 20 °C until used for molecular identifications.

PCR amplification and species identification by restriction fragment length polymorphisms and nucleotide sequencing

Representative morphotype species of ectomycorrhiza-forming fungi were identified based on the nucleotide sequences of the internal transcribed spacer (ITS) region. The PCR amplification was completed with the ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) primer pair and a T100 Thermal Cycler (Bio-Rad, Hercules, California, USA). The 10-μl PCR mix contained 1 μl 10× Blend Taq buffer, 0.4 μM each primer, 0.2 mM dNTP mixture, 0.25 U Blend Taq polymerase (TOYOBO, Osaka, Japan), and 1 μl template DNA. The PCR cycling conditions were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; 72 °C for 5 min.

Isolates obtained from ECM root tips were classified based on colony morphology and restriction fragment length polymorphism (RFLP) analysis of the ribosomal ITS sequence. The NSA3 (5′-AAACTCTGTCGTGCTGGGGATA-3′) and NLC2 (5′-GAGCTGCATTCCCAAACAACTC-3′) primer pair (Martin and Rygiewicz 2005) was used to PCR amplify DNA extracts from fungal cultures prior to the RFLP analysis. The PCR cycling conditions were as follows: 95 °C for 7 min; 35 cycles of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 40 s; 72 °C for 5 min. The PCR products were subsequently used for RFLP analyses involving the restriction endonucleases *Alu*I and *Hinf*I. Diluted PCR products were digested with each enzyme at 37 °C for 6 h. The PCR products and their digested fragments were separated by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light to analyze their banding patterns.

Representative ectomycorrhiza-forming fungi and one to nine samples from each RFLP type were analyzed by DNA sequencing. For ECM fungi identification, each of 1 to 12 ECM root tips was separately used for DNA extraction for each ECM morphotype. The PCR products were purified by gel electrophoresis and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) with the ITS1F and ITS4 primer pair and the 3130xl Genetic Analyzer (Thermo Fisher Scientific). To identify isolates, we searched for the most similar sequences using the BLAST online tool (http://www.ncbi.nlm.nih.gov/) (GenBank, NCBI). For the sequences from the ECM root tips, we searched the UNITE database (http://unite.ut.ee/). PCR products with different lengths, i.e., samples representing two or more bands were analyzed separately. Putative non-ECM fungi, e.g., Hyaloscyphaceae sp. 1 or Helotiales sp. 1 were excluded from the ECM species candidates. Also, samples that generated multiple PCR products which could not be separated were excluded from further analysis.

Statistical analysis

Data were analyzed using the R software. The fungal community structures (especially the proportions of Hyaloscyphaceae sp. 1 and Helotiales sp. 1) of the ectomycorrhizae of *Cortinarius obtusus*, *Russula* spp., and *Lactarius* sp. were obviously different. Thus, the proportions of Hyaloscyphaceae sp. 1 (i.e., the target species for in situ visualization) for all root tips used for fungal isolations were compared using a chi-squared test. A pairwise comparison with Bonferroni correction was then completed for each combination of the abovementioned three ECM groups.

Design of oligonucleotide probes

Because the isolation test results revealed the predominance of Hyaloscyphaceae sp. 1 at the study site, this species was targeted during the in situ visualization experiment. Nucleotide sequences of the D1/D2 region of the 26S rRNA region were determined using the NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primer pair (Vilela et al. 2005) as described above. The D1/D2 sequences from major putative-helotialean species (i.e., LC189052: Hyaloscyphaceae sp. 1, LC189055: Helotiales sp. 1, LC189053: Hyaloscyphaceae sp. 2, and LC189054: Helotiales sp. 2) were aligned using the ClustalW program. About 20 bases of a species-specific region were used to design the specific probe (i.e., G2-2: 5′-GTGCACCAGTGAGAACACCG-3′). The complementary sequence was used to design the negative control probe (i.e., non-G2-2: 5′-CGGTGTTCTCACTGGTGCAC-3′). The probes were synthesized by Fasmac (Kanagawa, Japan). The G2-2 probe was unable to discriminate between Hyaloscyphaceae sp. 1 and Hyaloscyphaceae sp. 2 because they were too closely related (i.e., 97.4% similarity in the ITS2 sequence), with no detectable sequence difference in the selected region. A fungal universal probe (i.e., R898: 5′-ATCCAAGAATTTCACCTCT-3′) (Tanaka 2009) targeting 18S rRNA was used as a positive control in the probe specificity test. Oligonucleotides were labeled with digoxigenin (DIG) using the DIG Oligonucleotide 3′-end Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Probe specificity test and in situ hybridization

