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IDENTIFICATION AND SYMBIOTIC ABILITY OF PSATHYRELLACEAE FUNGI ISOLATED FROM A PHOTOSYNTHETIC ORCHID, *CREMASTRA APPENDICULATA* (D. DON) MAKINO (ORCHIDACEAE)

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ABSTRACT

Premise of the study: Photosynthetic orchids found in highly shaded forests are often mixotrophic, receiving part of their carbon energy via ectomycorrhizal fungi that had originally received carbohydrate from trees. A photosynthetic orchid, *Cremastra appendiculata* is also found under highly shaded forest, but our preliminary data suggested that its associated fungi were not ectomycorrhizal. We suspected this might be an unusual example of a mixotrophic orchid associating with saprobic fungi.

***Methods*:** For isolated mycobionts of *C. appendiculata* plants, two regions of nuclear ribosomal DNA, the internal transcribed spacer (ITS) and the large subunit (LSU), were sequenced and fruiting bodies of the one isolate, SI1-1 were induced. In addition, the two fungal isolates, SI1-1 and KI1-1 were grown in symbiotic cultures with *C. appendiculata* to verify their ability as mycobionts.

Key results: In phylogenetic analyses, all isolates clustered with fungi belonging to *Coprinellus* in Psathyrellaceae of Agaricales. Phylogenetic analyses of these DNA sequences showed that five fungal isolates from *C. appendiculata*, including SI1-1 and two mycobionts isolated from the mycoheterotrophic orchid *Epipogium roseum*, have very similar ITS sequences. The fungal isolate, SI1-1 was identified as *Coprinellus domesticus* based on the morphological characteristics of the fruiting body. The two fungal isolates, SI1-1 and KI1-1 induced seed germination of *C. appendiculata* as mycobionts.

***Conclusions*:** This is the first report of relationships between fungi in Psathyrellaceae and photosynthetic orchids, in which new pathway to fully mycoheterotrophy could be revealed. The newly found mycorrhizal symbiosis could contribute to our understanding of evolution of mycoheterotrophy.

Key Words: *Coprinellus domesticus*; mycorrhizal symbiosis; Orchidaceae; photosynthetic orchids; saprobic fungi

INTRODUCTION

Photosynthetic orchids mainly associate with teleomorphic fungal families, Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae (Smith & Read 2008), which belong to the anamorphic polyphyletic form-genus, "Rhizoctonia". These orchids depend on saprobic "Rhizoctonia" mycobionts for the nutrients including carbohydrates from seed germination to protocorm development, and some species gain autotrophic feature after green leaves development. On the other hand, several photosynthetic terrestrial orchids growing under highly shaded conditions were found to obtain carbon not only from photosynthesis, but also from associating mycobionts even in adulthood (Gebauer & Meyer 2003; Bidartondo et al., 2004; Selosse et al., 2004; Julou et al., 2005). This nutritional mode is termed mixotrophy or partial mycoheterotrophy (reviewed in Selosse & Roy 2009). The mixotrophic orchids have been reported in *Cephalanthera* and *Epipactis* spp. of the tribe Neottieae (Bidartondo et al., 2004; Julou et al., 2005), in *Cymbidium* spp. of the tribe Cymbidieae (Motomura et al., 2010) and *Platanthera minor* of the tribe Orchideae (Yagame et al. 2012). These species all associate with ectomycorrhizal fungi, such as Thelephoraceae, Russulaceae, non-saprobic Ceratobasidiaceae, Sebacinaceae. The nutritional mode, mixotrophy may represent an evolutionary step towards fully mycoheterotrophic plants (Julou et al., 2005). Actually, this symbiotic feature between orchids and ectomycorrhizal fungi was also found in mycoheterotrophic species, including Russulaceae with *Corallorhiza* spp. (Taylor & Bruns, 1997, 1999), the Thelephoraceae with *Cephalanthera austinae* Heller (Taylor & Bruns, 1997) and the Sebacinaceae with both *Neottia nidus-avis* Rich. (McKendrick et al., 2002) and *Hexalectris spicata* Barnhart (Taylor et al., 2003). The

orchids associating with ectomycorrhizal fungi are usually found under ectomycorrhizal forest trees (Julou et al., 2005; Yagame et al. 2012; McKendrick et al., 2002; etc.). On the other hand, several saprobic fungi are also detected from various mycoheterotrophic orchid species: *Mycena* spp. with *Gastrodia confusa* Honda & Tuyama (Ogura-Tsujita et al., 2009), mycenoid fungi with *Wullschlaegelia aphylla* Rchb. f., *Resinicium* sp., and *Gastrodia similis* Bosser (Martos et al., 2009), Psathyrellaceae with *Eipogium roseum* (D.Don) Lindl. (Yamato et al. 2005) and *Psathyrella candolliana* (Fr.: Fr.) Maire in Psathyrellaceae with *Eulophia zollingeri* (Rchb.f.) J J Sm (Ogura-Tsujita et al. 2008). These studies have clarified that certain saprobic fungi in Agaricales have an ability to support the growth of mycoheterotrophic orchids. However, mixotrophic orchids associating with saprobic Agaricales fungi have not been hitherto confirmed. In fact, the process of mycoheterotrophy development with association of saprobic fungi had not been revealed.

The genus *Cremastra* (tribe Calypsoeae, Orchidaceae) comprises seven species (Yukawa, 1999), with three species distributed in Japan. Among them, *Cremastra appendiculata* (D. Don) Makino is the most common and widely distributed species in Japan with the wider distribution from Sakhalin Island in Russia to Taiwan and the Himalayas (Maekawa, 1971). This species has relatively wide green leaves, suggesting higher photosynthetic ability, but is usually found in the understory of humid and highly shaded forests. This suggests that it is unlikely to exert the photosynthetic ability to a sufficient level to support its own growth, and that the mycobionts alternatively may support the growth of this orchid in its natural habitat. Furthermore, this orchid is often found to grow in non-ectomycorrhizal tree forests such as plantation of *Cryptomeria*

japonica (thunb. ex L. f) and/or *Chamaecyparis obtusa* Sieb. & Zucc. in Japan. The above-mentioned characteristics, therefore, clearly indicate that the associating non-ectomycorrhizal fungi support the growth of this orchid under highly shaded condition in various types of forests.

The aim of this study is to unravel the ecological features of this common orchid species and provide another story of the evolution of mycoheterotrophy/mixotrophy in orchids. In order to achieve the aim, the mycobionts associating with *C. appendiculata* were identified by molecular phylogenetic analysis and the morphological characteristics of the fruiting body induced under artificial conditions. The latter procedure could reveal the identity of mycobionts at the species level. In addition, the fungal isolates were grown in symbiotic cultures with *C. appendiculata* to verify their abilities as mycobionts.

