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SHORT COMMUNICATION

The mycorrhiza of *Schizocodon soldanelloides* var. *magnus* (Diapensiaceae) is regarded as ericoid mycorrhiza from its structure and fungal identities

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Abstract Structure and fungal identities were examined in the mycorrhizal roots of *Schizocodon soldanelloides* var. *magnus* (Diapensiaceae) to determine the mycorrhizal category. Previous studies had suggested the mycorrhizae of Diapensiaceae could be categorized as ericoid, but the mycorrhizal fungi have never been identified. The diameter of the fine lateral roots, in which coiled hyphae were found in epidermal cells, was mostly less than 100 μm. Molecular analyses identified the fungal isolates to be Helotiales and *Oidiodendron*. From the structure and fungal identities, we confirmed that the mycorrhiza of *S. soldanelloides* is an ericoid mycorrhiza.

Keywords Angiosperm Phylogeny Group · Ericales · Helotiales · ITS rDNA · *Oidiodendron*

Ericoid mycorrhizae are generally recognized in Ericoideae, Cassiopoideae, Vaccinioideae, and Styphellioideae in Ericaceae (Smith and Read 2008). The ericaceous plants have specialized narrow lateral roots, called "hair roots," that consist of one or two layers of cortical cells and an epidermal layer of enlarged cells (Peterson et al. 2004). Ericoid mycorrhizae are characterized by hyphal coils in the epidermal cells. The mycorrhizal fungi enable host plants to utilize complex organic nitrogen, which is unavailable to the plants themselves (Stribley and Read 1980). This mechanism is advantageous for the growth of plants in cool-temperate climates because decomposition of soil organic materials is usually slow (Straker 1996). Fungal isolation and molecular studies of ericoid mycorrhizae have identified a diverse assemblage of fungi from hair roots of ericaceous plants, and most of them are in Helotiales or *Oidiodendron* in Ascomycota (Smith and Read 2008).

Diapensiaceae is a small plant family consisting of five genera, and most species are restricted to eastern Asia and the Appalachian Mountain system of eastern North America (Scott and Day 1983; Rönblom and Anderberg 2002). This family belongs to the order Ericales and is closely related to Symplocaceae and Styracaceae (Angiosperm Phylogeny Group 2003). These two plant families are known to form arbuscular mycorrhiza (Janse 1897; Zangaro et al. 2003); however, the ericoid mycorrhiza was reported in *Diapensia lapponica* L. in Diapensiaceae by Hesselman (1900), as cited by Harley and Harley (1987). Kagawa et al. (2006) surveyed mycorrhizal formation in an alpine plant community in the Japanese South Alps, in which they classified the mycorrhiza of *Shortia soldanelloides* f. *alpina*, a synonym of *Schizocodon soldanelloides* Sieb. et Zucc. f. *alpinus* Maxim., in Diapensiaceae as ericoid mycorrhiza. They determined the mycorrhizal category according to the criteria of Harley (1989) by observation of hyphal coils in roots stained with 0.05% trypan blue. However, no structural description of the mycorrhiza was shown, and no mycorrhizal fungi were identified in their study.

In the present study, we examined the structure and fungal identities in mycorrhizae of *S. soldanelloides* var. *magnus* (Makino) Hara in Diapensiaceae to confirm the mycorrhizal category of *S. soldanelloides*.

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The sampling site was in a deciduous broad-leaved forest in Yazu-cho, Tottori Prefecture, approximately 740 m above sea level. The dominant tree species of the forest was *Fagus crenata* Blume, with *Quercus crispula* Blume and *Abies firma* Sieb. et Zucc. sparsely intermixed. In vegetation of the understory, *S. soldanelloides* var. *magnus* was dominant. Soil core samples including stem and roots of *S. soldanelloides* var. *magnus*, 10 cm × 10 cm × 5 cm deep, were collected. In total, ten samples (SC1-10) were collected in October 2008, April 2009, and June 2009 (Table 1). The collected samples were kept in plastic bags in cool conditions until processed. Within 2 days, the fine roots attached to the stem were collected and washed with running water to remove soil particles. For each collected sample, a small portion of fine roots were stained with Clorazol black E according to Yamato and Iwasaki (2002) to observe hyphal coils under a differential interference contrast microscope (Eclipse 80iRT-DIC-1; Nikon, Tokyo, Japan).