Fungal isolates were cultured in liquid Modified Norkran's C medium for 2 weeks prior to the probe specificity test. In

addition to Hyaloscyphaceae sp. 1 (LC189052), isolates at the experimental site (i.e., Helotiales sp. 1, LC189053 and Hyaloscyphaceae sp. 3 LC190974) and another five helotialean isolates which were isolated from surface-sterilized roots of *Castanopsis cuspidata* and *Quercus* spp. collected in Tottori and Kyoto prefecture in 2015 by the authors (i.e., Hyaloscyphaceae sp. 5: LC314065, Hyaloscyphaceae sp. 6: LC314066, Hyaloscyphaceae sp. 7: LC314067, Helotiales sp. 4: LC314068 and *Lachnum* sp.: LC314069) were used as reference samples. The in situ hybridization procedure was conducted as described by Tanaka et al. (2016) with a slight modification. The hybridization was conducted in a 1.5-ml microtube. The pretreatment reagents were treated with diethylpyrocarbonate and autoclaved. Approximately 0.5–1 mg (wet weight) of cultivated hyphae fixed in 4% PFA in PBS was deaerated for 10 min. The hyphae were then treated with 0.2 N HCl at room temperature for 20 min, and proteinase K (10 μg/ml) at 37 °C for 45 min. Samples were dehydrated with an ethanol series and dried using the CC-105 centrifugal concentrator. The tubes containing hyphae were placed in an HL-2000 HybriLinker hybridization chamber (UVP, Upland, CA, USA), and samples were saturated with hybridization reagent, which included hybridization buffer, DIG-labeled probe, and DNA, MB grade (Roche Diagnostics) as the carrier DNA. Samples were incubated at 45 °C for 16 h. The hybridization buffer contained the following components: 25% formamide, 4× SSC, 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0), 1 mM EDTA, and 5× Denhardt's solution. After the hybridization, samples were washed twice for 5 min with 2× SSC containing 0.1% sodium dodecyl sulfate at room temperature. They were then washed twice for 45 min with 0.5× SSC containing 0.1% sodium dodecyl sulfate at 45 °C while being rotated. The samples were analyzed using the DIG Nucleic Acid Detection kit (Roche Diagnostics) according to the manufacturer's protocol.

The permeability of cells for the probes may differ among fungal strains, which can affect the hybridization and staining results. Therefore, a dot blot hybridization and in silico similarity test was used to confirm the specificity of the probe for the Hyaloscyphaceae sp. 1 sequence. Fungal genomic DNA extracted using the CTAB method was dissolved in TE buffer. The DNA solution was heated at 100 °C for 5 min and cooled on ice. A 1-μl aliquot was blotted on the Biodyne PLUS 0.45 μm nylon membrane (PALL Corporation, Port Washington, NY, USA), which was then exposed to UV irradiation for cross-linking. The hybridization steps were completed as described above. The probe specificity was also tested using the BLAST online tool (http://www.ncbi.nlm.nih.gov/).

For in situ hybridizations, ectomycorrhizae sections were prepared again for five of the 12 subplots used for the isolation test. The ECM root tips were embedded in Tissue-Tek (Sakura Finetek Japan), frozen with liquefied carbon dioxide, and sliced into segments (25–30 μm thick) using the HM 400 R sliding microtome (Microm Laborgeräte, Walldorf, Germany) with a C35-type microtome blade (FEATHER Safety Razor, Osaka, Japan). Sliced sections were immediately fixed in 4% PFA in PBS. The pretreatment of sections, hybridization, and detection were conducted in a 1.5-ml tube as described above.

The frequency of hybridization signals was determined by dividing the number of ECM sections that produced signals by the total number of analyzed ECM sections. Ectomycorrhizae formed by the most dominant ECM species at the study site (i.e., *C. obtusus*) were identified according to morphological characteristics. We collected 5-mm tips from five ECM roots at each subplot. The hybridization procedure was conducted as described above. The number of the sections that produced hybridization signals was counted with a tally counter.

Results

Species composition of the ectomycorrhiza-associated fungal community

To determine the major fungal species associated with ECM roots, fungi were isolated from ECM root tips. We identified 359 of the 421 cultures isolated from 834 ECM root tips (Table 1). With 155 isolates, Hyaloscyphaceae sp. 1 was the most common fungus (i.e., > 36% of all isolates). Thus, this species was selected as the target for the in situ hybridization localization test. Additionally, 105 and 26 isolates corresponded to Helotiales sp. 1 and Hyaloscyphaceae sp. 2, respectively. Thus, about 68% of all isolates were one of these three Helotiales species. Furthermore, the sequences of 15, 10, and 8 isolates were similar to those of *Nemania*, *Oidiodendron*, and *Cladophialophora* species, respectively.

Varying ectomycorrhiza-associated fungal community with ectomycorrhiza-forming species

The ECM root tips of each subplot were classified into three to seven morphotypes based on color, surface texture, and density of extraradical mycelium. The 31 root tips that generated ITS1–5.8S–ITS2 fragments were used for sequencing analyses. We observed that the ECM root tips successfully analyzed were formed by the following 14 species (in descending order of root tip number): *Cortinarius obtusus*, *Cortinarius* sp., *Russula* sp. 1, *Russula* sp. 2, *Russula* sp. 3, *Russula* sp. 4, *Lactarius* sp., Thelephoraceae sp., *Gymnascella* sp., *Boletellus* sp., Boletaceae sp., *Xerocomus* sp., *Coltriciella* sp., and *Tylopilus felleus*. Morphological characters by which ectomycorrhizae was categorized and the number of ECM root tips successfully used for DNA identification (in parentheses) are shown for each ECM morphotype as follows.