MATERIALS AND METHODS

Sample collection

Rhizomes and roots of *C. appendiculata* were collected from one individual (AF1) in a habitat (AF) in Sagamiko Town, Kanagawa Prefecture, about 235 m above sea level, two individuals (SI1 and SI2) in a habitat (SI) in Fujinomiya City, Shizuoka Prefecture, about 100 m above sea level, and three individuals (KM1, KM2, and KM3) in a habitat (KM) in Maizuru City, Kyoto Prefecture, about 20 m above sea level. At the meteorological stations nearest to the sampling sites, the annual mean temperature and annual total precipitation recorded between 1979 and 2000 were 15.1°C and 1,669 mm, respectively, at Ebina Meteorological Station in Kanagawa Prefecture; 16.3°C and 2,322 mm, respectively, at Shizuoka Meteorological Station in Shizuoka Prefecture; and

14.3°C and 1,786 mm, respectively, at Maizuru Meteorological Station in Kyoto Prefecture. The dominant tree species in the habitats were *Quercus serrata* Thunb. in AF and SI or *Castanopsis sieboldii* (Makino) Hatus. ex T. Yamaz. et Mashiba in KM. In these habitat, *Cryptomeria japonica* (Thunb. Ex L.f.) D.Don was sparsely found.

Fungal isolation

Mycobionts were isolated from collected rhizomes according to the methods of Warcup & Talbot (1967), with slight modifications as follows. The surface of the rhizome was washed in tap water and sterilized by immersion in 70% ethanol for 30 s and subsequently in sodium hypochlorite solution containing 1% available chlorine for 30 s. The surface-sterilized rhizome was then cut into small pieces approximately 5 mm in length. The pieces were placed in a Petri dish (9 cm diameter) with 1 ml sterilized distilled water and crushed with a sterilized glass rod to disperse the intracellular hyphal coils (pelotons). 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 g, distilled water 1000 ml) was cooled to 45°C, poured into the Petri dishes (approx. 20 ml per dish) and mixed well before solidification to disperse the pelotons throughout the medium. The plates were incubated at 25.0 ± 0.5°C in the dark for 3 days. Fungal colonies growing from the pelotons were isolated using a sterilized scalpel and cultivated on potato dextrose agar (PDA, Difco) medium.

Molecular phylogenetic analysis of isolates

DNA was extracted from each of the cultures and isolated fungal coils obtained from cultured protocorms using PrepMan Ultra Reagent (Applied Biosystems, Foster City,

CA, USA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) and the large subunit (LSU) regions of nuclear ribosomal DNA (rDNA) were amplified from the extracted DNA by polymerase chain reaction (PCR) with the primers ITS1F/ITS4 (Gardes & Bruns, 1993) and NL1/NL4 (O'Donnell, 1993), respectively, using TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 5 μl template DNA, 0.75 units *Taq* polymerase, 0.25 μM each primer, 200 μM each dNTP, and 3 μl supplied PCR buffer in a total volume of 30 μl. The amplification of the ITS region was performed on a Program Temp Control System PC-818S (Astec, Fukuoka, Japan) as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min, then a final elongation step at 72°C for 5 min. The amplification of the LSU region was performed as follows: initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final elongation step at 72°C for 10 min. All PCR products were cloned using the pT7Blue Perfectly Blunt Cloning kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions, and plasmid DNA was extracted from the cloned products using MagExtractor Plasmid (Toyobo, Osaka, Japan). Plasmid inserts were sequenced using the dye terminator method with sequencing primers T7 and SP6. All sequences were subjected to BLAST searches (Altschul et al., 1997), and the related sequences were downloaded from the DDBJ/EMBL/GenBank nucleotide sequence database. Alignment of the sequenced and downloaded data was performed using CLUSTAL W (Thompson et al., 1994). For phylogenetic analyses, Bayesian phylogenetic analyses were further conducted using the MRBAYS 3.1.2 program (Ronquist & Huelsenbeck, 2003) with additional neighbor-joining (NJ) or maximum parsimony (MP) analyses performed by MEGA

version 5 (Tamura et al., 2011) with bootstrap analysis of 1000 replications (Felsenstein, 1985). For the NJ analysis, evolutionary distances were estimated using γ-distributed rates. In the Bayesian analysis, the general time-reversible model, assuming a discrete γ-shaped rate variation with a proportion of invariable sites (GTR + I + G), was estimated as the best-fit likelihood model for the ITS and nuclear large subunit (nLSU) datasets with MRMODELTEST 2.2 (Nylander, 2004) in PAUP* 4.0b10 (Swofford, 2003). Posterior probabilities (PP) were approximated by a Metropolis-coupled Markov chain Monte Carlo (MCMC) method. Two parallel runs were each conducted with one cold and seven heated chains for 5 000 000 generations, starting with a random tree. The seven chains were heated at 0.2 for both datasets. Trees were saved to a file every 100th generation. We judged that the two runs reached convergence when the average standard deviation of split frequencies (ASDSF) fell below 0.01. Trees obtained before reaching convergence were discarded using the 'burn-in' command and the remaining trees were used to calculate a 50% majority consensus topology, and to determine PP for individual branches. Furthermore, the ITS and LSU datasets were analysed by the maximum likelihood (ML) method using PhyML version 3.0 (Guindon et al., 2010). The best-fit ML tree was inferred using a GTR + GAMMA model. To evaluate statistical support for tree topology, the bootstrap option was used (1000 replications). The phylogenetic trees obtained in these analyses were drawn with TREEVIEW software (Page 1996).

Fruiting body formation and morphological observations

The two isolates, SI1-1 and KM1-1, were induced to form fruiting bodies using the methods of Yagame et al., (2008), as follows. The isolates were inoculated into a

medium consisting of a mixture of broad-leaved tree sawdust and wheat bran [17:10 (w/w)] with 55% moisture content after autoclaving at 120°C for 60 min. The culture was incubated at 25.0 ± 0.5°C in the dark for 4 weeks to generate spawns of SI1-1 and KM1-1. A plastic container (55.0 × 35.0 × 11.5 cm), sterilized by spraying with 70% ethanol, was used to make a mushroom bed. At the bottom of the container, 1,500 ml autoclaved Hyuga soil (a volcanic soil, particle size 15–20 mm in diameter) was added to form a drainage bed. Then, 600 ml spawn was spread over the soil layer and 2,000 ml autoclaved (120°C for 40 min) leaf mold collected from a mixed forest of *Q. serrata* and *C. sieboldii* at Koyama-Minami, Tottori Prefecture, Japan was placed on top to make the mushroom bed. The pH and moisture content of the leaf mold after autoclaving were 4.5 and 80%, respectively. The container was kept in the dark at 15.0 ± 0.5°C and 80.0% relative humidity for 2 months to induce fruiting body formation. All descriptions of macro-and microscopic characteristics were of cultivated fruiting bodies. Color notations used in the species description were according to Kornerup & Wanscher (1978). Anatomical observations and measurements were made using specimens mounted in 10% KOH preparations using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Dried (60°C for 24 h) voucher specimens, TUMH 40454 and TUMH 40455 were kept in the Tottori University Mycological Herbarium (TUMH) and the isolates SI1-1 (TUFC 13516) are stored in the Tottori University Fungal Culture Collection (TUFC).

