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FULL PAPER

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Isolation and identification of mycorrhizal fungi associating with an achlorophyllous plant, *Epipogium roseum* (Orchidaceae)

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Abstract The identity of mycorrhizal fungi associated with the achlorophyllous orchid *Epipogium roseum* was investigated by DNA analysis. The fungi were isolated from each coiled hypha (peloton), and the ITS region of nuclear rDNA was sequenced. Phylogenetic analysis based on the neighbor-joining method showed that all the isolates clustered with fungi belonging to *Psathyrella* or *Coprinus* in Coprinaceae. Those fungi are known as saprobes, using dead organic materials for a nutritive source. Large colonies of this orchid were frequently found around tree stumps or fallen logs. In such colonies, these decaying wood materials would be used as a large and persistent carbon source for the growth of this orchid. This is the first report of Coprinaceae as an orchid mycorrhizal fungi.

Key words Coprinaceae · *Coprinus* · ITS · Mycorrhiza · *Psathyrella*

Introduction

Epipogium roseum (D. Don) Lindl. is an achlorophyllous orchid species that distributes in Japan, Southeast Asia, Australia, India, and Africa (Tsuyama 1967). Colonies of this orchid can be seen in groves in shrines and temples in Japan (Chuma 1982; Konta et al. 2000). Because this orchid is categorized as a near-threatened species (NT) in the Red Data Book of the Ministry of Environment in Japan, conservation of the colonies is required in some habitats. It is well known that the growth of orchids, especially in the protocorm stage, depends on the associated mycorrhizal fungi for their supply of nutrients, including organic carbon. Such behavior of plants is termed myco-heterotrophy (Leake 1994). The achlorophyllous orchids seem to be myco-heterotrophic throughout their life cycle. Therefore, clarification of the mycorrhizal fungi would be important for the conservation and propagation of those orchids. However, little is known about the mycorrhizal fungi of *E. roseum*.

In most photosynthetic orchids, their mycorrhizal fungi have been known to belong to the form-genus *Rhizoctonia* (Smith and Read 1997). On the other hand, other basidiomycete species have been described in many achlorophyllous orchids as the mycorrhizal fungi. Fungal isolation and inoculation experiments have revealed the association of *Armillaria* spp. in *Galeola septentrionalis* (Terashita 1985; Terashita and Chuman 1987; Cha and Igarashi 1996) and in *Gastrodia elata* (Lan et al. 1994), and the association of *Erithromyces crocicreas* in *Galeola altissima* (Umata 1995). Recently, DNA analysis has been applied to identify mycorrhizal fungi in orchids, and identity with ectomycorrhizal fungi have been reported in some achlorophyllous orchids, i.e., Russulaceae with *Corallorhiza* spp. (Taylor and Bruns 1997, 1999), Therepholaceae with *Cepharanthera austinae* (Taylor and Bruns 1997), and Sebacinaceae with both *Neottia nidus-avis* (McKendrick et al. 2002) and *Hexalectris spicata* (Taylor et al. 2003).

In this study, fungi were isolated from mycorrhizal roots of *E. roseum* collected from three sites, and the isolated fungi were identified by DNA analysis.

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Materials and methods

Sample collection

Root samples of *E. roseum* were collected by excavating the rhizomatous system under the flowering shoots in groves at

three sites, Meiji Shrine in Tokyo (139°42′ E; 35°40′ N), Kashihara Shrine in Kashihara City, Nara Pref. (135°47′ E; 34°29′ N), and Shimogamo Shrine in Kyoto City (135°46′ E; 35°02′ N) in the beginning of July in 2001, 2002, and 2003. Relatively larger colonies having more than ten flowering shoots were selected for each sampling.

Microscopic observation

Freehand sections of the mycorrhizal roots of *E. roseum* were examined by differential interference-contrast microscopy using a Leitz DMR microscope (Leica Microsystems, Heerbrugg, Switzerland).

Fungal isolation

The mycorrhizal fungi were isolated according to Warcup and Talbot (1967) with slight modification. Surface of the roots were washed in tap water and sterilized by immersion in 70% ethanol for 3 min. Each of the sterilized roots was put into 1 ml sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse the intracellular hyphal coils (pelotons). About 20 ml autoclaved modified Czapek Dox agar medium (sucrose 0.5 g, $NaNO_3$ 0.33 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, KCl 0.1 g, yeast extract 0.1 g, agar 15.0 g, distilled water 1000 ml), which was cooled to ~45°C, was poured into the Petri dish and mixed before solidification to disperse the pelotons in the medium. The plates were incubated at 25.0° ± 0.5°C overnight in the dark. The fungal colonies growing from the pelotons were isolated by a sterilized scalpel under the inverted microscope (Nikon, Tokyo, Japan) and subcultured on potato dextrose agar (PDA) medium (Difco, Detroit, MI, USA).

