FULL PAPER

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Diversity of ericoid mycorrhizal fungi isolated from hair roots of *Rhododendron obtusum* var. *kaempferi* in a Japanese red pine forest

Abstract The diversity of ericoid mycorrhizal fungi of *Rhododendron obtusum* var. *kaempferi* was examined in a stand of *Pinus densiflora* at Tsukuba, Japan. In total, 153 slow-growing fungal isolates were obtained from roots of *R. obtusum* var. *kaempferi*, in which 113 isolates formed an ericoid mycorrhizal structure in vitro. Among them, 53 isolates were morphologically identified as *Oidiodendron maius*, but the others were not identified due to their sterilities. PCR-RFLP analysis in the rDNA-ITS region divided them into four different RFLP types. Phylogenetic analysis from sequence data of the region suggested that the four RFLP types belonging to distinct taxa and one sterile type are considered to be *Hymenoscyphus ericae*. This study is the first report of ericoid mycorrhizal fungi in a natural habitat in Japan.

Key words Ericaceae · Ericoid mycorrhizae · *Hymenoscyphus ericae* · ITS · *Oidiodendron maius*

Introduction

In nature, ericaceous plants are found in various kinds of vegetation. They are dominant in the vegetation of heathland, or occur as understory shrubs in forests mixed with various other plants. The ericoid mycorrhizal association is considered to be important for ericaceous plants to grow in such habitats (Read 1996). *Hymenoscyphus ericae* (Read) Korf & Kernan and several *Oidiodendron* species have been recognized as typical ericoid mycorrhizal fungal partners. *Hymenoscyphus ericae*, which has *Scytalidium vaccinii* Dalpé, Sigler & Litten as its anamorph (Dalpe et al. 1989; Egger and Sigler 1993; Hambleton et al. 1999), has been reported as the most common mycorrhizal fungus in heathlands (Read 1991; Currah et al. 1999) and alpine zones (Hambleton and Currah 1997). Mycorrhizae have an ecologically significant role for the establishment of the host plant in heathland by facilitating transfer of nutrition to the host plant and contributing to detoxification of the root environment (Read and Kerley 1999). Several *Oidiodendron* species have been confirmed to form ericoid mycorrhizae with ericaceous plants in synthesis experiments (Couture et al. 1983; Dalpé 1986, 1989, 1991; Xiao and Berch 1995). Among them, *Oidiodendron maius* Barron, which was first isolated from cultivated *Rhododendron* by Douglas et al. (1989), has been described as a typical ericoid mycorrhizal species in forest soils in Italy (Perotto at al. 1996; Martino et al. 2000), western Canada (Hambleton and Currah 1997), and Finland (Currah et al. 1999). Its distribution is considered to be worldwide.

Numerous sterile fungi have been also reported from Ericaceae (Burgeff 1961; Perroto et al. 1990; Stoyke and Currah 1991; Xiao and Berch 1996; Hambleton and Currah 1997) and Epacridaceae species (Hutton et al. 1994). Recent molecular analysis of the rDNA–internal transcribed spacer (ITS) region have clarified their phylogenetic relationship and exhibited the genetic diversity of ericoid mycorrhizal fungi (Liu et al. 1998; Chambers et al. 1999, 2000; McLean et al. 1999; Monreal et al. 1999; Sharples et al. 2000).

In Japan, various ericaceous species are distributed widely in various types of forest vegetation. However, no fungal isolates have been reported from the roots of those plants. Currah et al. (1993) isolated *O. periconioides* Morrall from the hair roots of *Rhododendron brachycarpum* G. Don growing in pots, which is the only report of ericoid mycorrhizal fungi from ericaceous plants in Japan.

An ericaceous plant, *R. obtusum* var. *kaempferi* Planchon Wilson, is distributed in Japan as a common species in understory shrubs in red pine (*Pinus densiflora* Sieb. et Zucc) forests. The purpose of this study was to identify

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the fungal species forming ericoid mycorrhizae in a natural habitat in Japan. We isolated the fungi from roots and conducted mycorrhizal synthesis experiments. We also conducted polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequence analysis in the rDNA-ITS region to investigate fungal diversity and the relationship between our isolates and other ericoid mycorrhizal fungi in the GeneBank database.