Table 1 Fungal species obtained during the isolation test from *Castanopsis cuspidata* ECM root tips. Isolates were identified using a BLAST nucleotide sequence comparison of the ITS1–5.8S–ITS2 region. Species are listed in descending order of isolation frequency

Identity	No. of isolates	Accession number	Closest GenBank match	Similarity (%)	Coverage (%)
Hyaloscyphaceae sp. 1	155	LC189021	JQ272392 Hyaloscyphaceae 1 RB-2011 voucher AM3BB2E2	97	100
Helotiales sp. 1	105	LC189022	JQ272398 Ascomycota sp. 9 RB-2011 voucher AM3BB9E12	93	100
Hyaloscyphaceae sp. 2	26	LC189023	JQ272392 Hyaloscyphaceae 1 RB-2011 voucher AM3BB2E2	98	100
Nemania sp.1	9	LC189032	KP050582 *Nemania* sp. HD-2014 isolate DO27	99	91
Mucoromycotina sp.	6	LC189046	HQ406814 Mucoromycotina sp. 1277	98	78
Oidiodendron sp. 1	6	LC189545	AF062811 *Myxotrichum cancellatum*	98	100
Cladophialophora sp. 1	5	LC189025	AB986333 *Cladophialophora* sp. KO-groupA 2014	98	98
Helotiales sp. 2	5	LC189028	JQ272398 Ascomycota sp. 9 RB-2011 voucher AM3BB9E12	90	95
Hyaloscyphaceae sp. 3	5	LC189030	KP723459 Helotiales sp. D102	99	96
Umbelopsis nana	4	LC189036	KU516641 *Umbelopsis nana* isolate 356J14	99	100
Xylariaceae sp. 1	4	LC189045	AB741584 Xylariaceae sp. 4Y-Dg3-3	94	89
Chloridium sp.	2	LC189044	AB734790 *Chloridium* sp. A6-2	99	91
Cladophialophora sp. 2	2	LC189547	AB986415 *Cladophialophora* sp. KO-groupD 2014	99	100
Nemania sp. 2	2	LC189037	LC030441 Fungal sp. tuti-3	100	91
Nemania sp. 3	2	LC189038	KJ957776 *Nemania* sp. 11G008	100	100
Nemania sp. 4	2	LC189034	KP689109 *Nemania* sp. N155	99	100
Oidiodendron sp.2	2	LC189043	AF062811 *Myxotrichum cancellatum*	98	100
Scopulariopsis sp.	2	LC189049	LN850773 *Scopulariopsis* sp. BMU03910	100	96
Sordariomycetes sp.	2	LC189039	AY699691 Fungal sp. R47	98	90
Annulohypoxylon sp.	1	LC189050	KC345694 *Annulohypoxylon* sp. ZJLQ494	99	100
Ascomycota sp. 1	1	LC189035	FJ999654 Ascomycota sp. 528	87	92
Ascomycota sp. 2	1	LC189040	KU640382 *Pyrenochaeta ligni-putridi* isolate ZT 92163	98	97
Cladophialophora sp. 3	1	LC189029	GQ996076 Fungal sp. mh4293.4	98	92
Conlarium sp.	1	LC189031	AB847000 *Conlarium* sp. KO-2013	97	91
Helotiales sp. 3	1	LC189042	FN394702 Fungal endophyte	96	89
Hyaloscyphaceae sp. 4	1	LC189048	AB986370 Hyaloscyphaceae sp. KO-groupA 2014	98	94
Oidiodendron sp. 3	1	LC189051	AF062800 *Oidiodendron maius* strain UAMH 8921	100	100
Oidiodendron sp. 4	1	LC189546	EU888920 *Oidiodendron maius strain* EYR71	99	99
Phaeomoniella sp.	1	LC189041	KX908576 Eurotiomycetes sp. genotype 801 JMUR-2016 voucher ARIZ:NC0838	100	90
Rhytismataceae sp.	1	LC189026	JQ272405 Rhytismataceae sp. RB-2011	89	100
Xylariaceae sp. 2	1	LC189027	KT289576 Fungal sp. voucher Robert L. Gilbertson Mycological Herbarium 6606	95	100
Xylariaceae sp. 3	1	LC189033	AB741584 Xylariaceae sp. 4Y-Dg3-3	96	89

C. obtusus: white to brown, extraradical mycelium developed densely around ectomycorrhiza, irregularly branching, partly hydrophobic, (5). *Cortinarius* sp.: white, rhizomorphs abundant, irregularly branching, hydrophobic, (1). *Russula* spp.: white to brown, extraradical mycelium abundant or sparse, monopodial pinnate or no branching, (12). *Lactarius* sp.: light brown, no extraradical mycelium, no branching, smooth surface, (4). Thelephoraceae sp.: white, no extraradical mycelium, no branching, cystidia present, (2). *Gymnascella* sp.: dark brown, extraradical mycelium sparsely present, no branching, smooth surface, (1). *Boletellus* sp.: white, no extraradical mycelium, no branching, hydrophobic, (1). Boletaceae sp.: white, no extraradical mycelium, no branching, hydrophobic, (1). *Xerocomus* sp.: white, rhizomorphs present, no branching, hydrophobic, (1). *Coltriciella* sp.: dark brown, no extraradical mycelium, no branching, cystidia present, (1). *Tylopilus felleus*: brown, no extraradical mycelium, no branching, hydrophobic, (2). Although molecular analyses revealed clear differences among the four *Russula* species, it was difficult to differentiate between these species based on morphological features. Therefore, we treated the *Russula* species as one group. The ectomycorrhizae of *Gymnascella* sp., *Boletellus* sp., Boletaceae sp., *Xerocomus* sp., and *Coltriciella* sp. were not used for fungal isolations because of an insufficient number