Molecular identification of the isolated fungi

DNA was extracted by the CTAB method (Weising et al. 1995) from each of the isolated fungi cultured on PDA medium. The internal transcribed spacer (ITS) region of nuclear rDNA was amplified by polymerase cham reaction (PCR) for each of the isolated DNA using the primer ITS 1F and ITS 4B (Gardes and Bruns 1993) and TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR products were cloned using a pT7Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions. The cloned products were sequenced using a DYEnamic ET dye terminator kit (Amersham Biosciences, Piscataway, NJ, USA) with sequencing primers M13-47 and RV-M. Obtained sequence data were deposited in the GenBank nucleotide database. All the sequence data were subjected to BLAST searches, and analogous data were downloaded from GenBank database. For all the sequenced and obtained data, multiple sequence alignment was carried out using CLUSTAL W version 1.82 (Thompson et al. 1994). The aligned sequences were analyzed by the neighbor-joining method (Saitou and Nei 1987) using NEIGHBOR in PHYLIP version 3.5c (Felsenstein 1993), and the topology was tested with 1000 bootstrap trials. The phylogenic tree was drawn using Treeview (Page 1996).

The classification of the fungi corresponds to Kirk et al. (2001).

Results

Root morphology and mycorrhizal colonization

The colonies of *E. roseum* were found on the understory of evergreen broad-leaved trees, *Castanopsis* spp., *Quercus* spp., or *Cinnamomum camphora*, where heaps of litter or fallen logs were usually found. Larger colonies were likely to distribute around tree stumps or fallen logs. Belowground of the colonies, rhizomatous systems consisted of stolons, and two morphologically different swollen organs were found in the litter layer. The first swollen organs had brownish skin and contained starch granules, which were found at the tip of the stolons (Fig. 1). Flowering shoots were growing from some of the well-developed organs (Fig. 2); therefore, these were regarded as tubers. No mycorrhizal colonization was found in the tubers. The other swollen organs, found at the nodes of the stolons (Fig. 3), had a central vascular bundle and were colonized by mycorrhizal fungi in the cortex. This organ is defined as a tuberous root.

One tuberous root sample was collected from each colony to give 13 root samples in total from three collection sites (Table 1).

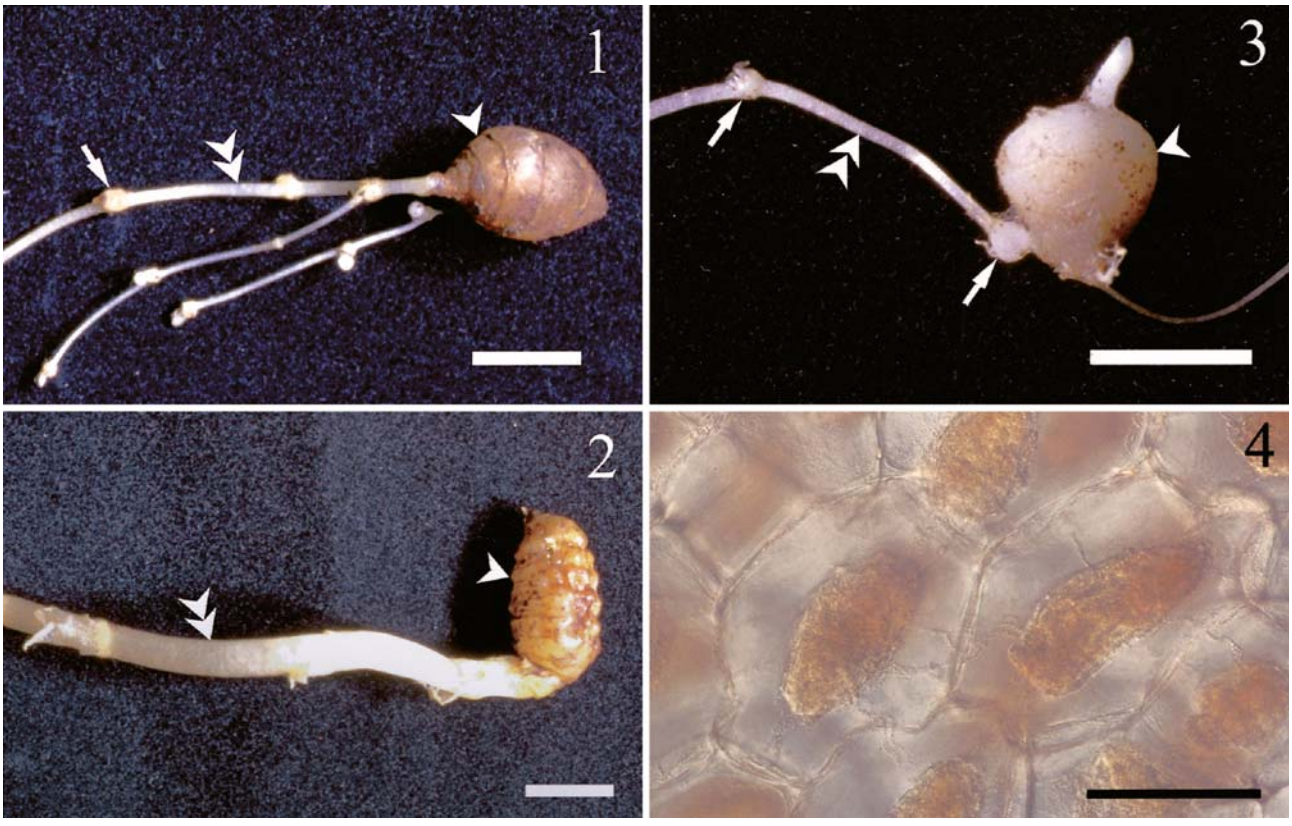
Most of the cortical cells were colonized by coiled hyphae in all the examined root samples. The fungal coils were mostly degenerated (Fig. 4).