Symbiotic cultivation

For symbiotic cultivation, autoclaved oatmeal agar (OMA: oatmeal agar (Difco) 2.5 g, agar 6.5 g distilled 1,000 ml, pH: 7.0) was poured into disposable plastic Petri dishes (9 cm diameter; approx. 20 ml OMA per dish). Seeds were removed from mature fruits of

the orchids, which were collected at Yazu, Tottori Prefecture, Japan, on 20 October 2009. The seeds were sterilized by immersion for 5 min in sodium hypochlorite solution containing 1% available chlorine, and were then rinsed thoroughly with sterilized distilled water. About 100 rinsed seeds were placed on OMA medium. For symbiotic cultivation, we used the two fungal isolates SI1-1 and KM1-1 because they grew well on PDA medium and belonged to the different clades in the phylogenetic analyses. The fungal inocula (each approx. 0.5 × 0.5 cm), which were pre-grown on PDA, were transferred onto the medium at a short distance from the sown seeds. Five replicates were used for each fungal isolate and for the non-inoculated control. The cultures were incubated at 20±0.5°C in the dark and observed weekly to assess seed germination, which was defined as the emergence of a swollen embryo from the seed coat. To maintain robust growth, the seedlings were transferred to the new OMA medium every month. In order to confirm fungal colonization, free hand sections of the formed rhizomes of *C. appendiculata* were examined by an optical microscope (Axio Imager A1, Zeiss) after 12 weeks after sowing.

RESULTS

Root morphology and mycorrhizal colonization

Cremastra appendiculata plants were collected near decayed fallen trees. Only the leaf stem was visible above the ground. The underground organs of *C. appendiculata* consisted of three parts: a bulb, roots, and a rhizome (Fig. 1). The leaf stem above the ground originated from the bulb, the rhizomes spread deep into the ground, and the roots elongated between the litter and the ground. Mycobionts were not found in the bulb, but were found in both the rhizomes and roots, where cortical cells were colonized.

The extent of colonization was, however, different, very limited in the roots, but abundant in the rhizomes.

Fungal isolation and fruiting body formation of SI1-1

We attempted to isolate mycobionts from pelotons both in the roots and rhizomes, but failed from the roots. However, we could isolate fungi from all of the collected rhizomes to obtain 13 isolates in total (Table 1). The fungal sequences were deposited in the DDBJ database under the following accession numbers: AB597769–AB597781 for the ITS sequences and AB685744–AB685756 for the LSU sequences (Table. 1). Among the 13 isolates, SI1-1 and KM1-1 grew well on PDA medium and were used to induce fruiting body formation. The isolate KM1-1 formed primordia of fruiting bodies but they did not mature, whereas SI1-1 formed mature fruiting bodies about 2 months after inoculation (Fig. 2). Detailed comparisons of the morphological characteristics of the fruiting bodies with those described in the literature (Orton & Watling, 1979; Breitenbech & Kränzlin, 1995; Uljé 2005) resulted in the identification of isolate SI1-1 as *Coprinellus domesticus* Vilgalys, Hopple & Johnson. The morphological features of the fruiting body of SI1-1 and the taxonomy are described below.

Coprinellus domesticus (Bolton : Fr.) Vilgalys, Hopple & Jacq. Johnson in Redhead, Vilgalys, Moncalvo, Johnson & Hopple, Taxon 50: 233, 2001.

≡ *Agaricus domesticus* Bolton : Fr., Syst. Mycol. 1: 311, 1821.

≡ *Coprinus domesticus* (Bolton :Fr.) Gray, Nat. Arr. Brit. Pl. (London) 1: 635, 1821.

Figs. 2a–g

Pileus ellipsoid to ovoid when young, expanding to conical or convex and then 36–60

mm (45.5±7.5 mm: mean±standard deviation (SD) *n*=11), broad, 16–31 mm high (22.6±3.9, n=11), radially sulcate almost to disc, when old the edge somewhat recurved and eroded irregularly (Fig. 2a), surface white when very young, soon becoming grayish orange to brownish yellow with paler margin (Fig. 2a), at first covered with felty white veil, later the veil breaking into small woolly flocks (Fig. 2a) that become grayish orange at center of pileus with age and are easily detachable. Flesh very thin, fragile, white; taste mild; odorless. Lamellae free, crowded (number of lamellae reaching stipe = 70–80), narrow (1 mm wide), white at first, then grayish, finally blackish, deliquescent. Stipe 48.9–103.3 (69.5±16.1, n=11) × 4.1–7.5 (6.0±1.1, n=11) mm, cylindrical, equal or somewhat tapering upward, base clavate, not rooting, hollow, fragile, surface white. Ozonium absent (Fig. 2a).

Basidiospores black in mass, dark red-brown under the microscope, subcylindrical to ellipsoid in face view, at times phaseoliform in side view, 7.0–9.0 (7.6±0.1, n=24) μm long, 4.0–6.3 (4.9±0.7, n=24) μm broad in face view, 4.6–4.8 (4.7±0.1, n=24) μm in side view with central germ pore 2.1–3.0 (2.4±0.5, n=24) μm wide (Fig. 2b). Basidia 17–25 (20.1±2.2, n=19) μm long, 7.2–10.1 (8.3±0.9, n=19) μm wide, 4-spored clavate to cylindric-clavate with a narrowed base, thin-walled, hyaline (Fig. 2c). Pleurocystidia 91–251 (172.1±48.3, n=10) μm long, 34–79 (59.7±13.1, n=10) μm wide, oblong-ellipsoid to ovoid, thin-walled, hyaline (Fig. 2d). Cheilocystidia 23-55 (35.8±9.5, n=10) μm long, 19–51 (35.8±9.5, n=10) μm wide, subglobose, numerous, thin-walled, hyaline (Fig. 2e). Caulocystidia 96–232 (178.3±44.8, n=10) μm long, 23–37 (29.4±6.3, n=10) μm wide, lageniform with a narrow, prolonged neck, 5–10 (7.0±2.0, n=10) μm wide, thin-walled, hyaline (Fig. 2f). Veil on the pileus surface composed of chains of

cylindrical to ellipsoid, fusiform or (sub)globose cells, if (sub)globose then towards end of the chain, thin-walled to somewhat thick-walled and grayish orange towards end of chain, 24–59 (41.7±8.7, n=24) μm long, 19–42 (30.0±6.2, n=24) μm wide (Fig. 2g).