Materials and methods

Isolation of fungi

The experimental plot ($\sim$225 m^2) was set up in a stand of *P. densiflora* and *Quercus* spp. at Tsukuba, Ibaraki, Japan. In this plot, *R. obtusum* var. *kaempferi* was grown sparsely as a shrub. Six plants of *R. obtusum* var. *kaempferi* were randomly selected in April and May 1998. Root samples of the plants were collected from the upper 15 cm of the soil and were kept in plastic bags at 4°C until use.

Fungi were isolated from hair roots by a modified maceration method (Pearson and Read 1973). Hair root segments (1 cm) were cut and transferred into a test tube (18 mm × 20 cm). The segments were washed three times in 10 ml sterile distilled water (SDW) with 0.005% (v/v) Tween 80 using a vortex mixer and then rinsed 20 times in 10 ml SDW. After washing, the epidermal cells of the root segments were detached from the central stele of the roots using a fine glass needle under the dissecting microscope (Olympus SZH10). The epidermal cells were suspended with 0.5 ml SDW in a Petri dish; then, 20 ml of malt extract agar (MEA; 2% malt extract, Difco) was decanted and mixed before solidification. The Petri dishes were incubated at 18°C in the dark. After 1–5 weeks, the fungal colonies growing from the epidermal cells were isolated and subcultured on MEA medium. Fast-growing fungi, such as species of *Penicillium*, *Trichoderma*, and *Cladosporium* species, were excluded from further study because they are apparently not ericoid mycorrhizal fungi. Thirty hair roots segments per plant were used for the isolation of fungi.

Ericoid mycorrhizal synthesis

Fungal isolates were examined for their ability to form ericoid mycorrhizae by inoculating seedlings of *R. obtusum* var. *kaempferi* in vitro. The fungi were cultured on the surface of slants of modified Melin Norkans medium (Marx 1969) with 0.1% glucose in a test tube (4 × 9 cm) at 18°C under dark conditions for 2 weeks. Approximately 50 ml autoclaved (40 min, twice) soil mixture [Kanuma tsuchi : vermiculite : peat moss, 1 : 2 : 2 (v/v), moisture content approximately 70%, pH 5.1) was overlaid on the fungal colony, and axenic seedlings of *R. obtusum* var. *kaempferi* were transplanted on the soil mixture. The tubes were kept in the growth room at $\sim$22°C with a 16-h photoperiod. Two months after inoculation, roots of the plants were examined for fungal colonization. Root segments were washed with SDW, stained with lactofuchsin, and observed under a microscope at 100–1000 × magnification.

DNA extraction and PCR-RFLP analysis

All mycorrhizal isolates were analyzed by PCR-RFLP in the rDNA-ITS region to divide them into genetically different fungal types. DNA was extracted from small fungal plugs 0.5 cm in diameter using the CTAB method of Gardes and Bruns (1993). The ITS region was amplified by PCR for each isolate using the primers ITS 1 and ITS 4 (White et al. 1990). Diluted samples were amplified on a Zymoreactor (model AB 1820; Atto, Tokyo, Japan). The reaction components and cycling conditions were those of Gardes and Bruns (1993). Amplified products were digested with five restriction endoucleases (Nippon Gene, Toyama, Japan), *Hae*III, *Hin*fI, *Rsa*I, *Msp*I, and *Taq*I, according to the manufacturer's recommendation. The restriction fragments were size-fractionated in 3% agarose gel by electrophoresis. The base-pair (bp) lengths of individual fragments were determined against a 100-bp ladder. No fragments smaller than 100 bp were scored.