Table 1 Plant samples of *Schizocodon soldanelloides* var. *magnus* with sampling date and identified fungal isolates with DDBJ accession numbers

Plant no.	Sampling date	Fungal isolate	Fungal identity	Accession no.
SC1	5/10/2008	SC1-1	Helotiales	AB598082
		SC1-2	Helotiales	AB598083
SC2	5/10/2008	SC2-1	Helotiales	AB598084
		SC2-2	Helotiales	AB598085
		SC2-3	Helotiales	AB598086
		SC2-4	Helotiales	AB598087
		SC2-5	Helotiales	AB598088
		SC2-6	Helotiales	AB598089
SC3	5/10/2008	SC3-1	Helotiales	AB598090
		SC3-2	Helotiales	AB598091
		SC3-3	Helotiales	AB598092
		SC3-4	Helotiales	AB598093
SC4	5/10/2008	SC4-1	Helotiales	AB598094
		SC4-2	Helotiales	AB598095
		SC4-3	Helotiales	AB598096
		SC4-4	Helotiales	AB598097
SC5	5/10/2008	SC5-1	Helotiales	AB598098
SC6	25/4/2009	SC6-1	Helotiales	AB598099
SC7	25/4/2009	SC7-1	Helotiales	AB598100
SC8	17/6/2009	SC8-1	Helotiales	AB598101
SC9	17/6/2009	SC9-1	*Oidiodendron*	AB598102
		SC9-2	*Oidiodendron*	AB598103
		SC9-3	Helotiales	AB598104
		SC9-4	*Oidiodendron*	AB598105
		SC9-5	*Oidiodendron*	AB598106
		SC9-6	*Oidiodendron*	AB598107
SC10	17/6/2009	SC10-1	Helotiales	AB598108
		SC10-2	Helotiales	AB598109

All collected samples were used for fungal isolation. The mycorrhizal fungi were isolated from fine roots according to Leake and Read (1991) with slight modifications as follows. In each sample, the washed fine roots were cleaned thoroughly by ultrasonication; then, a small portion of the fine roots, about 20 fine roots around 2 cm in length randomly collected, were put into a Petri dish with 1.0 ml sterilized distilled water. The root surface was scraped with the back side of a scalpel to peel off the epidermal cells. From the suspension of the epidermal cells, those having hyphal coils were selected under a dissecting microscope (Leica MZ125; Leica, Tokyo, Japan) to transfer onto 1.5% water agar plates. The plates were incubated at 25°C in the dark, and hyphal growth from the hyphal coil was observed after 2 or 3 days. The growing hyphae were transferred onto plates of potato dextrose agar (PDA) medium (Difco, Detroit, MI, USA) and incubated at 25°C in the dark to obtain fungal cultures.

DNA was extracted from each of the obtained fungal cultures using a PrepMan Ultra Reagent (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of nuclear ribosomal RNA gene (rDNA) was amplified by polymerase chain reaction (PCR) for each of the extracted DNA using the primer ITS1F and ITS4 (Gardes and Bruns 1993) in TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 2 μl template DNA, 0.75 units of *Taq* polymerase, 0.25 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate, and 3 μl supplied PCR buffer in 30 μl of the total amount. The PCR program performed on Program Temp Control System PC-818S (Astec, Fukuoka, Japan) was as follows: initial denaturation step at 94°C for 2 min, followed by a step of 30 cycles at 94°C for 25 s, 55°C for 30 s, 72°C for 1 min, then a final elongation step at 72°C for 10 min. The amplicons were directly sequenced using BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystem) using ITS1F and ITS4 as sequencing primers. If the sequence data were not available by direct sequencing, the PCR products were cloned using a pGEM-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturer's instruction, and plasmid DNAs were extracted from the cloned products using MagExtractor Plasmid (Toyobo, Osaka, Japan). The cloned products were sequenced with T7 and SP6 promoter primers. The obtained data were subjected to BLAST searches (Altschul et al. 1997), and homologous data were downloaded from the GenBank database. To compare with previous ericoid mycorrhizal studies, some sequence data of "*Hymenoscyphus ericae* aggregate," a fungal group containing many ericoid