Note*: *Coprinellus domesticus belongs to the subsection Domestici Singer of section Veriformes (Fr.) Penn. (Noordeloos et al., 2005). Within this group, *C. domesticus* is similar to *C. ellisii* (P.D. Orton) Redhead, Vilgalys & Moncalvo and *C. xanthothrix* (Romagn.) Vilgalys, Hopple & Jacq. Johonson. However, the fruiting bodies of *C. domesticus* are larger than those of *C. ellisii* and *C. xanthothrix,* and the basidiospores of *C. domesticus* are larger than those of *C. ellisii.* (*C. domesticus* (SI1-1)= Q: 1.42–1.75, 7.0–9.0 μm; *C. ellisii* = Q: 1.60–2.05, 5.5–7.0 μm) (Noordeloos et al., 2005). Also, *C. domesticus* (SI1-1) has subcylindrical, ellipsoid, in face view, at times phaseoliform basidiospores, but those of *C. xanthothrix* are ellipsoid. *C. domesticus* is very common and widespread in Europe, America, Asia, and North Africa and fruiting bodies of this species are found on or around trunks and dead trees (Noordeloos et al., 2005).

Molecular phylogenetic analysis of isolates

Phylogenetic relationships of the 13 isolates were analyzed based on nuclear ribosomal DNA sequences. Full-length sequence data of the ITS region including the 5.8S region and LSU of rDNA were obtained from these samples. BLAST searches showed that the obtained sequences are similar to those of *Coprinellus* in Psathyrellaceae family of Agaricales. Mycobionts of *C. appendiculata* formed two clades in the same genus, *Coprinellus* (Fig. 3a,b). Phylogenetic analysis of the ITS sequences showed that the five isolates including the isolate SI1-1 in this study and the mycobionts ME2-1

(AB176569) and ME2-2 (AB176570) of the mycoheterotrophic orchid, *Epipogium roseum* (D.Don) Lindl, belonged to the same phylotype, I2, showing a high degree of sequence identity (98.8–99.9%) (Fig. 3a). *Coprinellus domestics* (JN159580) formed other clade with *C. radians* (Desm.) Vilgalys, Hopple & Jecq. Johnson and *C. xanthothrix* (Romagn.) Vilgalys, Hopple & Jacq. Johnson (Fig. 3a), but the sequence identity between SI1-1 and *C. domestics* (JN159580) was 95.4%, lower than those in the former clade. Eight isolates in phylotype I1 showed high sequence identity with each other and grouped into the same clade as *C. callinus* (FN396105). The sequence identity between these eight isolates and *C. callinus* was considerably higher 99.5–99.7%. For analysis of the LSU of nrDNA region, fungal sequences of the five isolates including SI1-1 were 100% identical to that of *C. domesticus* (GU048622) in a phylotype L2 (Fig. 3b). In a phylotype L1, eight isolates and those of *C. callinus* AF041520 and FN396158 grouped into the same clade and the sequence identity ranged from 99.6% to 100%. Based on the sequences of the ITS and LSU, the LSU phylotypes L1 and L2 corresponded to ITS phylotypes I1 and I2, respectively. The close relationship between *C. callinus* and the eight isolates was confirmed in the phylogenetic analyses of both ITS and LSU sequences.

Symbiotic cultivation of C. appendiculata

Seeds inoculated with the fungal isolates SI1-1 or KM1-1 germinated 3 weeks after sowing (Fig. 4a), with a germination rate of 37.8±4.1% (mean ± standard error (SE)) and 33.3±5.9%, respectively, whereas uninoculated seeds did not germinate. In each inoculated treatment with SI1-1 or KM1-1, more than 30% seedlings grew vigorously

and developed a rhizome. Protocorms were colonized by fungi (Fig. 4b), which were identical to the inoculated isolates confirmed by their ITS of rDNA sequences in each inoculated condition. The sequences of the detected fungi from cultured rhizomes were deposited in the DDBJ database under the following accession numbers, AB817976 and AB817977, and were included in the phylogenetic tree (Fig. 3a). At 19 weeks after seed sowing, shoots developed on the rhizomes (Fig. 4c), with a formation rate of 59.0±10.7% and 20.0±6.7% with isolate SI1-1 and KM1-1, respectively. On the other hand, roots had not developed at this stage.

DISCUSSION

All of the fungi isolated from the rhizome of *C. appendiculata* were identified as *Coprinellus* in the Psathyrellaceae, Agaricales, by molecular phylogenetic analysis. Fruiting bodies were only formed by the isolate SI1-1. This is the first study of fruiting body formation from an Agaricales fungus isolated from a photosynthetic orchid, as far as we know. In previous studies, identifications of isolated fungi have been usually confined to the genus level because of the limitation of DNA sequence analysis. Several orchid mycorrhizal fungi have been identified at the species level based on morphological characteristics of the fruiting bodies taken from natural habitats, such as *Marasmius coniatus* var. *didymoplexis* Berk. et Br. with *Didymoplexis pallens* Griff (Burgeff, 1932), *Armillaria jezoensis* J.Y. Cha & Igarashi with *Galeola septentrionalis* Rchb. f. (Cha & Igarashi, 1996), *Erythromyces crocicreas* (Berk. & Broome) Hjortstam & Ryvarden with *Erythrorchis ochobiensis* (Hayata) Garay (*Galeola altissima* (Bl.) Reichenbach f.) (Umata, 1995), *Mycena orchidicola* L. Fan & S.X. Guo with *Cymbidium sinense* Willd. (Fan et al., 1996), and *Coprinellus disseminatus* (Pers.:Fr.)

S.F. Gray with *Epipogium roseum* (D. Don) Lindl. (Yagame et al., 2008). However, fruiting body formation of orchid mycobionts under artificial conditions is rarely successful. To our knowledge, this is only the second study of fruiting body formation from orchid mycobionts in the Psathyrellaceae (Yagame et al., 2008).