of samples. The ectomycorrhizae formed by *C. obtusus* yielded 124 fungal isolates, including 75 isolates of Hyaloscyphaceae sp. 1, six isolates of Helotiales sp. 1, 28 isolates of Hyaloscyphaceae sp. 2, and 28 isolates of other species. The ectomycorrhizae of *Russula* spp. yielded 158 isolates, including 36, 56, and 7 isolates of Hyaloscyphaceae sp. 1, Helotiales sp. 1, and Hyaloscyphaceae sp. 2, respectively. The ectomycorrhizae of *Lactarius* sp. yielded 105 isolates, including 40 Hyaloscyphaceae sp. 1 isolates, 36 Helotiales sp. 1 isolates, and a single Hyaloscyphaceae sp. 2 isolate. The ectomycorrhizae of the other species, including Thelephoraceae sp., *Tylopilus felleus*, and *Cortinarius* sp., yielded four isolates of Hyaloscyphaceae sp. 1, six isolates of Helotiales sp. 1, and two isolates of Hyaloscyphaceae sp. 2. Because most of the ectomycorrhizae were formed by three fungal groups (i.e., *C. obtusus*, *Russula* spp., and *Lactarius* sp.), we compared the corresponding ECM fungal communities (Fig. 1). The proportion of Hyaloscyphaceae sp. 1, which was the dominant species, was significantly different among the three ectomycorrhiza-forming species according to the chi-squared test ($p < 0.01$). Additionally, a pairwise comparison with Bonferroni correction revealed that the proportion of Hyaloscyphaceae sp. 1 in the *C. obtusus* ectomycorrhizae was significantly higher than that of the other two groups ($p < 0.01$). There was no significant difference in the proportion of Hyaloscyphaceae sp. 1 for the *Russula* spp., and *Lactarius* sp. ectomycorrhizae.

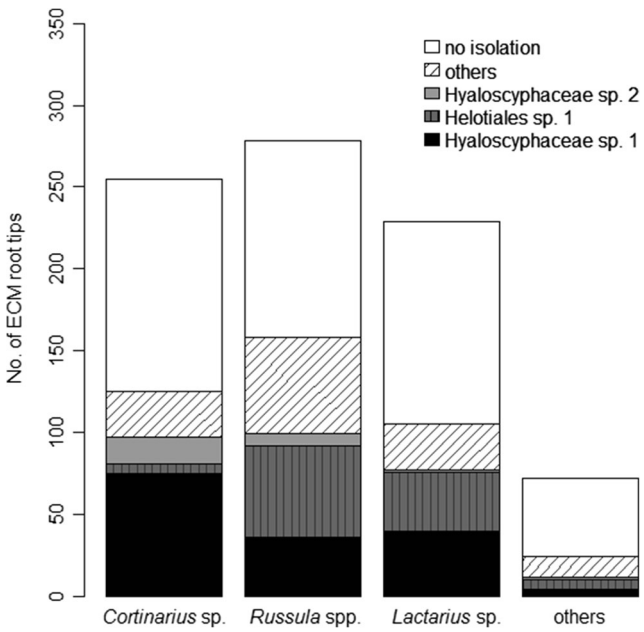

Fig. 1 Species composition of ectomycorrhiza-associated fungal isolates obtained from each ECM taxon. The number of isolates of three major ectomycorrhiza-associated fungi (i.e., Hyaloscyphaceae sp. 1, Helotiales sp. 1, and Hyaloscyphaceae sp. 2) and of other species are presented for each of the following three dominant ECM taxa at the study site: *Cortinarius obtusus*, *Russula* spp., and *Lactarius* sp. Regarding Hyaloscyphaceae sp. 1, *C. obtusus* ectomycorrhizae yielded 75 isolates from 255 root tips, *Russula* spp. yielded 36 isolates from 278 root tips, and *Lactarius* sp. yielded 40 isolates from 229 root tips. The proportion of Hyaloscyphaceae sp. 1 was significantly different among the three ectomycorrhiza-forming species (chi-squared test, $p < 0.01$), and that of *C. obtusus* was significantly different from the other two groups (pairwise comparison with Bonferroni correction, $p < 0.01$)

Specificity test

To visualize fungal localizations in ectomycorrhizae by in situ hybridization, we designed a probe targeting the most frequent isolate, Hyaloscyphaceae sp. 1, and evaluated the viability of the probe. The specificity and suitability of the probe were confirmed using fungal hyphae and dot blots on a nylon membrane. However, Hyaloscyphaceae sp. 2 produced the same signaling pattern as Hyaloscyphaceae sp. 1 (data not shown) because they share the same nucleotide sequences in the putative targeted rDNA region (GenBank accession: LC189052 and LC189053). In the specificity test using cultured fungal hyphae, the Hyaloscyphaceae sp. 1-specific probe (i.e., G2-2 probe) generated hybridization signals for Hyaloscyphaceae sp. 1 (Fig. 2a) and Hyaloscyphaceae sp. 2 hyphae. In contrast, the negative control probe (i.e., non-G2-2 probe) did not generate clear signals (Fig. 2b). Neither probe generated a signal for the reference species (i.e., Helotiales sp. 1, Hyaloscyphaceae sp. 3, Hyaloscyphaceae sp. 5, Hyaloscyphaceae sp. 6, Hyaloscyphaceae sp. 7, Helotiales sp. 4 and *Lachnum* sp.), all of which were isolated from healthy fagaceous roots (Fig. 2a, b). The dot blots involving DNA extracts from all eight fungal species revealed that the G2-2 probe hybridized only to the Hyaloscyphaceae sp. 1 target DNA, and no signal was detected in tests with non-target DNA samples (Fig. 3). The probe sequence was searched in the GenBank using the BLAST. The probe showed 100% similarity with targeted species but also with some species of lichen, leaf endophytes, and marine and arctic soil-inhabiting fungi.

