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Characterization of mycorrhizal fungi isolated from the threatened *Cypripedium macranthos* in a northern island of Japan: two phylogenetically distinct fungi associated with the orchid

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Abstract We isolated *Rhizoctonia*-like fungi from populations of the threatened orchid *Cypripedium macranthos*. In ultrastructural observations of the septa, the isolates had a flattened imperforate parenthesome consisting of two electron-dense membranes bordered by an internal electron-lucent zone, identical to the septal ultrastructure of *Rhizoctonia repens* (teleomorph *Tulasnella*), a mycorrhizal fungus of many orchid species. However, hyphae of the isolates did not fuse with those of known tester strains of *R. repens* and grew less than half as fast as those of *R. repens*. In phylogenetic analyses, sequences for rDNA and internal transcribed spacer (ITS) regions of the isolates were distinct from those of the taxonomically identified species of *Tulasnella*. On the basis of the ITS sequences, the isolates clustered into two groups that corresponded exactly with the clades demonstrated for other *Cypripedium* spp. from Eurasia and North America despite the geographical separation, suggesting high specificity in the *Cypripedium*–fungus association. In addition, the two phylogenetic groups corresponded to two different plant clones at different developmental stages. The fungi from one clone constituted one group and did not belong to the other fungal group isolated from the other clone. The possibility of switching to a new mycorrhizal partner during the orchid's lifetime is discussed.

Keywords *Cypripedium macranthos* var. *rebunense* · Mycorrhizal fungi · *Rhizoctonia* · Specificity · Threatened orchid · Tulasnellaceae

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Introduction

Orchids have unique mycorrhizal associations in that seed germination depends on carbon nutrition supplied from mycorrhizal fungi (Smith and Read 1997) because the very small seeds lack the reserves for germination. Orchids are dependent on their mycorrhizal fungi until the nonphotosynthetic young seedlings (i.e., protocorm) develop shoots and become photoautotrophic. Many fungi isolated from mycorrhizal orchid tissues have been classified as *Rhizoctonia* or *Rhizoctonia*-like fungi. When a teleomorph is occasionally induced in vitro (i.e., a basidiospore forms), the isolated fungi have been identified as basidiomycetes (*Ceratobasidium*, *Sebacina*, *Tulasnella*, and several other genera). For example, *Rhizoctonia repens*, the well-known orchid mycorrhizal fungus, with a wide host range from

terrestrial to epiphytic orchids, has the teleomorph *Tulasnella deliquescens* (Taylor et al. 2002; Sharon et al. 2008).

Because of difficulty in inducing basidiospore formation in orchid mycorrhizal species of *Rhizoctonia*, the fungi often have been identified by vegetative characteristics in culture (Warcup and Talbot 1971; Zelmer and Currah 1995) and by their hyphal anastomosis group (Sneh et al. 1991). In addition to such classical techniques, molecular identification has substantially advanced the taxonomy of orchid mycorrhizal *Rhizoctonia* and revealed previously undiscovered potential mycorrhizal associates. This technique particularly helps in the classification of fungal species that are difficult to grow in pure culture, and many researchers have adapted this molecular taxonomy technique to investigate the ecology of the orchid–fungal association (Dearnaley 2007). Many papers suggest that molecular identification is more accurate and reliable than morphological approaches (Taylor and Bruns 1997; Bidartondo et al. 2004; McCormick et al. 2004; Shefferson et al. 2007).

The lady-slipper orchids (*Cypripedium* spp.) are temperate terrestrial orchids that are distributed throughout Eurasia and North America. *Cypripedium macranthos* var. *rebunense* is endemic to Rebun Island (45°30′ N, 141°04′ E) in the northernmost part of Japan. This orchid is one of the most famous wild plants in Japan because it has become a symbol for the conservation of threatened plants. To conserve surviving wild populations and reintroduce plants into declining populations, we must understand the interaction between this orchid and its mycorrhizal fungi, but there have been few reports on the fungal isolation of mycorrhizal associates of *Cypripedium* species. By direct polymerase chain reaction (PCR) amplification of fungal ribosomal DNA (rDNA), several species of *Cypripedium* have been demonstrated to be exclusively associated with fungi in Tulasnellaceae based on the sequence homology of the nuclear (nrLSU) and mitochondrial large subunit rDNA (mtLSU; Shefferson et al. 2005, 2007), while most of fungal sequences in *Cypripedium* spp. differed from those of known Tulasnellaceae accessions in the phylogenetic position of the 5.8S rDNA (Shefferson et al. 2007).

Although molecular approaches have contributed to new findings on orchid mycorrhizae without pure isolation of the fungus, confirmation of the mycorrhizal status and artificial propagation of orchids through symbiotic germination does require the isolation of the fungus. In our previous work, we developed an efficient method to artificially induce symbiotic germination of orchid seeds using a fungus isolated from the roots of *C. macranthos* var. *rebunense* adult plants (isolate WO97, Shimura and Koda 2005). The first flowering of the plant was observed in June 2006 at our botanical garden (Hokkaido University).

The present study includes classical taxonomy of *Rhizoctonia* spp. using morphological and physiological characteristics as the first step in fungal identification. Using molecular phylogenetic studies based on nuclear rDNA, its internal transcribed spacer (ITS), and mtLSU sequences, we address the following questions: (1) What is the phylogenetic topology of the *Rhizoctonia*-like fungi associated with *C. macranthos* relative to other *Rhizoctonia*? (2) What is the degree of specificity of the orchid for its fungal partner? (3) Do phylogenetic assessments of fungal species correspond to biological assessment of species?

