Corresponding author's information

Takahiro YAGAME

Botanic Garden and Museum, Field Science Center for Northern Biosphere, Hokkaido University

Kita 3, Nishi 8, Chuo-ku, Sapporo, Hokkaido, 060-0003, Japan

Tel: +81-(11)-221-0066

Fax: +81-(11)-221-0664

E-mail: sebacina@gmail.com

Member of Botanical society of Japan

Subject area

(2) Ecology/Ecophysiology/ Environmental Biology

Information of number of tables and figures

Number of tables: 4

Black and white figures: 2

Color figures: 1

Title

Mycoheterotrophic growth of *Cephalanthera falcata* (Orchidaceae) in tripartite symbioses with Thelephoraceae fungi and *Quercus serrata* (Fagaceae) in pot culture condition

Authors

1) Takahiro YAGAME, 2) Masahide YAMATO

Addresses of authors

1) Botanic Garden and Museum, Field Science Center for Northern Biosphere, Hokkaido University Kita 3, Nishi 8, Chuo-ku, Sapporo, Hokkaido, 060-0003, Japan

2) Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University 4-101 Koyama-Minami, Tottori, 680-8553, Japan

Abstract

Mixotrophy, obtaining carbon by mycoheterotrophy and photosynthesis, has been suggested in *Cephalanthera* species (Orchidaceae) by analyses on stable isotopes of carbon. In this study, we examined the growth of *Cephalanthera falcata* in pot cultured tripartite symbioses with Thelephoraceae fungi and *Quercus serrata*. Mycorrhizal fungi were isolated from roots of *C. falcata* in natural habitats. Two fungal isolates identified as Thelephoraceae were cultured and inoculated to fine roots of non-mycorrhizal seedlings of *Q. serrata* (Fagaceae). After the ectomycorrhizal formation, non-mycorrhizal seedlings of *C. falcata* were co-planted. The pots with tripartite symbioses were cultured in greenhouse for 30 months, and growth of *C. falcata* seedlings was examined. Fresh weight of *C. falcata* seedlings was significantly increased by the tripartite symbioses even in those with no shoot, thus providing further evidence for the mycoheterotrophic nature of this orchid. The achievement of seedling culture in tripartite symbioses would be valuable for conserving many forest orchids and for conducting experiments to understand their physiology and ecology. .

Key words

Ectomycorrhiza, Microcosms, Mixotrophy, Mycoheterotrophy, symbiotic culture in pot

Introduction

Some orchids species known as mycoheterotrophic are achlorophyllous through of their lives and depend on mycorrhizal fungi for their carbon source (Leake 1994). For some chlorophyllous orchids in *Cephalanthera*, *Epipactis*, *Cymbidium*, *Platanthera*, etc., partial mycoheterotrophy was found (Bidartondo et al. 2004; Julou et al. 2005; Motomura et al. 2010; Yagame et al. 2012), and the nutritional mode is termed mixotrophy. Ectomycorrhiza (ECM) forming fungi are generally identified as the mycorrhizal fungi for such mixotrophic orchids (Rasmussen 2002; Dearnaley 2007), and the dependencies on fungi for the nutritions can be estimated by analyses on stable isotopes of carbon and nitrogen (Gebauer and Meyer 2003). For *Cephalanthera* species, Abadie et al. (2006) found the symbiosis between *C. longifolia* (L.) Fritsch. and Thelephoraceae fungi, and calculated the dependency on fungal carbon and nitrogen as 33% and 86%, respectively. Such dependency on ECM forming fungi were also reported in *C. damasonium* (Mill.) Druce and *C. rubra* (L.) L.C.M. Rich. (Bidartondo et al. 2004; Julou et al. 2005).

Actual growth of mycoheterotrophic orchids under tripartite symbioses was demonstrated in *Rhizanthella gardneri* R. S. Rogers. Warcup (1985) succeeded in formation of a tripartite symbiosis for subterranean mycoheteotrophic orchid, *Rhizanthella gardneri* R. S. Rogers with *Melaleuca uncinata* R. Br. and ectomycorrhizal *Rhizoctonia* fungus that was thereafter identified as Ceratobasidiaceae by molecular identification (Bougoure et al. 2009), in which a growth to flowering was achieved. Furthermore, Bougoure et al. (2010) confirmed carbon and nitrogen supply to the orchid in the tripartite symbiosis. Mycoheterotrophic orchid, *Corallorhiza trifida* Châtel which

is distributed in Eurasia and North America was cultivated and confirmed carbon transfer from host tree to this orchid via mycobionts by by Mckendick & Read (2000). However, mixotrophic orchids have never been cultivated under tripartite symbioses in pot culture conditions as far as we know.

Cephalanthera falcata (Thunb.) Blume is an orchid species distributed in Japan, Korean Peninsula and China (Kitamura et al. 1964). In Japan, it is known as an endangered species listed as vulnerable in the red data book by Ministry of the Environment. Yamato and Iwase (2008) planted asymbiotic seedlings of *C. falcata* into a natural habitat, and found the colonization of Thelephoraceae and Russulaceae fungi for the survived seedlings after four years. Matsuda et al. (2009) also identified Thelephoraceae and Sebacinaceae fungi as mycorrhizal fungi of *C. falcata,* in some natural habitats. In these studies, mixotrophic feature of *C. falcata* is suggested by the association with ectomycorrhizal fungi which are frequently detected from other mixotrophic *Cephalanthera* species (Abadie et al. 2006; Bidartondo et al. 2004).