Sequencing of rDNA-ITS region and phylogenetic analysis

PCR products were treated by automated DNA sequencing reactions using standardized methods, then analyzed by a ABI PRISM 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocols. A homology search was conducted using the FASTA 3.0 program, and the sequences of fungal taxa indicated to be most similar to our isolates were obtained from the GeneBank database; The sequence data of our 15 isolates and selected fungi from the database were applied to neighbor-joining analysis (1000 bootstrap resampling replicates) using the CLUSTAL W (version 1.7) software package (Thompson et al. 1994). *Neurospora crassa* was used for outgroup. The phylogenetic tree was plotted by TREEVIEW (Page 1996).
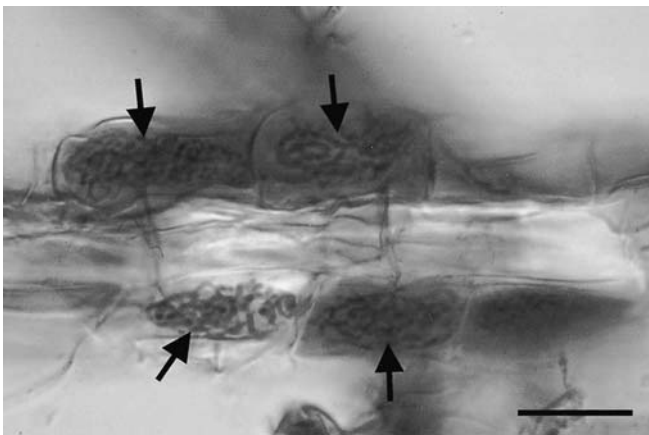
Results

Morphology and identification of mycorrhizal isolates

In total, 153 slow-growing fungal isolates were obtained from 180 root segments, in which 113 isolates formed ericoid mycorrhizal structures in epidermal cells of *Rhododendron* hair roots after 2 months. They formed hyphal coils (1–2 μm in diameter) in the epidermal cells of the host plant (Fig. 1). All inoculated plants grew in a healthy fashion, and no symptoms were observed during synthesis experiments. Among them, 53 isolates sporulated in the culture and were morphologically identified as *O. maius* (Fig. 2). They formed white to gray colonies and reached 1.3–2.0 cm in diameter after 10 days at 23°C on MEA. Thick-walled erect

dematiaceous conidiophores were mostly 250–400 μm, occasionally up to 600 μm, with strongly undulate fertile hyphae. Arthroconidia were subhyaline, smooth or slightly rough, cylindrical, ovoid, or subglobose, commonly about 2–2.5 × 2–4 μm (Fig. 2). The other 100 isolates were not identified from morphological observation because they did not form any reproductive structures on the media.

Grouping of mycorrhizal isolates by PCR-RFLP analysis

Mycorrhizal isolates (113 isolates) were analyzed by PCR-RFLP in the rDNA-ITS region. Amplified product size was 550–700 bp. After digestion with five restriction enzymes, *Hae*III, *Hin*fI, *Rsa*I, *Msp*I, and *Taq*I, the fungal isolates were divided into four different RFLP types (Table 1). All isolates (53 isolates) of *O. maius* were included in the same RFLP type. The colonial characteristics of isolates in the same RFLP type were similar to each other (Table 2).

Phylogenetic relationships of mycorrhizal isolates by rDNA ITS sequence analysis

Randomly selected, 15 isolates (Table 3) were further analyzed by sequencing of the ITS region. Full-length ITS sequences were obtained for 15 fungal isolates from *R. obtusum var. kaempferi* and submitted to the GeneBank Nucleotide Database. ITS sequences of isolates in the same RFLP types were identical to each other, except for a slight sequence divergence of 0.4% within 5 isolates of RFLP type 4. Sequence similarity between each RFLP type was lower then 84%. FASTA analysis revealed that our isolates of *O. maius* (RFLP type 1) have more than 90% sequence simi-

Fig. 1. Hair roots of seedling of *Rhododendron obtusum* var. *kaempferi* inoculated with *Oidiodendron maius*, 2 months after inoculation. *Arrows*, intracellular hyphal coils. *Bar* 50 μm

Fig. 2. Dematiaceous conidiophore (*arrowhead*) and cylindrical arthroconidia (*arrow*) of *Oidiodendron maius*. *Bar* 40 μm

Table 1. PCR-RFLP types of ericoid mycorrhizal isolates obtained from *Rhododendron obtusum* var. *kaempferi* and their fragment sizes