Materials and methods

Isolation of *Rhizoctonia*-like fungi from orchid roots and protocorms

Roots were collected from four plants of *C. macranthos* var. *rebunense* (two juvenile plants estimated to be 4–5 years old and two flowering adult plants estimated to be 10–11 years old) and two flowering adult plants of *C. macranthos* var. *speciosum* in Funadomari reserve (1.7 ha) in Rebun Island, with the permission of the Ministry of the Environment of Japan. Juvenile plants of *C. macranthos* var. *rebunense* had two to three leaves without any evidence of a floral bud. Adult plants of *C. macranthos* var. *rebunense* and var. *speciosum* had four to five leaves and they were at the blooming stage. The distance between adult plants of these varieties was about 1 m, and juvenile plants of *C. macranthos* var. *rebunense* were 5 m away from adult plants of both varieties. Root tips about 5 cm long were cut from healthy white roots of two *C. macranthos* varieties each June from 2002 to 2004. The fungi were isolated using acidic water agar (AWA; 18 g/l agar, pH was adjusted to 4.5 with lactic acid), chloramphenicol water agar (CWA; 50 mg/l chloramphenicol, 18 g/l agar), and oatmeal agar (OMA1; 2 g/l fine oatmeal powder [40 mesh], 18 g/l agar) as reported previously (Shimura and Koda 2005). Elongating hyphae for all isolates were examined with a light microscope, and isolates with *Rhizoctonia*-like morphology according to the description of Sneh et al. (1991) were selected. The criteria included branching near the distal septum of cells in young vegetative hyphae, constriction of hyphae with formation of septa just under the branching point, nothing of clamp connections, conidia or rhizomorphs, etc. Isolate WO97, which was isolated from the roots of flowering adult plants of *C. macranthos* var. *rebunense* in Funadomari reserve in June 1997, has the ability to induce efficient germination of *C. macranthos* var. *rebunense* (Shimura and

Koda 2005). Isolate WO97 appeared to be *Rhizoctonia*-like fungi according to the description of Sneh et al. (1991), but the isolate had been not identified further with analyses such as molecular phylogenetic analyses.

Fungi from naturally germinated protocorms were isolated from packets of previously buried seeds of *C. macranthos* var. *rebunense*. Ten packets of nylon mesh (50 μm opening) containing about 200 seeds each were buried in Funadomari reserve in September 2003. One year after burying the packets, we examined the packets and harvested all the protocorms that had developed to isolate any associated fungi. The protocorms were then surface-sterilized for 2 min with 10% sodium hypochlorite solution and placed in a 9-cm Petri dish containing 10 ml green pea agar (GPA; supernatant of boiled 200 g green pea plus 18 g agar/l). Fungi emerging from the protocorm were then selected as described earlier. All the isolates obtained from the roots and protocorms of *Cypripedium* (hereafter designated as *Cypripedium* isolates) were maintained on GPA and kept in the dark at 20°C.

Physiological and morphological characterization

For further characterization, 14 isolates including WO97 were selected based on their host origin and morphological character on GPA (Table 1). For the anastomosis test, 19 tester strains were used—17 binucleate *Rhizoctonia* (AG-A, Ba, Bb, C–I, K, L, N–R) and two *R. repens* (OR810 and N332, belonging to R.r.1 and R.r.2, respectively). All tester strains had been collected from various orchids, crops, and

Table 1 The fungal isolates from *C. macranthos* var. *rebunense* and *C. macranthos* var. *speciosum* used for the phylogenetic analysis

Host orchid	Isolate	Accession[a] (rDNA-ITS/mtLSU)	Mean growth rate ± SE (mm/day)[b]			Symbiotic germination (%)
			20°C	25°C	30°C	
Cypripedium macranthos						
(Adult plant)						
C. macranthos var. *rebunense*	WO97	AB369822/AB495225	2.68±0.06	2.56±0.11	0	21.1±3.3
C. macranthos var. *rebunense*	WO034	AB369823/AB495226	2.64±0.05	2.24±0.07	0	20.9±1.6
C. macranthos var. *rebunense*	WC035	AB369824/AB495227	2.61±0.05	2.29±0.15	0	24.1±3.9
C. macranthos var. *rebunense*	WO042	AB369923/AB495228	2.45±0.11	2.31±0.08	0	n.e.
C. macranthos var. *rebunense*	WC041	AB369925/AB495230	2.25±0.12	2.18±0.06	0	n.e.
C. macranthos var. *rebunense*	MO043	AB369928/AB495233	2.56±0.04	2.38±0.04	0	n.e.
C. macranthos var. *rebunense*	MC044	AB369929/AB495234	2.63±0.05	2.37±0.08	0	6.6±1.8
C. macranthos var. *speciosum*	RO02	AB369933/AB495238	2.62±0.03	2.28±0.08	0	1.1±0.2
C. macranthos var. *speciosum*	RO031	AB369934/AB495239	3.16±0.05	2.15±0.09	0	3.0±0.5
C. macranthos var. *speciosum*	RC033	AB369937/AB495242	2.73±0.05	2.28±0.11	0	n.e.
C. macranthos var. *speciosum*	RC043	AB369938/AB495243	2.73±0.03	2.48±0.09	0	4.2±1.1
(Juvenile plant)						
C. macranthos var. *rebunense*	SO035	AB369931/AB495237	2.15±0.08	2.68±0.07	0	0.6±0.3
C. macranthos var. *rebunense*	SC034	AB369932/AB495236	2.01±0.09	2.10±0.06	0	n.e.
(Protocorm)						
C. macranthos var. *rebunense*	FT061	AB373957	1.88±0.03	2.05±0.06	0	1.2±0.4
Spiranthes sinensis	OR810 (AG R.r.1)	AB369939/AB495244	4.61±0.05	4.93±0.23	4.26±0.12	0
Liparis kumokiri	N332 (AG R.r.2)	AB369940/AB495245	4.50±0.03	5.49±0.21	2.22±0.07	0

rDNA-ITS nuclear rDNA with the ITS region, *mtLSU* mitochondrial large subunit rDNA, *n.e.* not examined, *AG* anastomosis group