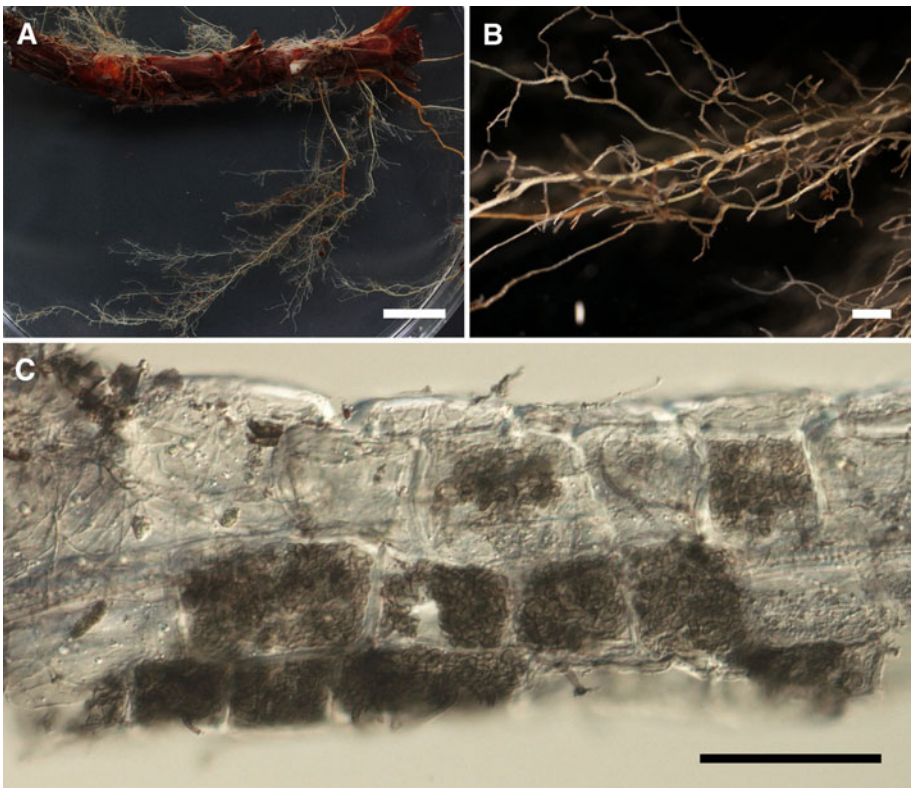

Fig. 1 Roots of *Schizocodon soldanelloides* var. *magnus*. **a** Intact thin roots growing from a thick root. **b** Intact thin root. **c** A thin root stained by Clorazol black E showing coiled hyphae in the epidermal cells. *Bars* **a** 1 cm; **b** 1 mm; **c** 50 μm

mycorrhizal fungi, were also downloaded from GenBank. For all sequenced and downloaded data, multiple sequence alignment was carried out using CLUSTAL X version 2 (Larkin et al. 2007). The aligned sequences data were analyzed by the neighbor-joining method (Saitou and Nei 1987), and the topology was tested with 1,000 bootstrap trials. The phylogenetic tree was drawn using TreeView (Page 1996).

Roots of *S. soldanelloides* var. *magnus* were mostly found in litter layers. No other plant roots were found in the collected soil cores except those of *F. crenata*. The roots of *S. soldanelloides* var. *magnus* were easily distinguished from the ectomycorrhizal roots of *F. crenata*. For the root morphology and fungal colonization, representative images from one sample (SC6) are shown in Fig. 1. The root system consists of main thick roots and highly branched fine lateral roots (Fig. 1a,b). Diameter of the fine roots was mostly less than 100 μm. Hyphal coils were present in epidermal cells (Fig. 1c). The same kind of root morphology and fungal structure were confirmed in all the other samples.

Mycorrhizal fungi were successfully isolated from peeled root epidermal cells on the water agar plate. From 10 individuals of *S. soldanelloides* var. *magnus*, 28 fungal isolates were obtained (Table 1). The ITS rDNA sequences of the fungal isolates were deposited to the DNA Data Bank of Japan (DDBJ) database with accession numbers AB598082–AB598109. Most of the isolated fungi were identified as Helotiales based on the DNA sequences. In the phylogenetic analysis (Fig. 2), the fungal isolates were shown to be multi-lineages, and most of them were closely related to some fungi isolated from ecto-, ericoid, or arbutoid mycorrhiza. No fungi were included in the *Hymenoscyphus ericae* aggregate.

The five other fungi collected from SC9 (see Table 1) were identified as *Oidiodendron* (Fig. 3). All the sequences formed a clade with *O. maius* including some ericoid mycorrhizal fungi.

The diameter of the ericaceous fine root is less than 100 μm, which is referred to as a hair root (Smith and Read 2008). The diameter of the fine lateral root of *S. soldanelloides* var. *magnus* was also mostly less than 100 μm. These lateral roots were highly branched, which was a feature different from the hair root of ericaceous plants.