RFLP type	PCR product size (bp)	Fragment size (bp)					Number of isolates
		*Hae*III	*Hin*fI	*Rsa*I	*Msp*I	*Taq*I	
1 (*Oidiodendron maius*)	570	260, 140, 100	280, 150	400, 130	280, 110	230	53
2	560	290, 160, 100	290, 270	380, 180	440, 100	230, 220	37
3	560	460, 100	280, 270	400, 160	440, 110	230, 220	10
4	550	290, 160, 100	280, 260	380, 180	550	230, 210	13

Table 2. Colonial characteristics of isolates of four RFLP types obtained from roots of *Rhododendron obtusum* var. *kaempferi* on malt extract agar at 18°C

RFLP type	Colonial morphology	Growth rate (mm/day)
1 (*Oidiodendron maius*)	Hyaline aerial hyphae, white–whitish gray; orange pigment produced	0.4–0.6
2	Lightly floccose at the margin, dark green with light olive-green zone at the middle	0.8–1.0
3	Smooth, waxy, white–pale yellow, pale beige, sometimes wrinkled radially; irregular margin	0.03–0.06
4	Smooth, olive green–dark green at center; white irregular margin; hyphal mat displays scalelike pattern	0.05–0.07

Table 3. Isolates of *Rhododendron* sequenced in this study and their GeneBank accession codes

Isolate number	RFLP type	Accession code
E97053	1	AB089654
E97154	1	AB089655
E97111	2	AB089656
E97112	2	AB089657
E97123	2	AB089658
E98262	2	AB089659
E98481	2	AB089660
E97020	3	AB089661
E97021	3	AB089662
E97022	3	AB089663
E98013	4	AB089664
E98014	4	AB089665
E98018	4	AB089666
E98053	4	AB089667
E98100	4	AB089668

larity with *Oidiodendron* species and 99% sequence identity with *O. maius* isolates obtained from GeneBank. RFLP type 4 had high sequence similarity with sequences of *H. ericae* obtained from GeneBank database, 98.4%–98.8% over 502–511 nucleotides. Isolates of RFLP type 2 showed sequence similarity (about 87% similarities over 520 nucleotides) with several *Pezicula* species (Dermateaceae in Helotiales), and isolates of RFLP type 3 had similarities with *Neofabraea malicorticis* H.S. Jackson (87.6% over 522 nucleotides) and *N. alba* (E.J. Guthrie) Verkley (Dermateaceae in Helotiales) (86.7% over 520 nucleotides).

Neighbor-joining trees clustered our ericoid mycorrhizal isolates into four distinct types, *Oidiodendron*, *H. ericae* and two unknown groups (Fig. 3). Our two *O. maius* isolates were included in *Oidiodendron* groups and formed a monophyletic clade with *O. maius* in GeneBank with 100% bootstrap support. Five isolates of RFLP type 4 were included in the *H. ericae* clade with 100% bootstrap support. Isolates of RFLP type 2 and RFLP type 3 were not clustered with other ericaceous isolates or GeneBank sequences in high similarity.

Discussion

PCR-RFLP analysis in the rDNA-ITS region showed that four genetically distinct fungal types were obtained from roots of *R. obtusum* var. *kaempferi*. Colonial morphology of each RFLP type was also distinct from each other. Thus, it seems that they are divided into different fungal taxa. Among them, RFLP type 1 was morphologically identified as *O. maius*, which was strongly supported by their ITS sequence similarities (99%) to the sequences of *O. maius* obtained from the database. *Oidiodendron maius* was the most abundant species (35% of all mycorrhizal isolates) in this study, suggesting that this species is the most common ericoid mycorrhizal fungi in this red pine forest. This species has been also isolated from roots of ericaceous plants in

Italy (Perroto et al. 1996), Canada (Douglas et al. 1989; Hambleton and Currah 1997), and Finland (Currah et al. 1999). In Japan, Tokumasu (1973) reported that *O. maius* is a common fungal species in the forest soil humus layer. However, this is the first report of *O. maius* as ericoid mycorrhizal fungi isolated from roots of an ericaceous plant in Japan.