In situ hybridization

The DIG-labeled G2-2 probe hybridized to the target DNA and generated clear signals in field-collected ECM samples (Fig. 4). In contrast, no signals were detected for the non-G2-2 complementary negative control probe (data not shown). In ECM sections, hybridization signals were observed on the surface of fungal mantle (Fig. 4a). Also, hyphal penetration into the intercellular space (Fig. 4b) as well as penetration into ECM hyphal cells (Fig. 4c–e) of the mantle were observed. Signals were rarely observed in the inner layer of the mantle (Fig. 4g, h) and the Hartig net under the mantle (Fig. 4h). The frequency of hybridization signals was calculated by dividing the number of tissues with hybridization signals by the total number of ECM sections. Approximately 2% of the analyzed ECM sections (i.e., 19 of 945 sections) exhibited clear

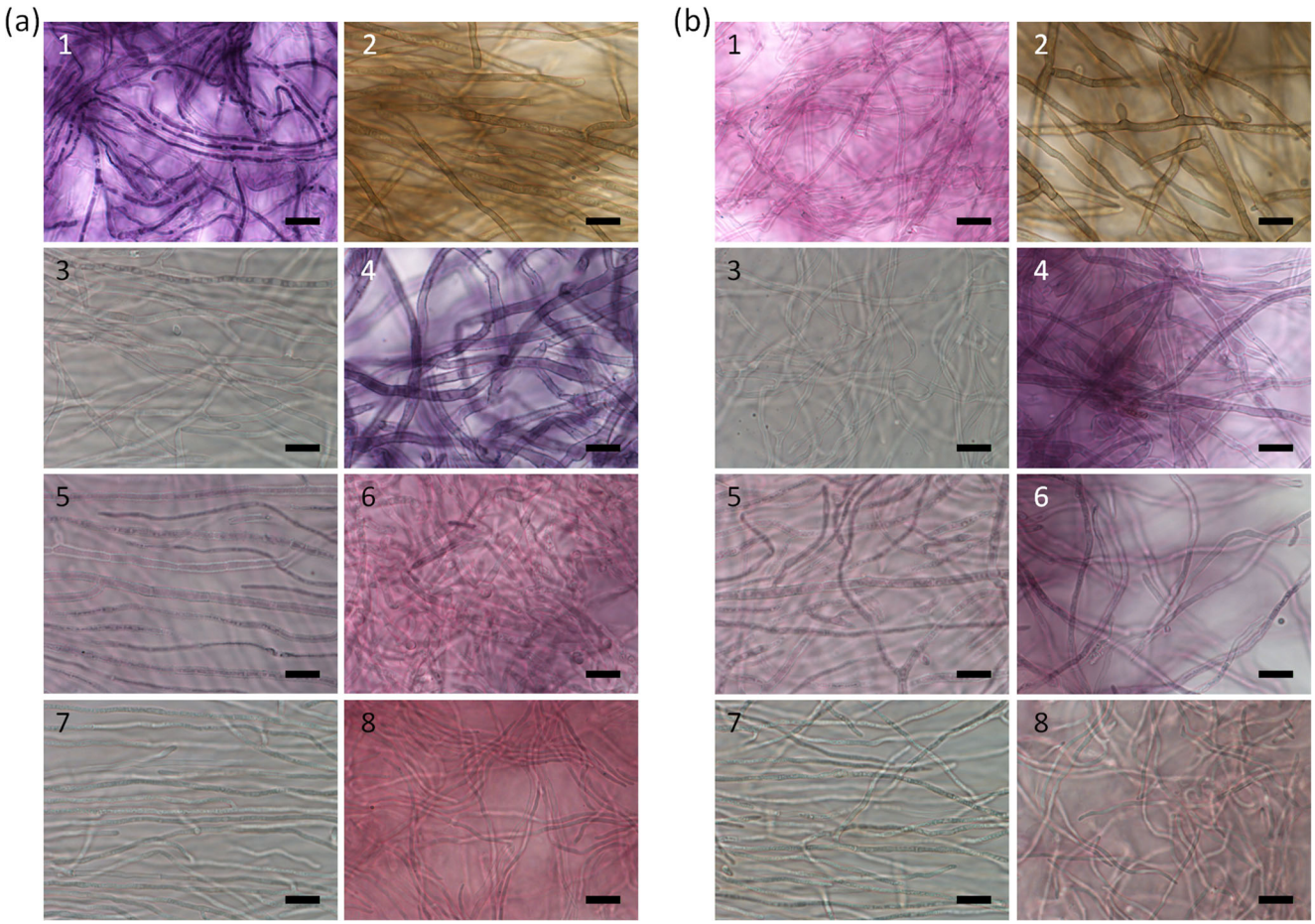

Fig. 2 Probe specificity test with cultured fungal bodies. Eight fungal isolates (i.e., 1: Hyaloscyphaceae sp. 1, 2: Helotiales sp. 1, 3: Hyaloscyphaceae sp. 3, 4: Hyaloscyphaceae sp. 5, 5: Hyaloscyphaceae sp. 6, 6: Hyaloscyphaceae sp. 7, 7: Helotiales sp. 4, and 8: *Lachnum* sp.) were used to optimize pretreatment conditions and evaluate the signal specificity of the newly-designed G2-2 probe (**a**) and the negative control non-G2-2 probe (**b**). Cytoplasmic hybridization signals were detected only when Hyaloscyphaceae sp. 1 was tested with the G2-2 probe. Bars correspond to 10 μm