The isolated and identified mycobionts, *C. domestics* and *C. callinus* are common saprobic fungi and widely distributed in warm to cold temperate region from Europe to Asia (Noordeloos et al., 2005). The ecological features of *C. appendiculata* growing under various types of forests and with rather wide distribution in Asia could be attributed to the above-mentioned nature of mycobionts. In the phylogenetic analysis of the mycobionts, ITS data clearly showed the relationship between mycobionts of photosynthetic *C. appendiculata* and those of mycoheterotrophic orchid, *E. roseum*. The ITS sequences of *C. domestics* (JN159580) and SI1-1 were not identical, but showed high similarity (more than 95 %). Even a single species of *Coprinellus domesticus* may have the ITS sequence with some variations. Actually, the mycobionts of *E. roseum* identified as *C. disseminates* with the accession numbers of AB176568 (Yagame et al. 2008) and AF345809 in GenBank were not identical and furthermore belonged to the different clade (Fig. 3a). The tree constructed using LSU data showed the relationships between mycobionts of *C. appendiculata* and *Coprinellus* species. The branching pattern was not identical between the phylogenetic trees constructed by ITS or LSU regions, probably because of differences in the rate of nucleotide substitution in those two regions. All of the mycobionts of orchids in Psathyrellaceae exclusively hitherto belonged to the *Psathyrella-Coprinellus* group, as defined by phylogenetic analyses of the Psathyrellaceae (Mahajabeen et al., 2008; Vašutová et al., 2008). Oliver et al. (2010) tested the degradation abilities of *Coprinellus radians* and *C. micaceus*, both of which

belong to the *Psathyrella-Coprinellus* group, and confirmed that both species showed high degradation abilities of brown or white rotted woods. Therefore, the mycobionts of *C. appendiculata* belonging to the same fungal group may also have a strong ability to decompose rotted woods. There found lots of decaying fallen logs and branches in the natural habitat of this orchid species. Those logs or branches could be the sources of carbohydrates supplied by mycobionts to those terrestrial orchids growing on the shaded forest floor.

When the fungal taxon was expanded to the Agaricales, saprobic fungi have been reported as mycobionts in mycoheterotrophic orchids (Hamada, 1939; Terashita & Chuman, 1987; Xu & Mu, 1990; Lan et al., 1994; Cha & Igarashi, 1996; Yamato et al., 2005; Ogura-Tsujita et al., 2009 etc). These findings indicate that several saprobic fungi of Agaricales may be able to support the growth of mycoheterotrophic orchids. In the present study, we found that saprobic Psathyrellaceae fungi in the Agaricales induced seed germination of the photosynthetic orchid *C. appendiculata*. As far as we know, this is the first report of Psathyrellaceae fungi as mycobionts associated with photosynthetic orchids. *Cremastora appendiculata* may depend on mycobionts for enough amounts of nutrients for their growth even in adult stage. This ecological feature of this orchid could contribute to survive under highly shaded forest stand.

The so-called “Rhizoctonia” fungi, which used to be detected from photosynthetic orchids, could not be found from the rhizome of *C. appendiculata*. We failed to isolate any mycobionts from the roots. There found, however, only a few pelotons in the roots, and those appeared to be degraded already at the time of isolation, which suggests that the main part of mycobionts colonization in this orchid is not roots but rhizomes. In the photosynthetic terrestrial orchid, *Cypripedium calceolus* Walter, the fungal colonization

was much different between the roots, heavily colonized in the first elongating root, but the subsequent elongating roots were more sparsely colonized (Fuchs & Ziegenspeck, 1925). Roots of photosynthetic orchids appear to have a specialized role to support the plant body and to absorb water and nutrients, while they may decrease their abilities of hyphal digestion and absorption of nutrients from mycobionts when the plant reaches the photosynthetic stage. In the present study, roots did not develop until 19 weeks under symbiotic culture conditions, whereas the rhizomes were already developed and heavily colonized with the fungus during the same period. Therefore, we assume that the primary roles of the roots in *C. appendiculata* are not to associate with mycobionts but to support the plant body and to absorb water and nutrients from the surrounding soil. Development of the rhizome was also observed in another mycoheterotrophic species in the same genus, *Cremastra aphylla* (Yukawa, 1999). Furthermore, a mycoheterotrophic species, *Collarhiza* spp., which belongs to the same tribe, develops only rhizomes with no roots (Leake, 1994). To maintain a mycorrhizal symbiosis throughout their whole life history, several orchids including *C. appendiculata* may develop a specialized organ of rhizome for mycorrhizal symbiosis.

Cremastora appendiculata is usually found on the shaded forest floor, suggesting that this orchid cannot perform high-level photosynthetic activity, despite having rather wide leaves. Insufficient carbon gain, therefore, could be compensated by the supply from the associating saprobic Agaricales mycobionts, indicating that this orchid is partially mycoheterotrophic (mixotrophic) in nature. This ecological feature indicates that *C. appendiculata* may be on the way to develop fully mycoheterotrophic feature in adapting to highly shaded condition. In present study, we could find new pathway to develop fully mycoheterotrophy. This mycorrhizal symbiosis could contribute to our

understanding of evolution of mycoheterotrophy and survival strategies of terrestrial orchids growing under highly shaded forests.

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Table. 1 Isolates of mycobionts from *Cremastra appendiculata*

Isolate number	Sampling date	Sampling site	Accession numbers in DDBJ of the isotates sequences	
			ITS	LSU
AF1-1	18-Jun-02	Sagamiko town Kanagawa Prefecture, JAPAN	AB597769	AB685744
AF1-2	18-Jun-02	Sagamiko town Kanagawa Prefecture, JAPAN	AB597770	AB685745
SI1-1	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN	AB597771	AB685746
SI1-2	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN	AB597772	AB685747
SI2-1	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN	AB597773	AB685748
KM1-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597774	AB685749
KM1-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597775	AB685750
KM1-3	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597776	AB685751
KM2-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597777	AB685752
KM2-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597778	AB685753
KM2-3	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597779	AB685754
KM3-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597780	AB685755
KM3-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN	AB597781	AB685756

Isolate numbers indicate the propereties of the isolates, e.g., AF1-2, sequentially the sampling site (AF), plant number (1) and isolate number (2).

FIGURE LEGENDS

Fig. 1 Root system of *Cremastra appendiculata* RH, rhizome; RO, root; B, bulb; LS, leaf stem. Bar=5 cm

Fig. 2 Morphological characteristics of fruiting bodies formed by cultivation of isolate SI1-1 obtained from the photosynthetic orchid *Cremastra appendiculata*.

a. External appearance of fruiting bodies *Bar*=3 cm. **b–f.** Micromorphology of SI1-1; **b.** basidiospores (TUMH40454) bar=5 μm; **c.** basidia (TUMH40454) bar=5 μm; **d.** pleurocystidia (TUMH40454) bar=30 μm; **e.** cheilocystidia (TUMH40454) bar=30 μm, **f.** caulocycstidia (TUMH40454) bar=30 μm. **g.** veils on the pileus (TUMH40454) bar=30 μm.