[a] GenBank accession numbers of the nuclear rDNA with the ITS region (rDNA-ITS) and the mitochondrial large subunit rDNA sequences. Isolate FT061 was not used in the sequencing analysis of mtLSU

[b] $n=5$

soils in Japan and have been maintained in the Laboratory of Plant Pathology of our Graduate School. Fourteen *Cypripedium* isolates were examined for the anastomosis reaction in all combinations. A *Cypripedium* isolate and a tester strain were precultured on GPA separately, and then an agar disk (5 mm diameter) was excised from the hyphal edge of each culture. These disks were then placed 1–2 cm apart on GPA or water agar (18 g/l agar) and cultured at 25°C in the dark. The zone of contact was excised, placed on a slide, stained with lactophenol cotton blue, and the hyphae examined for fusion using a light microscope.

The ultrastructure of the septal pore is considered to be valuable method for distinguishing higher taxa because unique forms of septal pores correspond closely with other characters at the generic level (Andersen 1996; Suárez et al. 2006). For morphological characterization of septal ultrastructure, four *Cypripedium* isolates (WO97, WO034, RO02, RC033) and a tester strain OR810 of *R. repens* were cultured separately on GPA for 7–10 days, then agar blocks (3×3 mm) with hyphae were fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 25 mM phosphate buffer (pH7.0). After four 15-min washes in the buffer, the blocks were postfixed in 1% osmium tetroxide in 25 mM phosphate buffer (pH7.0) for 3 h, washed with the same buffer four times for 5 min each, and dehydrated in an ethanol series for 15 min. After dehydration, ethanol was replaced with *n*-butyl glycidyl ether (QY-1; Nisshin EM, Tokyo, Japan) three times for 20 min each, then embedded in epoxy resin (Epon 812; Polyscience; Wako Pure Chemical Industries, Osaka, Japan). Briefly, the sample was treated with QY-1/Epon 812 (1:1) overnight, then with pure Epon 812 twice (for 5 h, then overnight). Samples were then polymerized at 60°C for 24 h. Thin sections (80 nm) were cut with glass knives and stained with 1% uranyl acetate in 50% ethanol (*w*/*v*) for 10 min, then with Reynolds lead citrate (Reynolds 1963) for 3 min. The sections were observed with a transmission electron microscope (H-800, Hitachi, Tokyo, Japan) at 200 kV.

Assessment of ability to induce symbiotic germination

We examined the ability of our fungal isolates to induce symbiotic germination using methods reported previously (Shimura and Koda 2005). Because a shortage of orchid seeds did not permit our testing all the *Cypripedium* isolates, we tested nine *Cypripedium* isolates and two tester strains (OR810 and N332) of *R. repens*. A mature capsule of *C. macranthos* var. *rebunense* was harvested in September 2005 from the Funadomari reserve, and the seeds were used for a germination test. The seeds were surface-sterilized with 10% sodium hypochlorite solution for 30 min and sown on modified oatmeal agar (OMA2; 2 g/l fine oatmeal powder, 10 g/l agar) in a petri dish, then held at 4°C for 12 weeks in the dark to break dormancy. The seeds were then inoculated by placing an agar disk of mycelium on the center of the plate, and the cultures were kept at 20°C for 16 weeks in the dark.

Molecular characterization

For the DNA isolation, another six *Cypripedium* isolates and the aforementioned 14 isolates and the two tester strains (OR810 and N332) of *R. repens* were cultured in liquid GPA for 2–6 weeks. After incubation, the GPA was removed by filtration, and the harvested mycelia were frozen with liquid nitrogen. DNA was extracted from the frozen mycelial pellets using the QIAGEN DNeasy Plant Mini kit (QIAGEN K.K., Tokyo, Japan). The PCRs were performed using primers ITS1 and ITS4 (White et al. 1990) to amplify the ITS1–5.8S–ITS2 region of the nuclear ribosomal repeat. To obtain sequence information beyond the ITS–5.8S region, we amplified the partial nrLSU and nuclear ribosomal small subunit (nrSSU) from fungal total DNA using the following primer pairs: Ctb6/cNL2F (Taylor et al. 2003) or Ctb6/Rm1R (5′-AGCCTCCACCAGAGTTTTCCT-3′) for the 5′ portions of nrLSU and UNT1F (5′-ATTGACGGAAGGG CACAAC-3′)/WO1R (5′-ATGCTCTCCGGAATACCAGA-3′) or UNT1F/ITS4 for the 3′ portions of nrSSU. To amplify the fungal mitochondrial large subunit, we used primers ML5 and ML6 as described by White et al. (1990). The PCR conditions were as follows: initial denaturation at 95°C for 9 min; 30 cycles of one step of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s; then a final extension at 72°C for 5 min. The PCR products were purified with QIAquick (QIAGEN) and sequenced at both directions. DNA sequencing was performed with an ABI 3100 Genetic Analyzer using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All sequences were deposited in DNA Data Bank of Japan (nuclear rDNA and ITS region, accessions AB369822–AB369824, AB369923–AB369940, AB373957; mtLSU region, accessions AB495225–AB495245). For phylogenetic analysis, multiple alignments were performed using the ClustalW program (Thompson et al. 1994) with minor manual adjustments. DNA substitution models and individual model parameters were estimated using the Akaike information criterion as implemented in the program Modeltest version 3.7 (Posada and Crandall 1998). Phylogenetic analysis by the maximum likelihood (ML) method was performed using parameter setting generated by Modeltest. The maximum parsimony (MP) and a ML methods were performed by the program PAUP* version 4.0 beta 10 (Swofford 2002). Bootstrap analysis used 1,000 random addition replicates with tree bisection–reconnection