Fungal isolation and molecular identification

Mycorrhizal fungi were isolated from all 13 root samples. All the isolated fungal colonies were morphologically identical in each root sample. One fungal isolate each, randomly selected, was examined for the DNA sequence (see Table 1). Full-length sequence data of ITS region including the 5.8S region were obtained from the 13 isolates. The BLAST searches showed that these sequences are analogous to those of *Coprinus* or *Psathyrella* in Coprinaceae. The phylogenetic analysis for these DNA sequences showed that eight isolates clustered with two species in *Psathyrella* and five isolates clustered with five species in *Coprinus* (Fig. 5).

Discussion

All the fungi isolated from root samples of *E. roseum* were identified to be nearly or closely related to *Psathyrella* or *Coprinus* in Coprinaceae in Agaricales, in spite of the distantly separated samplings. For Agaricales, *Armillaria* spp.



Figs. 1–4. Root system and mycorrhiza of *Epipogium roseum*. **1** A tuber (*arrowhead*) and stolons (*double arrowhead*). One of the nodes of stolons is indicated by *arrow*. **2** A tuber (*arrowhead*) with a flowering shoot (*double arrowhead*). **3** A tuberous root (*arrowhead*) developed from one of the nodes (*arrows*) of a stolon (*double arrowhead*). **4** Amorphous clumps of degenerated hyphal coils in the cortical cells of tuberous root. *Bars* **1–3** 1 cm; **4** 100 μm

Table 1. Isolates of mycorrhizal fungi from *Epipogium roseum* sequenced in this study

Isolate number[a]	Sampling date
ME1-1	20 June 2001
ME2-1	8 July 2002
ME2-2	8 July 2002
KA2-1	3 July 2002
KA2-2	3 July 2002
KA2-3	3 July 2002
KA2-4	3 July 2002
KA2-5	3 July 2002
KA3-1	9 July 2003
KA3-2	9 July 2003
KA3-3	9 July 2003
KA3-4	9 July 2003
SH3-1	7 July 2003

ME, Meiji Shrine; KA, Kashihara Shrine; SH, Shimogamo Shrine
[a] Individual isolates are indicated by site, sampling year, and colony number

in Tricholomataceae were already reported as mycorrhizal fungi in achlorophyllous orchids *Galeola septentrionalis* (Hamada 1939; Terashita and Chuman 1987; Cha and Igarashi 1996) and *Gastrodia elata* (Lan et al. 1994). In addition, *Mycena osmundicola* (Tricholomataceae) was also reported as a mycorrhizal fungus of *Gastrodia elata* in the protocorm stage (Xu and Mu 1990). This is the first report of Coprinaceae in orchid mycorrhizas. For the precise identification of the associated fungi at species level, further study is required to compare with sequence data obtained from basidiomata of Coprinaceae collected from the study sites.

Because all the fungal isolates from *E. roseum* were found to belong to Coprinaceae in this study, it is suggested that *E. roseum* is highly specialized to Coprinaceae in the mycorrhizal association in nature. However, it is also probable that other fungi that cannot grow in the culture condition in this study might be involved in the association. Furthermore, mycorrhizal fungi could be changed during the development of individual plants in some orchids such as in *Gastrodia ellata* from *Mycena osmundicola* in the protocorm stage to *Armillaria mellea* in subsequent growth (Xu and Mu 1990). The associating fungi in the early growth stage have been examined in some orchids by the field sowing techniques of seeds and protocorms (Rasmussen and Whigham 1993; Masuhara and Katsuya 1994). To identify the associated fungi without the bias of fungal isolation, it is also necessary to examine the fungal DNA extracted directly from mycorrhzal roots. Kristiansen et al. (2001) showed an identification method of mycorrhizal fungi using DNA analysis based on a single peloton isolated from orchid roots. The application of these promising techniques