Fig. 3 Bayesian 50% majority-rule consensus topology based on nuclear (a) internal transcribed spacer (ITS) and (b) large subunit (LSU) nrDNA sequences of

Psathyrellaceae mycobionts, including *Cremastra appendiculata*, obtained from 162023 to 34402 trees, respectively. Bayesian posterior probabilities (PP) and bootstrap (BS) values (%) of 1000 BS replications in the neighbor joining (NJ) analysis (NJBS) and of 1000 BS maximum parsimony (MP) analysis (MPBS) are indicated as PP/NJBS/MPBS above or below branches or nodes. Likelihoods (In L) of the best states for cold chains of the two runs were -3666.11 and -3724.14for ITS, and –1734.41 and –1736.82 for LSU sequences. Maximum likelihood (ML) analysis of identical dataset resulted in one ML tree (In L =-3352.16488) for ITS and one ML tree (In L = -1641.94743) for LSU sequences. Only BS values greater than 70% are shown. Closed circles in (**a**) indicate mycobionts of mycoheterotrophic orchids, *Epipogium roseum* or *Eulophia zollingeri*. Triangles show isolate SI1-1, which formed fruiting bodies. White and black squares and bold letters indicate detected sequences from rhizomes cultured with mycobionts, SI1-1 and KM1-1, respectively.

Fig. 4 Protocorms and seedling of *Cremastra appendiculata* grown in symbiotic cultivation with isolate SI1-1 on oatmeal agar. **a**. Protocorms (white arrow heads) with hyphae (black double arrow heads) of mycobiont, SI1-1, at 3 weeks after seed sowing. Bar= 400 μm. **b.** Longitudinal section of the protocom in *Cremastora appendiculata* at 12 weeks after seed sowing. Undigested fungal coil (black double arrow head), digested fungal coil (White double arrow head) Bar= 1 mm **c.** Shoot (double white arrow head) development on rhizome (white arrow head) with mycobiont, SI1-1, at 19 weeks after sowing. Bar= 1 cm.

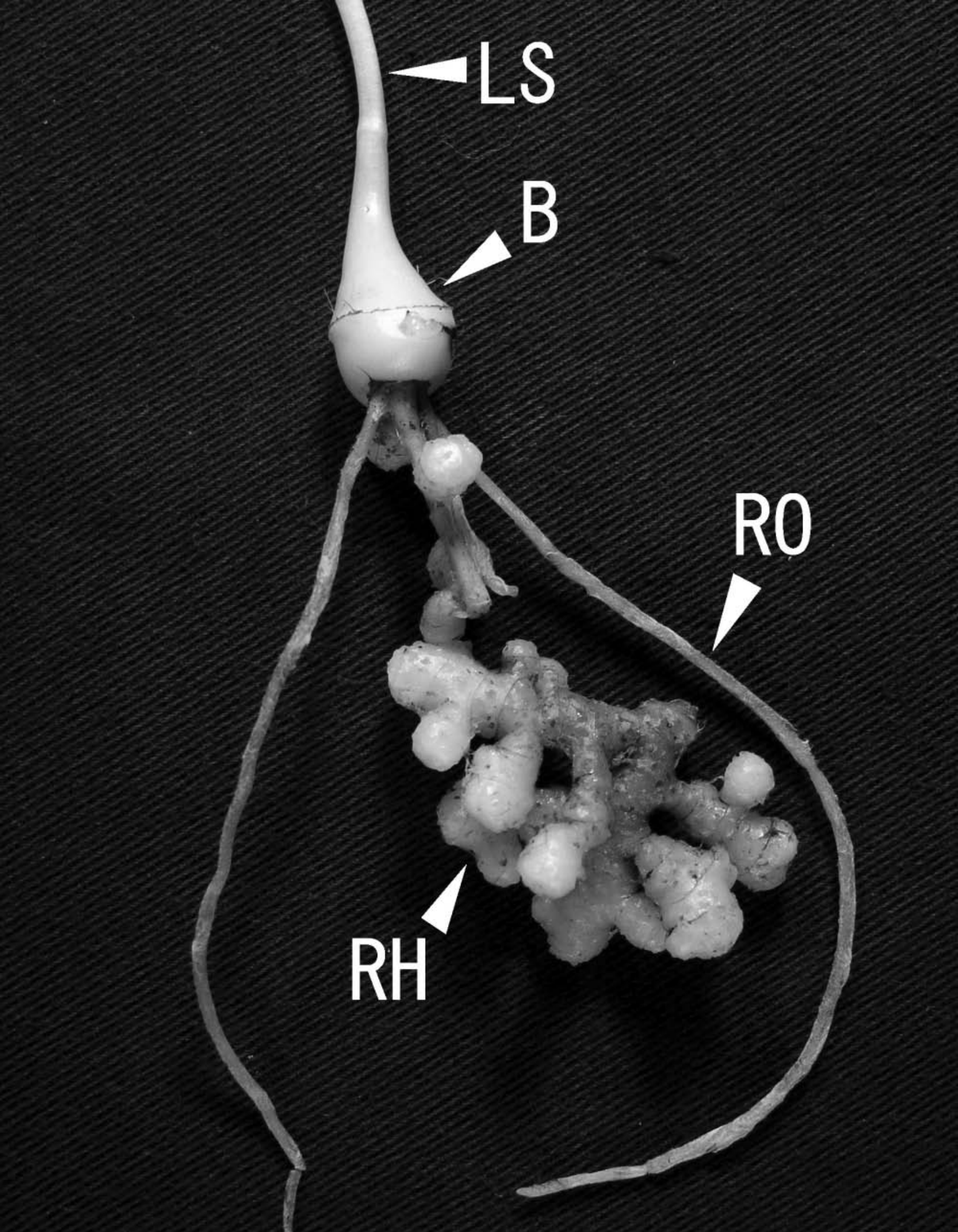
Table. 1 Isolates of mycobionts from *Cremastra appendiculata*