branch swapping. Branches with low bootstrap support (<50% or 70%) were collapsed.

Results

Physiological and morphological characterization

According to the description of Sneh et al (1991), *Rhizoctonia*-like fungi were not isolated on AWA from root segments of *Cypripedium* spp., while many hyphae grew out on CWA, OMA1, and GPA. We thus obtained 47 isolates as tentative mycorrhizal fungi. Among the 47 isolates, only three fungi were derived from juvenile plants and protocorms: two (SO035 and SC034) from juvenile plants and one (FT061) from protocorms in spite of intensive efforts at isolation. We attempted to induce basidiospore production of the *Cypripedium* isolates using the soil methods of Ogoshi (1975) and GPA, but the fungus did not grow or sporulate on the soil. The *Cypripedium* isolates could be divided into two groups based on cultural characteristics. Group I consisted of the isolates from adult plants of *C. macranthos* var. *rebunense* and *C. macranthos* var. *speciosum*, and the hyphal colonies were hyaline or white on GPA. Group II consisted of the isolates from juvenile plants and protocorms of *C. macranthos* var. *rebunense*, and the hyphal colonies were creamy or yellowish on GPA. Therefore, groups I and II morphologically differ in their hyphal color. Hyphal growth rates of all the *Cypripedium* isolates tested on GPA were less than 50% of those of *R. repens* at 25°C (Table 1). In addition, all isolates failed to grow at 30°C. The *Cypripedium* isolates did not grow well on sucrose-containing media such as potato sucrose agar (PSA; sucrose 2%) and Czapek's agar (sucrose 3%), both of which were favored by *R. repens* and *R. solani*.

The observations of septal ultrastructure revealed that the isolates derived from *C. macranthos* var. *rebunense* (WO97 and WO034) and *C. macranthos* var. *speciosum* (RO02 and RC033) had similar ultrastructure. They have smooth, flattened, and imperforate parenthesomes (Fig. 1a, b), which consisted of two electron-dense membranes bordered by an internal electron-lucent zone. These characteristics matched those of the tester strain OR810 of *R. repens* (Fig. 1c, d).

In anastomosis tests, no hyphal fusion was observed in any combination between the *Cypripedium* isolates and 19 tester strains of binucleate *Rhizoctonia* and *R. repens*. On the other hand, hyphae of isolates in group I fused with each other, and those in group II fused with each other, although no fusion was observed between groups.

In the in vitro germination test, all the *Cypripedium* isolates tested had some ability to induce symbiotic germination (Table 1). Of these, isolates WO034 and WC035 had a marked ability comparable to that of WO97 in inducing germination. The fungi isolated from *C. macranthos* var.