hybridization signals. Additionally, the proportion of sections with signals varied from 0 to 7.7% depending on the subplot. Of these sections, which originated from two of five subplots, approximately 15.8% (i.e., 3 of 19 sections) produced signals inside the ECM mantle. No signals were observed in the Hartig net or plant tissues.

Discussion

At the *Castanopsis*-dominated study site, the ectomycorrhiza-associated fungal community was predominantly composed of a few helotialean species, including Hyaloscyphaceae sp. 1 and Helotiales sp. 1. These two species accounted for over 60% of the community. This suggests that we succeeded in selectively isolating the ectomycorrhiza-associated fungi from a complex soil fungal community that included free-living saprobes. However, the detected population may have been biased because of differences in individual culturabilities. The major isolated helotialean species could not be identified at the species or genus level using a DNA database because the closest matches were ericoid root colonizers or broad-leaf tree colonizers. Some studies on fungal root endophytes, most of which focused on boreal forests, revealed the dominance of the *P. fortinii* s.l.–*A. applanata* species complex or the *Rhyzoscyphus ericae* aggregate (Vrålstad et al. 2002b; Menkis et al. 2005; Kernaghan and Patriquin 2011). However, at our study site, there were relatively few darkly pigmented fungi, and the lineages mentioned above were not isolated. These findings suggest the fungal community status differs between our *Castanopsis* forest and a boreal, mainly coniferous forest, supposedly because some putative root endophytic species exhibit host plant specificity (Kernaghan and Patriquin 2011; Toju et al. 2013a, 2014; Yamamoto et al. 2014).

Bergero et al. (2000) isolated an *Oidiodendron* species from the ECM root tips of *Quercus ilex* and determined that it formed ericoid mycorrhizae under laboratory conditions. The genus *Oidiodendron* includes a group of ericoid mycorrhizal fungi. In our study, nine *Oidiodendron* spp. isolates

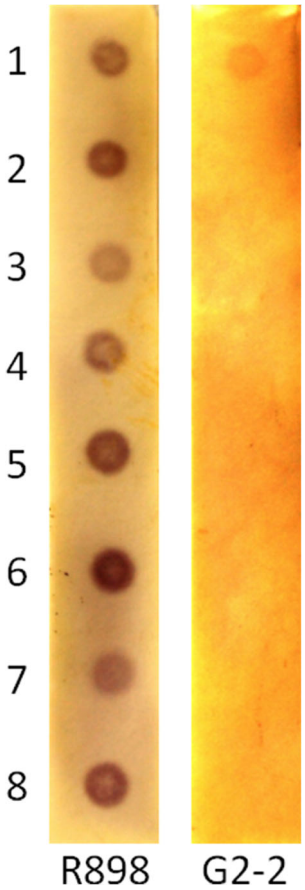

Fig. 3 Probe specificity test by dot blot hybridization with fungal genomic DNA. Genomic DNA extracts of eight fungal species (i.e., 1: Hyaloscyphaceae sp. 1, 2: Helotiales sp. 1, 3: Hyaloscyphaceae sp. 3, 4: Hyaloscyphaceae sp. 5, 5: Hyaloscyphaceae sp. 6, 6: Hyaloscyphaceae sp. 7, 7: Helotiales sp. 4, and 8: *Lachnum* sp.) were tested with a positive control probe R898 (left) and the newly-designed G2-2 probe (right). The G2-2 probe generated a clear hybridization signal only with the target DNA derived from Hyaloscyphaceae sp. 1. The brightness and contrast of the image was adjusted to improve visibility

were observed to inhabit the roots of *C. cuspidata*. The fact that we detected an ericoid mycorrhizal host (i.e., *Pieris japonica*) at our study site implies that the ericoid mycorrhizal fungi identified in ectomycorrhiza-forming host plants also potentially colonized ericaceous host plants. However, the possible saprotrophic activities of *Oidiodendron* spp. (Rice and Currah 2002) may have resulted in the ubiquitous presence of these species (Bergero et al. 2003).

The community structure of ectomycorrhiza-associated fungi depended on the fungal taxa that formed the ectomycorrhizae used for isolations (Fig. 1). Regarding the major species, Hyaloscyphaceae sp. 1 tended to occur at relatively high proportions in the ECM community of *C. obtusus*, but at relatively low proportions in the ECM communities of *Russula* spp. and *Lactarius* sp. This result is consistent with those of some studies that concluded that root-associated species were not randomly distributed, but tended to co-occur with certain ectomycorrhiza-forming species or an ECM morphotype (Urban et al. 2008; Tedersoo et al. 2009; Yamamoto et al. 2014). Our results imply that these two ecological types of fungi may interact with each other. Meanwhile, the reason for this biased occurrence should be carefully considered because the dense extraradical *C. obtusus* mycelium that formed the hyphal mats observed at our study site may have affected the microbial community in the hyphal mats via biochemical factors as described by Kluber et al. (2010, 2011). Environmental conditions surrounding ectomycorrhizae formed by *C. obtusus* may be considerably different from those surrounding the ectomycorrhizae of the other two groups because of enzymatic activities (Bödeker et al. 2009; Hobbie et al. 2013; Bödeker et al. 2014). This difference might be responsible for the diversity in the associated fungal community.