Fungal hyphae grew from coiled hyphae in some root epidermal cells of *S. soldanelloides* var. *magnus* on water agar medium. This feature was also similar to that of ericoid mycorrhizae. The fungi grown from coiled hyphae were the subjects of this study, which avoided including other fungal endophytes and surface-colonizing fungi. Most of the obtained fungi were identified as belonging to Helotiales based on the ITS rDNA sequences. The fungi were shown to be multi-lineages in the phylogenetic

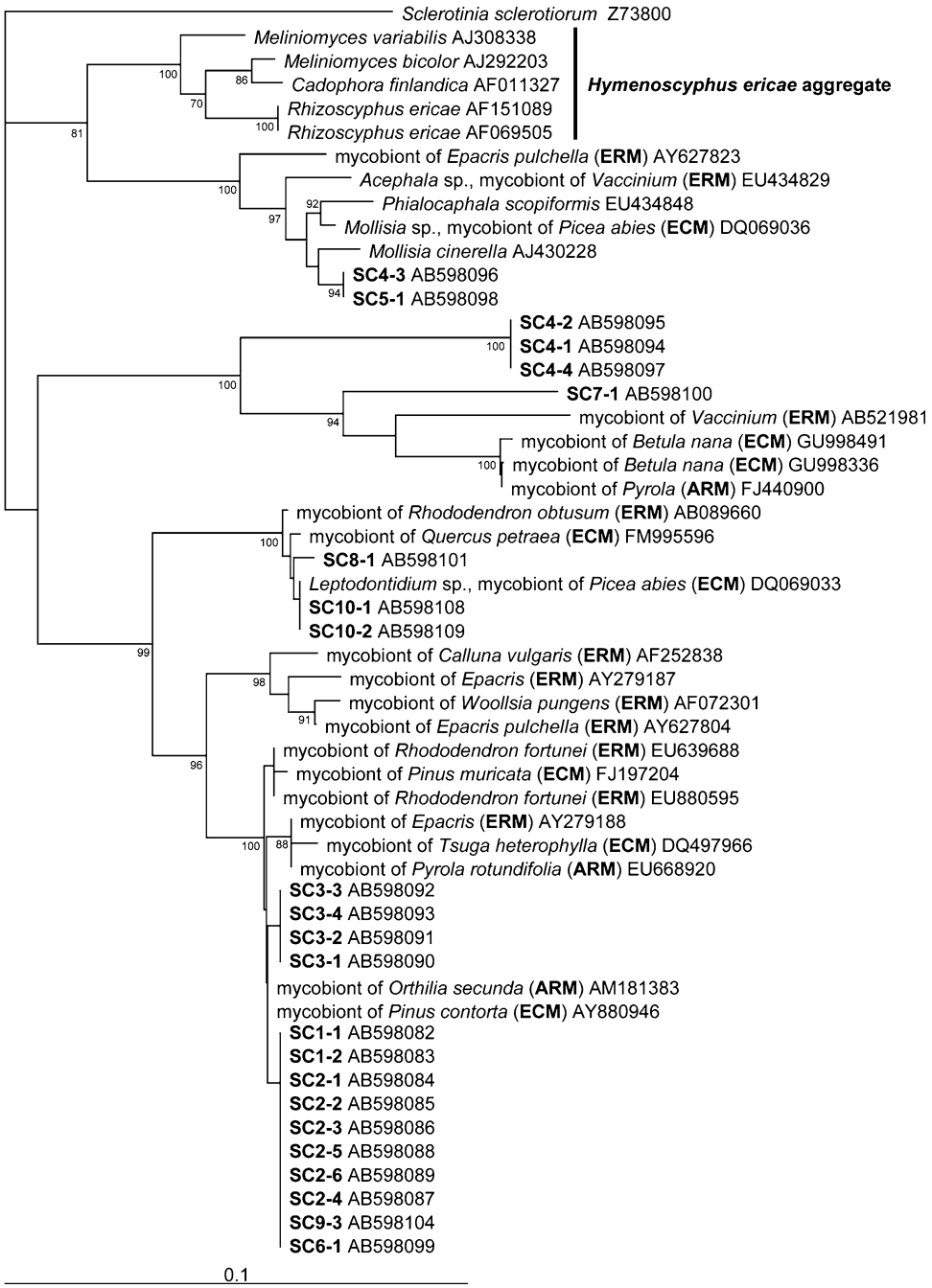

Fig. 2 A neighbor-joining phylogenetic tree based on partial internal transcribed spacer (ITS) sequence of rDNA of Helotiales. The DNA sequences of cultured fungi obtained from roots of *Schizocodon soldanelloides* var. *magnus* in this study are examined with those in the Genbank database to infer the phylogenetic relationships. The tree is rooted to *Sclerotinia sclerotiorum* (Sclerotiniaceae). The obtained fungal DNA sequences are shown with fungal number. The identifiers of the fungal number are shown in Table 1. Bootstrap values are shown where they exceed 70% (1,000 replicates). A *scale* is shown to infer the evolutionary distances. Accession numbers are given for all sequences. *ECM*, ectomycorrhiza; *ERM*, ericoid mycorrhiza; *ARM*, arbutoid mycorrhiza

analysis, suggesting relatively low specificity in this plant–fungal relationship. Some of them were closely related to the fungi isolated from ectomycorrhizae in the phylogenetic analysis, which suggested these fungi could connect between the roots of *S. soldanelloides* var. *magnus* and the ectomycorrhizal root tips of surrounding trees.