Although isolates of RFLP type 4 did not produce the zigzag chain of arthroconidia characteristic of *Scytalidium* and remained sterile in culture, they were considered to be a typical ericoid mycorrhizal fungi, *Hymenoscyphus ericae*, by analysis of ITS sequences. Sequence divergences of the complete ITS region between five isolates of RFLP type 4 and *H. ericae* in the database were very low (<1.6%), which indicates they are within the divergence between strains of *H. ericae* and its anamorph *S. vaccinii*, 1.2%–3.5% (Egger and Sigler 1993). The divergence was much lower than the divergence (up to 24%) between *H. ericae* and *H. monotropae* Kernan et Finocchio (Egger and Sigler 1993). Low sequence divergence of the ITS region at the intraspecific level was also reported in other species, e.g., *Laccaria* spp., 1%–2% (Gardes et al. 1991) and *Suillus* spp., 3% (Baura et al. 1992). Chambes et al. (1999) also identified their sterile isolates from rhizoids of liverwort in Australia and Antarctica as *H. ericae*, although they showed slight sequence divergence (<2.1%) within the ITS region. These data strongly support that our five sterile isolates of RFLP type 4 are *H. ericae*. However, RFLP type 4 was not isolated abundantly in this experiment, suggesting that this species was distributed less commonly in this forest compared to *O. maius*. *Hymenoscyphus ericae* has been often described as a ubiquitous mycorrhizal fungus of ericaceous plants in heathland (Read and Kerley 1999). Sharples et al. (2000) reported that the majority of fungal isolates from roots of *Calluna vulgaris* L. Hull in heathland in southwest England were identified as *H. ericae*, and *Oidiodendron* species were not found. It seems that fungal communities of ericoid mycorrhiza in this red pine forest of Japan are different from those of heathland, and that *H. ericae* is not always the dominant mycorrhizal species. Perroto et al. (1996) and Xiao and Berch (1996) also reported that *H. ericae* was not obtained from roots of *C. vulgaris* in Italy and roots of *Gaultheria shallon* Pursh in Canada, respectively.

Neighbor-joining analysis suggested that mycorrhizal isolates of RFLP type 2 and RFLP type 3 were not identical to ericoid mycorrhizal fungi, *Oidiodendron* species and *H. ericae*. In FASTA analysis, RFLP type 2 and 3 were closest to *Pezicula* and *Neofabraea* (Dermateaceae, Helotiales; differing from these by 12.8% and 12.4% respectively). The similarities were, however, not enough to indicate that they are closely related to these fungal taxa. Thus, their taxonomic positions are still unclear. These results suggest that at least four genetically distinct fungal taxa form ericoid mycorrhizae with *R. obtusum* var. *kaempferi* in this forest. These diverse fungal taxa that form ericoid mycorrhizae in a single ericaceous species are also reported in roots of *C. vulgaris* in England (Sharples et al. 2000), *G. shallon* in Canada (Monreal et al. 1999), and *Woollsia pungens* (Cav.) F. Muell (Chambers et al. 2000), *Epacris impressa* Labill,