Cultured hyphae were used for the specificity test to examine the probe efficiency under conditions similar to those of the ensuing analysis. Moreover, considering the possibility that a pretreatment of cultured hyphae for in situ hybridizations may affect the apparent signal selectivity, we conducted dot blot hybridizations to confirm the specificity of the probe for the genomic DNA of the target isolates. Hyaloscyphaceae sp. 1 and Hyaloscyphaceae sp. 2 were specifically visualized during the specificity test with cultured hyphae. Additionally, the dot blot hybridization results confirmed the probe specificity. These observations suggest that the newly-designed G2-2 probe is suitable for practical use in the selective detection of Hyaloscyphaceae sp. 1 and/or Hyaloscyphaceae sp. 2 by hybridizing 26S rRNA, and not 26S rDNA. Unfortunately, Hyaloscyphaceae sp. 1 and Hyaloscyphaceae sp. 2 could not be distinguished by in situ hybridization because of their molecular similarity. However, these two species are close relatives that share habitats. Thus, they can be assumed to have the same or similar ecological status. Regarding this point, our results presented herein may be relevant to discussions of the ecology of a very limited spectrum of root-associated fungal species. Unfortunately, we could not eliminate the probability of cross-reactivity because the probe showed 100% similarity with some non-targeted fungal species in the database. However, none of these non-targeted species were reported as root colonizer and none was detected in the study site. Also, the majority of the isolates in the study site was Hyaloscyphaceae species. Therefore, we considered that the hybridization signals could be regarded as the targeted species, Hyaloscyphaceae sp. 1 and/or sp. 2.

Detectable in situ hybridization signals were concentrated mainly around the fungal mantle and partly observed in the mantle tissues (Fig. 4). This indicates that Hyaloscyphaceae sp. 1 and/or Hyaloscyphaceae sp. 2 colonized the mantle tissues and likely survived the surface sterilization process completed before the isolation test. There were a limited number of sections whose signals apparently penetrated into the mantle tissues (i.e., 15.8%; 3 of 19 sections with signals, Fig. 4b–h).