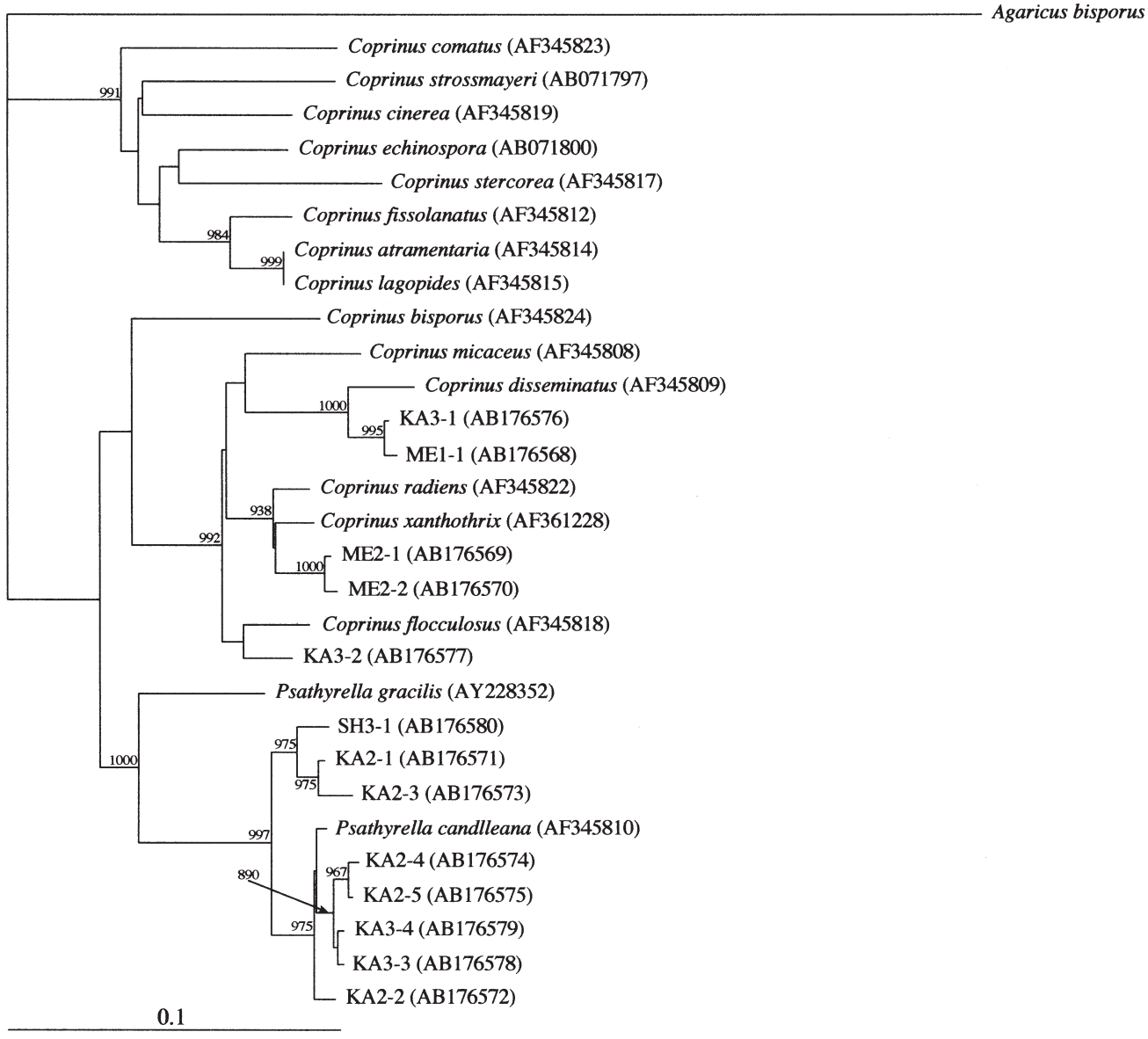


Fig. 5. Neighbor-joining phylogenetic tree showing the relationship between mycorrhizal fungi isolated from *Epipogium roseum* and related fungi in Coprinaceae based on the sequences of ITS region of nuclear rDNA. *Agaricus bisporus* (AY484694) is the relevant outgroup species. All bootstrap value more than 80% are shown (1000 replicates). Accession numbers of GenBank nucleotide database are given for all sequences

would be useful in evaluating the details of the mycorrhizal association in *E. roseum*.

Large colonies of this orchid were frequently found around tree stumps or fallen logs. Because the fungi in Coprinaceae are saprobes, these decaying wood materials would supply a large and persistent carbon source to the orchid.

The information obtained in this study would be useful to consider in the conservation and propagation of *E. roseum* in natural habitats.

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