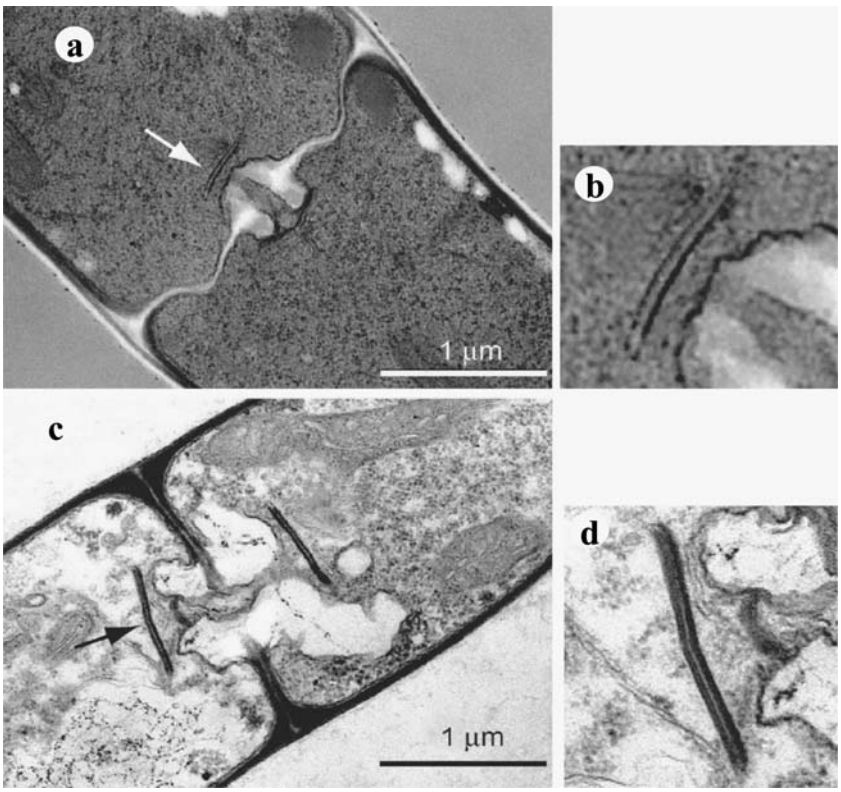

Fig. 1 Ultrastructure of the septal pore of fungi isolated from *C. macranthos* var. *rebunense* (WO97; **a**, **b**) and a tester isolate of *Rhizoctonia repens* (OR810; **c**, **d**). **a** The septum of WO97 has a dolipore structure with imperforate parenthesome (*arrow*), and the septal walls have prominent swellings around a narrow pore channel. **b** Close-up of parenthesome of WO97. The parenthesome consists of two electron-dense layers separated by an electron-transparent zone. **c** The septum of OR810 has a dolipore structure with imperforate parenthesome (*arrow*). **d** Close-up of parenthesome of OR810. The parenthesome consists of two electron-dense layers separated by an electron-transparent zone. The margins of the parenthesome are recurved

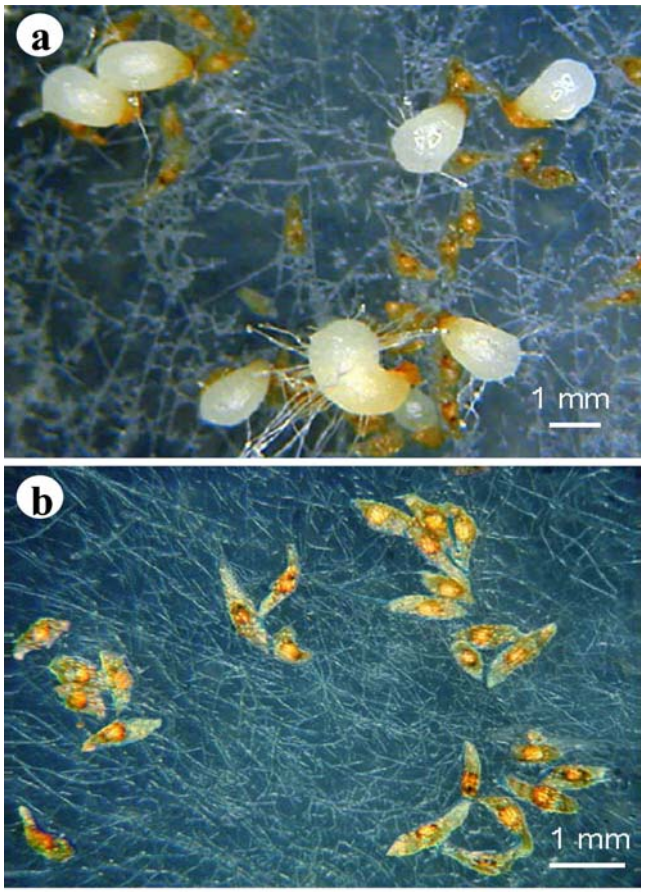

Fig. 2 Comparison of the ability of fungal isolates from *C. macranthos* var. *rebunense* to induce germination, including a strain WC035 and a tester strain OR810. **a** White protocorms developed after the symbiotic germination with isolate WC035 at 16 weeks after inoculation. **b** Seeds failed to germinate with OR810. Actively growing hyphae are visible on the medium inoculated with each fungus (**a**, **b**)

speciosum induced symbiotic seed germination of *C. macranthos* var. *rebunense* although the germination rates were lower compared to those with the fungi from *C. macranthos* var. *rebunense*. In contrast, the two strains of *R. repens* did not induce seed germination (Fig. 2).

Molecular characterization

In all the *Cypripedium* isolates tested here, one fragment (650 bp) was detected in the PCR amplification with primer pair ITS1/ITS4. In restriction fragment length polymorphism (RFLP) analyses of the ITS-5.8S region using *Hha*I, *Hpa*II, and *Hae*III, the banding patterns of all *Cypripedium* isolates differed from those of *R. repens* (OR810 and N332) for all restriction enzymes (data not shown). Among the isolates, the PCR-RFLP results for group I differed from those for group II. Sequence homologies between the two groups were 93% for 5.8S rDNA, 92% for nrSSU, and 87% for nrLSU. The ITS1 and ITS2 sequences between the two groups differed markedly (homology value 63%), while homology within each group was 98–100% for the ITS and nuclear rDNA sequences. In a comparison of the partial sequences of mtLSU, the homology between the two groups was 77%, and the homology within each group was 98–100%. Sequences of the three isolates (WO97, WO035, and WC034) that efficiently induced germination were almost identical.

The ITS–5.8S sequences of the *Cypripedium* isolates were distinct from those of the taxonomically identified members in genus *Tulasnella*. On the other hand, the sequences had high homology with those from unidentified tulasnelloid fungi, which were detected by PCR in various *Cypripedium* species including *C. macranthos* var. *rebunense* (Shefferson et al. 2007). A similar sequence homology with several unidentified mycorrhizal associates of European terrestrial orchids such as *Orchis* and *Ophrys* was also found. Although the ITS–5.8S sequence of isolates in group I shared no homology with morphologically identified Tulasnellaceae, those in group II shared homology with *Epulorhiza* sp. (Taylor and McCormick 2008) and *Epulorhiza calendulina*-like *Rhizoctonia* from tropical orchid mycorrhizae (Ma et al. 2003). Partial nrLSU and nrSSU sequences shared homology with *Tulasnella calospora* (AY15240) and various basidiomycetes. Partial mtLSU sequences of our samples shared homology with several *Tulasnella* spp. and unidentified tulasnelloid fungi, including the isolated fungal sequences from *C. macranthos* var. *rebunense* used for a similar analysis by Shefferson et al. (2007).