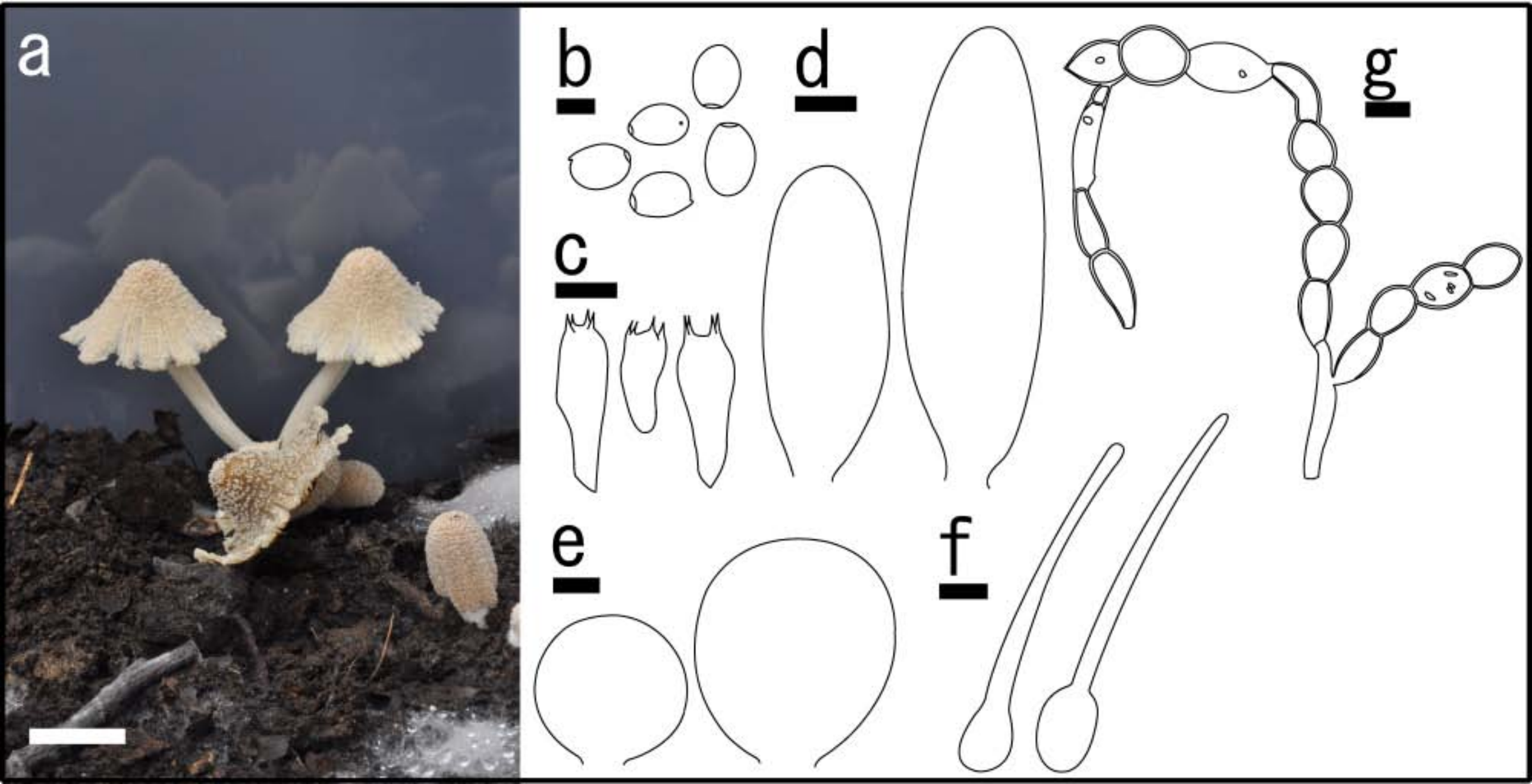
Isolate number	Sampling date	Sampling site
AF1-1	18-Jun-02	Sagamiko town Kanagawa Prefecture, JAPAN
AF1-2	18-Jun-02	Sagamiko town Kanagawa Prefecture, JAPAN
SI1-1	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN
SI1-2	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN
SI2-1	16-May-05	Fujinomiya city Shzioka Prefectre, JAPAN
KM1-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM1-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM1-3	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM2-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM2-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM2-3	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM3-1	15-May-06	Maizuru city Kyoto Prefectre, JAPAN
KM3-2	15-May-06	Maizuru city Kyoto Prefectre, JAPAN

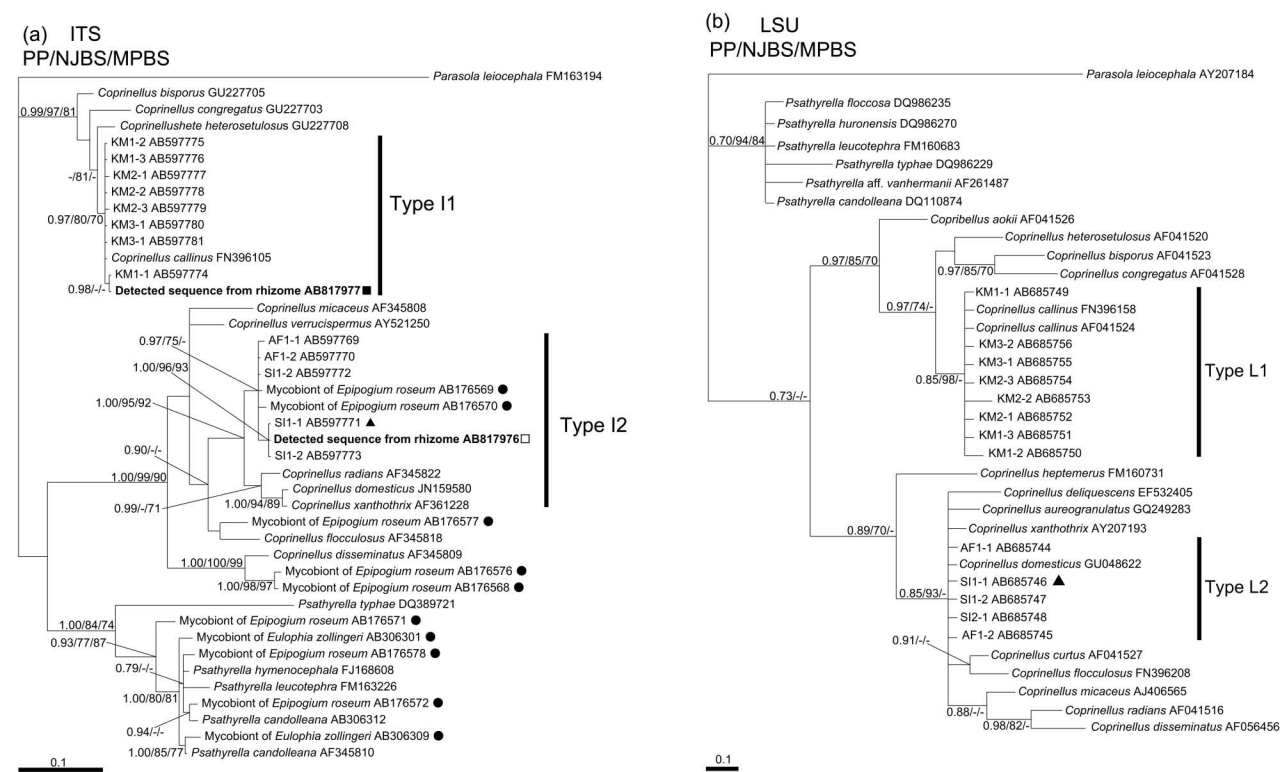
Isolate numbers indicate the propereties of the isolates, e.g., AF1-2, sequentially plant number (1) and isolate number (2).