In many ericaceous plants, *H. ericae* has been identified as the fungal symbiont (Straker 1996). Vrålstad et al. (2000) showed that the genus *Hymenoscyphus* (*Rhizoscyphus*) forms a clade with genetically related fungi in a phylogenetic study based on the ITS rDNA region, and this grouping is called the *Hymenoscyphus* aggregate. The fungi in this aggregate are ecologically diverse; some are known to form ectomycorrhizae (Vrålstad et al. 2002). No fungi belonging to the *H. ericae* aggregate were detected in *S. soldanelloides* var. *magnus* in this study, but this result does not deny the possibility that further studies may detect the fungal group from Diapensiaceae.

A few mycorrhizal fungi were found to belong to the genus *Oidiodendron*, and all of them were closely related

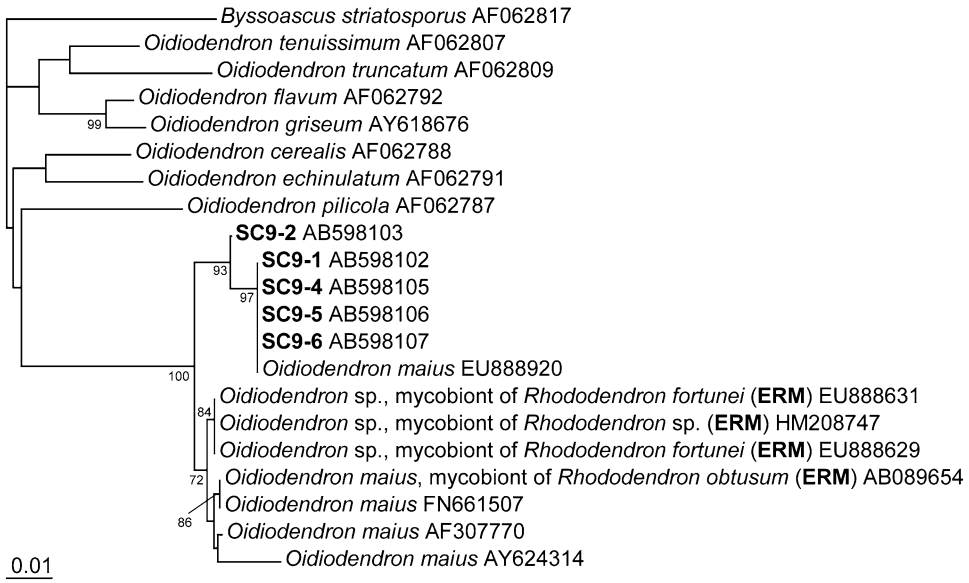

Fig. 3 A neighbor-joining phylogenetic tree based on partial ITS sequence of rDNA of *Oidiodendron*. The DNA sequences of cultured fungi obtained from roots of *Schizocodon soldanelloides* var. *magnus* in this study are examined with those in Genbank database to infer the phylogenetic relations. The tree is rooted to *Byssoascus striatosporus* (Myxotrichaceae). The obtained fungal DNA sequences are shown with fungal number; the fungal numbers are identified in Table 1. Bootstrap values are shown where they exceed 70% (1,000 replicates). A *scale* is shown to infer the evolutionary distances. Accession numbers are given for all sequences. *ERM*, ericoid mycorrhiza

to *O. maius* (see Fig. 3). Some species in *Oidiodendron*, such as *O. maius*, have been frequently recorded as ericoid mycorrhizal fungi in Ericaceae (Usuki et al. 2003; Addy et al. 2005; Bougoure and Cairney 2005). In this study, *Oidiodendron* fungi were isolated from only one sample of *S. soldanelloides* var. *magnus* (SC-9) among the ten samples examined, but they were dominantly detected in this sample (see Table 1).

The structure of the mycorrhizae having coiled hyphae in epidermal cells of thin lateral roots is very similar to that of ericoid mycorrhizae. The affiliation of the mycorrhizal fungi Helotiales and *Oidiodendron* was also the same as the ericoid mycorrhizae. From the structure and fungal identities, we confirmed that ericoid mycorrhizae occur in the roots of *S. soldanelloides*.

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