The *Cypripedium* isolates in groups I and II were distinct from the major clusters of *Tulasnella* spp. (Fig. 3). Most recently, Sharon et al. (2008) reviewed the classification of *Rhizoctonia* spp. and showed a phylogenetic tree for binucleate *Epulorhiza* spp. (syn. *Rhizoctonia* spp.; teleomorph *Tulasnella* spp.) using rDNA-ITS sequences. Because *Cypripedium* isolates are closely related to *Epulorhiza* spp. in morphological characteristics of septal ultrastructure, we included in our phylogenetic analysis the same fungal isolates available in GenBank as those analyzed in their review. Phylogenetic analysis of the ITS–5.8S sequences of *Cypripedium* isolates compared with those of binucleate *Epulorhiza* spp. isolates showed that *Cypripedium* isolates were located in separate clusters (Supplementary Fig. S1), which were distant from the clusters of *Tulasnella* and *Epulorhiza* groups (clade TG-A to TG-F; Sharon et al. 2008). Some uncultured fungi from nonphotosynthetic liverworts were classified into Tulasnellaceae and found to have an ability to form ectomycorrhizae (Bidartondo et al. 2003), but the *Cypripedium* isolates were placed in a clearly different clade from such *Tulasnella* spp. of liverworts (clade TG-E, AY192441 and AY192442; Supplementary Fig. S1).

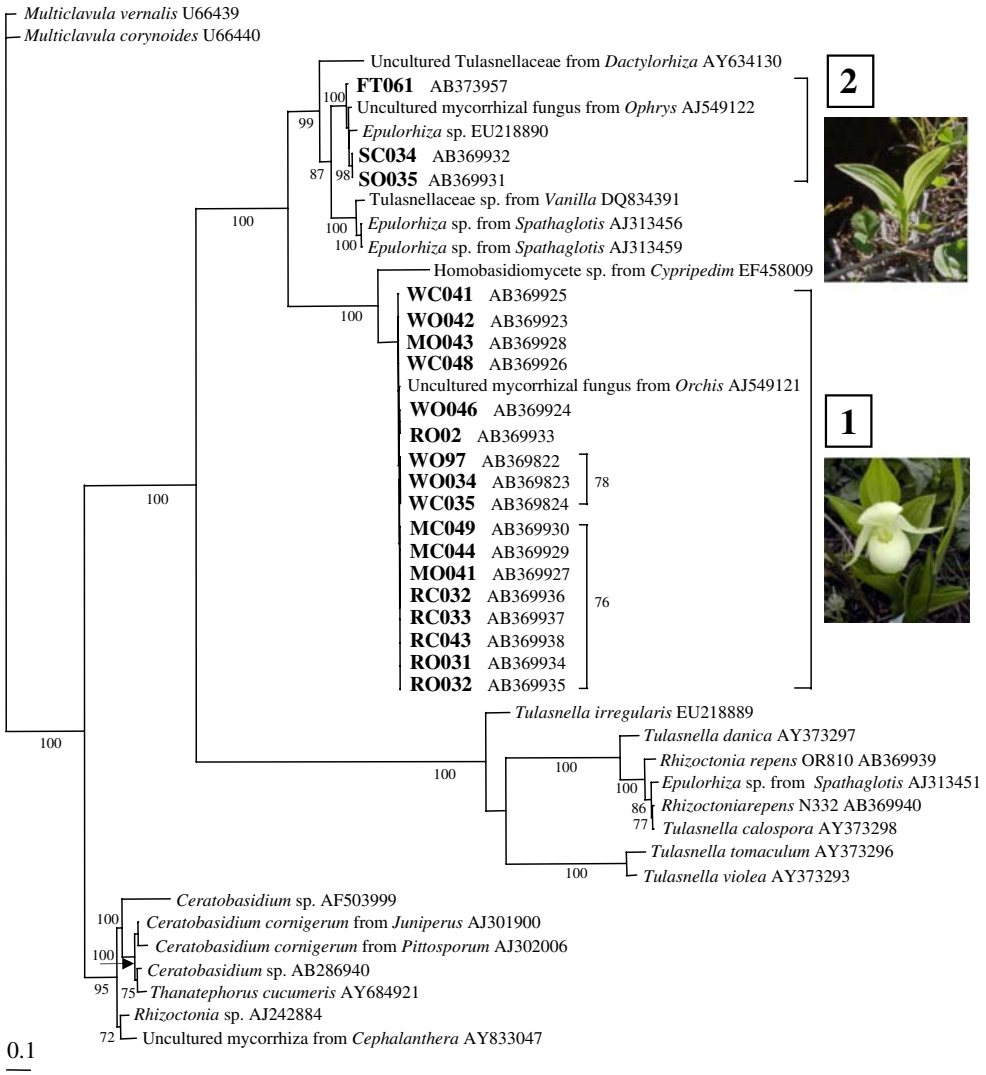

Fig. 3 Phylogenetic placement of the ITS1–5.8S–ITS2 sequences derived from fungi isolated from *C. macranthos* var. *rebunense* and *C. macranthos* var. *speciosum*. The best tree resulting from heuristic maximum likelihood analysis in PAUP* is presented with support values derived using 1,000 bootstrap ML replicates (values >70% are shown). The tree was rooted with *Multiclavula* spp. Fungi isolated from *C. macranthos* varieties are indicated by *bold face*. All fungi isolated from adult plants grouped in clade 1, and the fungi isolated from young plants and germinating protocorms formed a distinct clade (clade 2)