Fig. 3. Neighbor-joining tree based on sequences of rDNA-ITS region from ericoid mycorrhizal isolates of *Rhododendron obtusum* var. *kaempferi* and selected fungi obtained from GeneBank database. *Numerical values above the branches* are the confidence levels from 1000 replicate bootstrap samplings
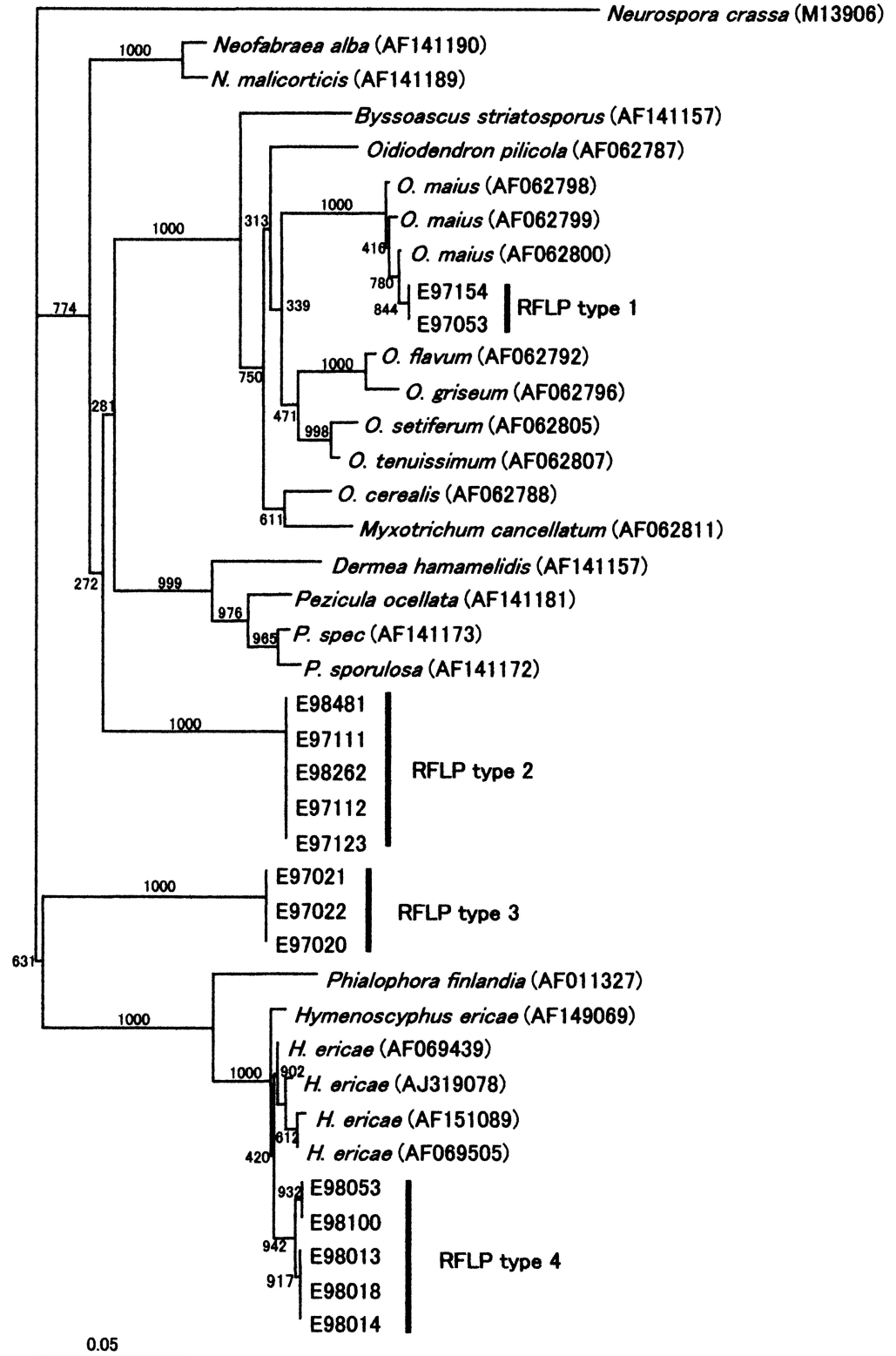
and *Astroloma pinifolium* (R. Br.) Benth. (McLean et al. 1999) in Australia. In these reports, sterile mycorrhizal isolates collected from a single host plant species were divided into several genetically distinct groups that were different from known ericoid mycorrhizal species and were shown to belong to fungal taxa with affinity to Helotiales by ITS sequence analysis. None of our isolates exhibited high sequence similarities with those isolates in the GeneBank database, suggesting that they do not belong to the same taxa. These data imply that a considerable diverse group of fungi, not only *H. ericae* and *Oidiodendron* species, form ericoid mycorrhizae in the natural habitat. More diverse

fungal taxa that form ericoid mycorrhizae might be found if more studies were conducted in a different habitat.

In our experiments, *O. maius* and RFLP type 2 were isolated most frequently. It was suggested that they might be a common mycorrhizal species and play an ecologically significant role in this vegetation. *Hymenoscyphus ericae* has been known to improve the growth of a host plant by enhancing nutritional uptake or by detoxification of the root environment under acidic and nutrient-limiting soil conditions (Read 1996). However, most of the reports of other mycorrhizal fungi only described the morphological features without discussing any functional aspects (Douglas et al. 1989; Xiao and Berch 1995, 1996). Further work is required to demonstrate the role of mycorrhizal fungi in the wider range of fungal taxa.

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