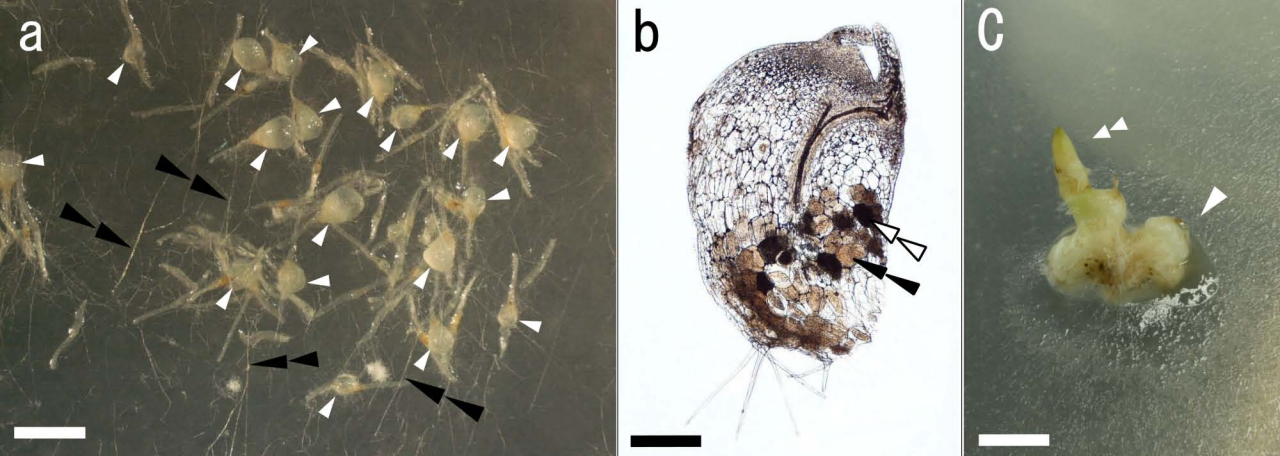
Accession numbers in DDBJ o the isotates sequences	
ITS	LSU
AB597769	AB685744
AB597770	AB685745
AB597771	AB685746
AB597772	AB685747
AB597773	AB685748
AB597774	AB685749
AB597775	AB685750
AB597776	AB685751
AB597777	AB685752
AB597778	AB685753
AB597779	AB685754
AB597780	AB685755
AB597781	AB685756

y the sampling site (AF),


LS
B
RO
RH


a
b
c
d
e
f
g


(a) ITS
PP/NJBS/MPBS
Parasola leiocephala FM163194
Coprinellus bisporus GU227705
0.99/97/81
Coprinellus congregatus GU227703
Coprinellushete heterosetulosus GU227708
KM1-2 AB597775
KM1-3 AB597776
-/81/-
KM2-1 AB597777
KM2-2 AB597778
KM2-3 AB597779
0.97/80/70
KM3-1 AB597780
KM3-1 AB597781
Coprinellus callinus FN396105
KM1-1 AB597774
0.98/-/-
Detected sequence from rhizome AB817977
Type I1
Coprinellus micaceus AF345808
Coprinellus verrucispermus AY521250
0.97/75/-
AF1-1 AB597769
AF1-2 AB597770
1.00/96/93
SI1-2 AB597772
Mycobiont of Epipogium roseum AB176569
1.00/95/92
Mycobiont of Epipogium roseum AB176570
SI1-1 AB597771
Detected sequence from rhizome AB817976
SI1-2 AB597773
Type I2
0.90/-/-
Coprinellus radians AF345822
1.00/99/90
Coprinellus domesticus JN159580
1.00/94/89
Coprinellus xanthothrix AF361228
0.99/-/71
Mycobiont of Epipogium roseum AB176577
Coprinellus flocculosus AF345818
1.00/100/99
Coprinellus disseminatus AF345809
Mycobiont of Epipogium roseum AB176576
1.00/98/97
Mycobiont of Epipogium roseum AB176568
Psathyrella typhae DQ389721
1.00/84/74
Mycobiont of Epipogium roseum AB176571
0.93/77/87
Mycobiont of Eulophia zollingeri AB306301
Mycobiont of Epipogium roseum AB176578
0.79/-/-
Psathyrella hymenocephala FJ168608
Psathyrella leucotephra FM163226
1.00/80/81
Mycobiont of Epipogium roseum AB176572
Psathyrella candolleana AB306312
0.94/-/-
Mycobiont of Eulophia zollingeri AB306309
1.00/85/77
Psathyrella candolleana AF345810
0.1
(b) LSU
PP/NJBS/MPBS
Parasola leiocephala AY207184
Psathyrella floccosa DQ986235
Psathyrella huronensis DQ986270
0.70/94/84
Psathyrella leucotephra FM160683
Psathyrella typhae DQ986229
Psathyrella aff. vanhermanii AF261487
Psathyrella candolleana DQ110874
Copribellus aokii AF041526
Coprinellus heterosetulosus AF041520
0.97/85/70
Coprinellus bisporus AF041523
0.97/85/70
Coprinellus congregatus AF041528
KM1-1 AB685749
0.97/74/-
Coprinellus callinus FN396158
Coprinellus callinus AF041524
KM3-2 AB685756
KM3-1 AB685755
0.85/98/-
KM2-3 AB685754
KM2-2 AB685753
KM2-1 AB685752
KM1-3 AB685751
KM1-2 AB685750
Type L1
0.73/-/-
Coprinellus heptemerus FM160731
Coprinellus deliquescens EF532405
Coprinellus aureogranulatus GQ249283
Coprinellus xanthothrix AY207193
0.89/70/-
AF1-1 AB685744
Coprinellus domesticus GU048622
SI1-1 AB685746
SI1-2 AB685747
0.85/93/-
SI2-1 AB685748
AF1-2 AB685745
Type L2
0.91/-/-
Coprinellus curtus AF041527
Coprinellus flocculosus FN396208
Coprinellus micaceus AJ406565
0.88/-/-
Coprinellus radians AF041516
0.98/82/-
Coprinellus disseminatus AF056456
0.1


a
b
c