In our phylogenetic analysis, *Cypripedium* isolates were divided into two distinct clades (clades 1 and 2) and the phylogenetic topology was essentially similar to the topology of Shefferson et al. (2007). Our two phylogenetic groups corresponded exactly with the clades demonstrated for other *Cypripedium* spp. from Eurasia and North America (Shefferson et al. 2007) despite the geographical separation. Intriguingly, the isolates from adult plants (i.e., fungi in group I) were positioned in a different clade from the isolates from juvenile plants and the germinating protocorms (i.e., fungi in group II; Fig. 3). The phylogenetic tree constructed by MP methods for the ITS–5.8S sequences had similar topologies (data not shown). To verify this result further, we used the mtLSU sequences to construct another phylogenetic tree (Fig. 4), and the clade for the fungi in group I was positioned differently from the clade for fungi in group II. The mtLSU phylogenetic tree essentially agreed well with the mtLSU tree described in Shefferson et al. (2008). The isolates from adult plants of *C. macranthos* var. *speciosum* (isolate RO02, RO031, RC033, and RC043) were positioned in the same clade of the isolates from adult plants of *C. macranthos* var. *rebunense* (isolate MO043 and MC044), suggesting low mycorrhizal specificity between the two *C. macranthos* varieties.

Discussion

The *Rhizoctonia* spp. are generally soilborne saprophytes, and the hyphae grow well on common nutrition-rich media such as PSA. In contrast, all the fungi that we isolated from *C. macranthos* varieties grew well on nutrition-poor media such as GPA and OMA that contain only a small amount of starch as a carbon source, suggesting that the nutritional demands of *Cypripedium* isolates differ from those of typical saprophytic *Rhizoctonia*. For long-term storage of the *Cypripedium* isolates, we inoculated barley seeds, commonly used to store binucleate *Rhizoctonia*, with the

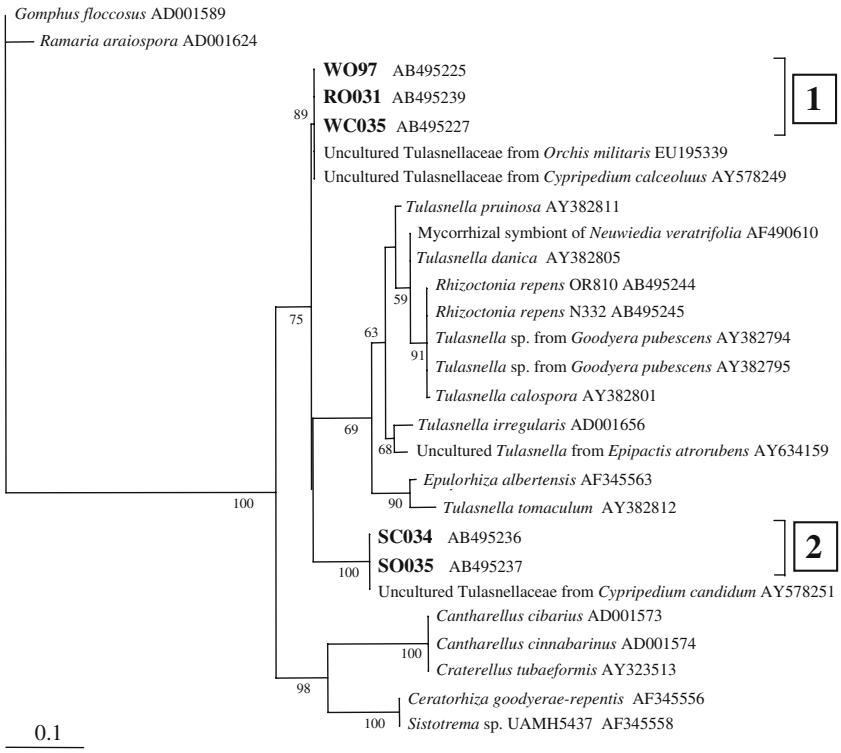

Fig. 4 Phylogenetic tree based on the mitochondrial large subunit rDNA sequences from the fungi isolated from *C. macranthos* var. *rebunense* and *C. macranthos* var. *speciosum*. The best tree resulting from heuristic maximum likelihood analysis in PAUP* is presented with support values derived using 1,000 bootstrap ML replicates (values >50% are shown). Fungi isolated from *C. macranthos* varieties are indicated by *bold face*. The tree was rooted with *Gomphus floccosus* and *Ramaria araiospora*. Note the fungi isolated from adult plants grouped in clade 1 and the fungi isolated from young plants were positioned in another clade (clade 2). Most the sequences were taken from Shefferson et al. (2008)

isolates, but the fungi failed to invade the seeds. These results suggest that the *Cypripedium* isolates are less saprobic and are dependent on another source of nutrients.