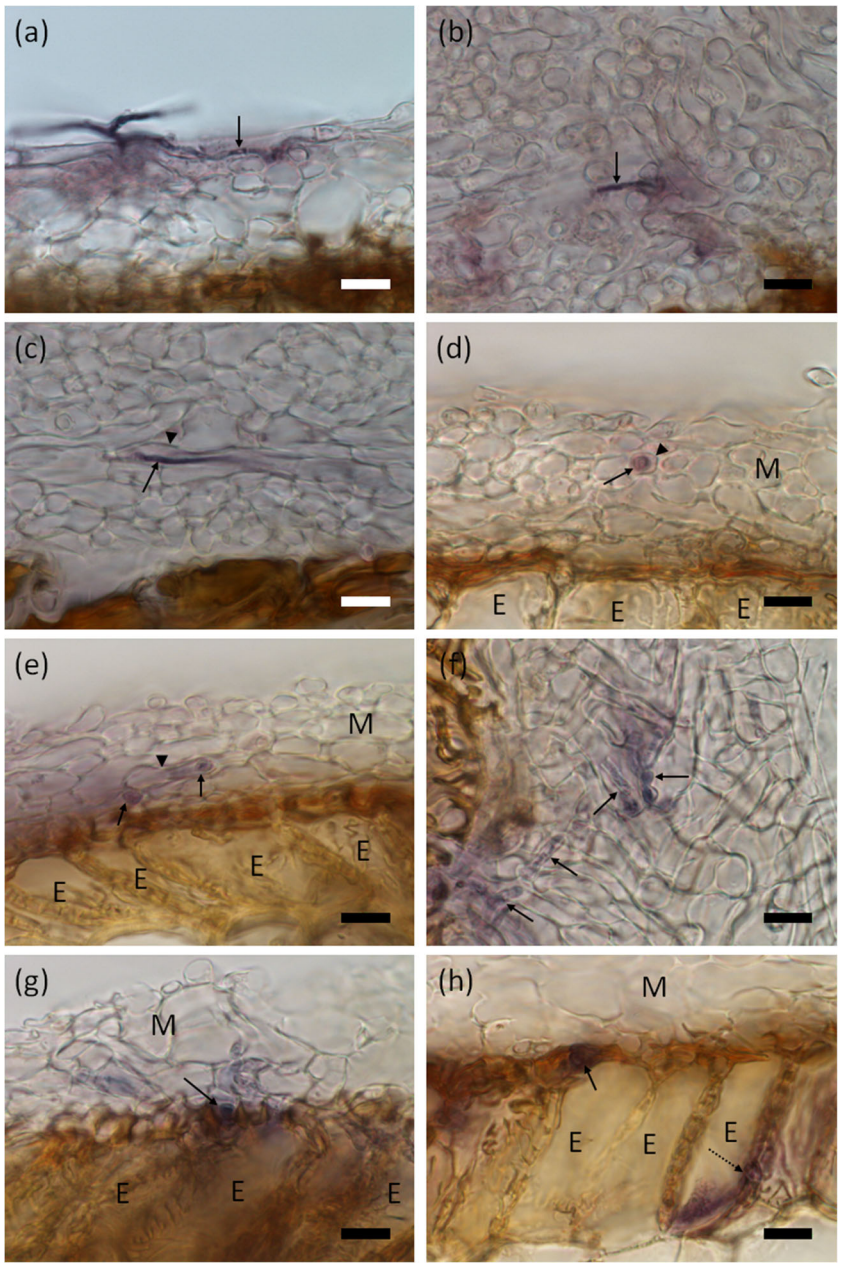

Fig. 4 Optical micrographs of in situ hybridization of ECM sections with the taxon-specific G2-2 probe. All presented ECM sections are 25–30 μm thick. Signals indicating the presence of Hyaloscyphaceae sp. 1 or Hyaloscyphaceae sp. 2 appear as a blue–purple stain on the surface of ECM fungal mantle (**a**) and in the intercellular space of the mantle (**b**). Signals were also detected within mantle cells (**c**–**e**) but sometimes unclear whether signals were inter- or intracellular space of the mantle tissue (**f**). In rare cases, signals were observed around plant epidermal cells, inner mantle (**g** and **h**, arrow), and in the Hartig net (h, broken line arrow). Arrows and arrowheads indicate hybridization signals of hyphal cells and ectomycorrhizal fungal cell walls, respectively. **a**–**c**, **f** ECM mantle tissues. **d**, **e**, **g**, **h** Mantle tissues and the Hartig net around plant epidermal cells. The brownish parts in **d**, **e**, **g**, and **h** correspond to plant tissues. M, mantle; E, plant epidermal cell. Bars correspond to 10 μm

The other signals appeared to be on the surface of the mantles (Fig. 4a) or it was difficult to distinguish whether they penetrated into the mantle tissues. Hyphal penetration into mantle tissues was relatively common among subplots (i.e., two of three subplots signals were detected) despite the rarity of signals in the sections (i.e., 2.0%; 19 of 945 sections). The proportion of hyphal penetration was not determined because the sections which clearly showed signal localization were very limited, and in many cases, it was difficult to recognize whether the signals were inter- or intrahyphal (Fig. 4f). Additionally, signals penetrated into the fungal mantle, but rarely reached the Hartig net (Fig. 4h) and absent in the plant tissue surrounded by the mantle. Vohník et al. (2013) reported that non-mycorrhizal mycelium occurred inside and around plant cells in senescent ectomycorrhizae, whereas it was usually absent in the healthy and young ectomycorrhizae of Norway spruce. This observation is consistent with our in situ hybridization results, which revealed that signals specific to Hyaloscyphaceae sp. 1 and Hyaloscyphaceae sp. 2 were often observed in the mantle tissues and that fungal colonizations of plant tissues did not occur in healthy-looking ectomycorrhizae.

The observed hyphal penetration into ECM hyphae may indicate possible direct interaction between these fungi within the ECM tissues. Also, their biased occurrence depending on the ECM taxa may suggest there is an ecological association

between these Hyaloscyphaceae species and ECM fungi. So far, the physiological evidence for mycoparasitism in this group is lacking. However, the information of chitinolytic ability in helotialean root endophytes (Heinonsalo et al. 2016) and fungicolous tendency of some hyaloscyphaceous teleomorphs (Hosoya 2002; Hosoya 2013; Huhtinen et al. 2008) implies a potentially complex lifestyle including mycoparasitism and endophytism in these species. A certain dark septate endophyte parasitizes arbuscular mycorrhizal fungi in plant roots and penetrates into the arbuscular mycorrhizal hyphae (Mandyam and Jumpponen 2008). The phenomenon that we observed resembles their observation, during which fungal cells of other species are penetrated. Although mycoparasitism has been reported in ECM roots (Summerbell 1987; Pöder and Scheuer 1994; Olsson et al. 2000), it is difficult to distinguish mycoparasites from other modes of life. Additionally, there are some complex modes related to nutrient acquisition of saprophytic, mycoparasitic, and even nematophagous status (e.g., Rubner 1996; Komon-Zelazowska et al. 2007). In the present study, we can only speculate about the biological mechanisms regulating ectomycorrhiza-associated fungi because of their wide host range from plants to ECM fungi. Additionally, although we observed that the hyphae of ectomycorrhiza-associated fungi penetrate ECM fungal cells, we must emphasize that these species are usually isolated from surface-sterilized non-mycorrhizal roots as well (data not shown). In the present study, over 18% of ectomycorrhizae harbored Hyaloscyphaceae sp. 1. To clarify the reasons for the prevalence of this species at the research site, the ecological significance of this species on the ECM community will need to be characterized.

In conclusion, our findings revealed the prevalence of helotialean species in ectomycorrhizae formed on *C. cuspidata* roots as well as the potential of these species to interact with ECM fungi. In contrast to our understanding of their behavior as endophytes, the dominant Hyaloscyphaceae species at the study site colonized the inter- and intracellular spaces of the ECM fungal mantle tissues and hyphal penetration into ECM hyphal cells was observed. Although we focused only on a limited number of culturable species from the ectomycorrhiza-associated fungal community, our findings should help to characterize the ecological status of hyaloscyphaceous root-associated fungi and the complex network of root-associated fungal communities in forest soil ecosystems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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