Nonphotosynthetic orchids are known to associate with ectomycorrhizal fungi, and a tripartite symbiosis between orchid, fungus, and woody plant has been demonstrated (Taylor et al. 2003). Molecular-based analyses have shown that putatively autotrophic orchids, which live in a shaded habitat, were also associated with various basidiomycetes and ascomycetes that form ectomycorrhizae with woody plants (Bidartondo et al. 2004; Ogura-Tsujita and Yukawa 2008). Recently, we found that seedling recruitment of *C. macranthos* var. *rebunense* in natural habitats was poor near an adult colony compared to areas where shrubby plants such as creeping juniper (*Juniperus conferta*) were present, but few adult *Cypripedium* colonies were found. In a preliminary in vitro experiment, we observed the formations of thin hyphal sheaths and Hartig nets on and within the roots of *J. conferta* seedling after inoculation of *Cypripedium* isolates (WO97), but our phylogenetical analysis failed to confirm any relevance between fungal isolates of *Cypripedium* and *Ceratobasidium* spp. derived from shrubs such as *Juniperus* (Fig. 3). Uncultured Tulasnellaceae fungi associated with nonphotosynthetic liverworts have been demonstrated to form ectomycorrhizae with roots of woody plants (Bidartondo et al. 2003), but the *Cypripedium* isolates were phylogenetically distinct from such ectomycorrhizal *Tulasnella* sp. (Supplementary Fig. S1). There are probably other as yet unknown ectomycorrhizal associates of juniper, some of which are closely related to *Cypripedium* isolate and may be able to provide carbon to *C. macranthos* var. *rebunense*.

Previous studies regarding the specificity of orchid mycorrhizal relationships have demonstrated that the degree of specificity is rather variable among species and that orchid–fungus interactions were not specific (Muir 1989; Otero et al. 2002). However, terrestrial orchids *Goodyera pubescens* and *Lilaris lilifolia* have a very specific fungal association, and both protocorms and adult plants associate with a closely related fungal group (McCormick et al. 2004). Similarly, phylogenetic analyses by Shefferson et al. (2005, 2007) revealed that *Cypripedium* spp. have a highly specific association with fungi within the family Tulasnellaceae, just like the specific associations of nonphotosynthetic orchids (Taylor and Bruns 1997). We also investigated here the degree of specificity in the interaction between the threatened Japanese *Cypripedium* and the newly isolated *Rhizoctonia*-like fungi. Our phylogenetic analyses showed that the *Rhizoctonia*-like fungi isolated from *Cypripedium* were divided into two distinct clades (clades 1 and 2), and the two clades exactly correspond with the two prominent fungal groups demonstrated in the phylogenetic trees of Shefferson et al. (2007, 2008; Figs. 3 and 4; Fig. S1). These results indicate that the *Cypripedium* on a northern island of Japan shares common fungal groups with other *Cypripedium* spp. distributed throughout the northern hemisphere despite the geographical separation, and thus, its fungal association is indeed specific.

Shefferson et al. (2007) previously constructed a phylogenetic tree containing a large number of *Cypripedium* mycorrhizae based on the 5.8S rDNA sequences, revealing two main clades although the bootstrap support was not very high. To assess whether young- and adult-associated fungi are phylogenetically distinct, we here conducted a rather compact analysis using closely related fungi among *Tulasnella* spp. expecting distinct branching with high bootstrap support. Instead of the 5.8S rDNA, we used the 5.8S–ITS sequences with primers ITS1 and ITS4, which have been successfully used for phylogenetic analyses of fungi (McCormick et al. 2004; Suárez et al. 2006). In the results, the ITS tree we obtained was indeed supported by very high bootstrap values, and the topology was essentially similar to that of the 5.8S tree drawn by Shefferson et al. (2007), suggesting that the isolates belong to two different phylogenetic groups.

These two phylogenetic groups of fungi were isolated from orchid plants at different developmental stages: group I from adult flowering plants and group II from juvenile plants and protocorms. In previous phylogenetical analyses of fungal isolates, a protocorm and the adult plants of photosynthetic terrestrial orchid *Tipularia discolor* were shown to be associated with different fungi (McCormick et al. 2004). For *Cypripedium*, groups I and II fungi differed in colony color (white or yellow) and hyphal growth rate (see Table 1). Furthermore, the anastomosis tests revealed that the two phylogenetic groups were actually biologically distinct. These results indicate that our molecular characterization of the mycorrhizae isolated from *C. macranthos* var. *rebunense* corresponds to their biological characterization; groups I and II correspond to clades 1 and 2, respectively. This observation is supported by other reports that the differences in the ITS region of several *Rhizoctonia* spp. appear to correlate with those of their biological properties (Kuninaga et al. 1997; Salazar et al. 1999).

Our results also suggest that the plant–fungus specificity may be determined by the developmental stages of the plants. As shown in Table 1, groups I and II fungi have different biological characteristics in germination tests; the orchid seeds can germinate in vitro more efficiently with group I than group II, suggesting that the infectivity of the fungi in group I is perhaps higher. Such differences in fungal infectivity would influence the specificity and eventual orchid–fungus association. Furthermore, *C. macranthos* var. *rebunense* could have changed its fungal partner in accordance with its development (possible succession of fungi). Also in good agreement with this stage specificity, mycorrhizal colonization of several *Cypripedium* taxa has been shown to vary ontogenetically (Shefferson et al. 2007). McCormick et al. (2006) recently suggested that a terrestrial orchid *G. pubescens* might switch its fungal partner with extreme environmental perturbations, but this idea has not been proved. It will be interesting to verify whether the plant associated with group II as a juvenile does indeed switch to associate with group I as an adult. To conclude whether such a succession occurs in the *Cypripedium*–fungus interaction, we need to observe other plant colonies also. We now are undertaking an extensive and long-term investigation. If the orchid indeed requires different fungi within its lifetime, such knowledge about the fungal partner will certainly be helpful for habitat recovery, especially for rare orchids like *C. macranthos* var. *rebunense*.

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