In the present study, we examined growth of seedlings of *C. falcata* in the tripartite symbiosis with Thelephoraceae fungi and *Q. serrata* in pot culture condition to confirm the mycoheterotrophic or mixotrophic features of this orchid.

Material and methods

Isolation of mycobionts from *Cephalanthera falcata*

One individual of *C. falcata* (S1) was collected from *Q. serrata* and *Pinus densiflora* Siebold & Zucc forest located at Sayama (35º 49’ 06’’N, 139º 25’ 49’’E) in Saitama Prefecture, and three (K1,

K2, and K3) were from *Q. serrata* forest located at Mizuho (35º 12’ 41’’N, 135º 24’ 05’’E) in Kyoto Prefecture, Japan (Table 1). The collected plant roots were kept cool in individual plastic bag until processed within one day after sampling. From each individual, two or three brown roots indicating fungal colonization were chosen for the fungal coil isolation. The roots were washed with tap water, and cut into 5 mm in length. The surface was sterilized by immersion in 70% ethanol for 30 seconds and in sodium hypochlorite solution with 1% available chlorine for 30 seconds. The root fragment was put into a mortar with 1 ml of sterilized distilled water and crashed by a sterilized pestle to disperse the fungal coils. After rinsing the fungal coils in sterilized distilled water for five times, they were transferred onto medium plates of a modified Melin Norkrans (glucose 10.0 g, $(CH(OH)COONH_4)_2$ 1.0 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $FeCl_3$ 1.2 mg, thiamine hydrochloride 0.1 mg, malt extract 3.0 g, yeast extract 2.0 g, vegetable juice (V8) 10 ml, agar 15.0 g, distilled water 1,000 ml, pH 6.5) and cultured at 25.0 ± 0.5°C in the dark for 1 week. Fungal hyphae growing from the pelotons were picked up and subsequently cultured on the modified Melin Norkrans medium plate.

Preparation of ECM host trees

Because *Q. serrata* was found in the sampling sites of *C. falcata*, this woody plant species was used as the ECM plant species for the tripartite symbiosis. *Q. serrata* seeds were sown in pots containing granulated volcanic soil mixture of Akadama and Kanuma soils (1:1 v/v) that was autoclaved at 121°C for 30 min. After one year of cultivation, 45 seedlings, 15-20 cm in height with 25-30 leaves,

were selected. Two fungal strains, S1-1 and K1-1, showing better hyphal growth on the medium plate were selected as inocula. They were grown in 15 ml of liquid modified Melin Norkrans medium at 25.0 ± 0.5°C in the dark for 3 months, and the cultured mycelia were buried on the fine roots of *Q. serrata.* Fifteen *Q. serrata* seedlings were prepared for each treatment, S1-1, K1-1 and non-fungal control. The pot was put on plastic dish, which was wrapped by Saran Wrap (Asahi Kasei, Tokyo) to cover the soil surface for avoiding contaminations of other ECM fungi. For watering, sterilized water was poured into the plastic dish once a week through a hole made on the wrap. The seedlings were cultivated in a green house under eight hours day time with light photon adjusted between 600 to 800 μmol $m^{-2}s^{-1}$ for another 3 months, and the ECM formation was confirmed by microscopic observation.

Seedling culture of *C. falcata*

In a natural habitat of *C. falcata* in Mizuho, artificial cross-pollination was conducted for 10 individuals in the beginning of May 2006. The pollinated inflorescences were covered with nonwoven cloth to avoid invasion of insects. After 65 days, one capsule each from randomly selected five individuals was collected. The surface of the capsules was sterilized by soaking in 70% ethanol and subsequent combustion of the ethanol covering on the surface (Yamato and Iwase 2008). After splitting the capsules with a sterilized scalpel, the seeds were dispersed in sterilized 0.05% Tween 20 solution. The seeds from five capsules were mixed at this step, and they were sown on five medium plates of BM_1 (Van Waes and Debergh 1986). One milliliter of suspension containing

around 300 seeds was spread onto each plate. The seeds were incubated at 25.0 ± 0.5°C in the dark. After 60 days from the seed sowing, the germination rate was determined under a dissecting microscope. The emergence of swollen embryo from the seed coat was defined as seed germination. After 5 months from the seed sowing, rooting rate was determined. The seedling with root elongation was transferred into a culture bottle (6 cm in diameter and 10 cm) containing 100 ml of the Hyponex agar medium (Kano 1968) with slight modification (2 g of Hyponex (N−P−K: 6.5−6−19), 25 g of sucrose, 1 g of peptone, 10 g of agar, 1,000 ml of distilled water, pH 5.8), and incubated at 25.0 ± 0.5°C in the dark.

Tripartite symbiotic cultivation

Eight months after the transfer to the culture bottles, 45 *C. falcata* seedlings were selected to have the similar levels of fresh weights and root lengths. Fresh weight, total root length and roots number of the selected seedlings were 0.79±0.03 g , 24.5±1.46 cm, and 9.67 ± 0.25 (means±SE), respectively. Fifteen *C. falcata* seedlings each were applied for 3 treatments, S1-1, K1-1 and non-fungal control, in which no significant differences were found in the fresh weight, the total root length and root numbers among the treatments. *Cephalanthera falcata* seedlings (Fig. 4a) and *Q. serrata* seedlings were co-planted in sterilized polystyrene pot with 30 cm in diameter and 40 cm in depth containing ca. 3500 ml of soil medium described above in September 2006. The pots were cultured under the same condition for that of ECM seedling preparation. During the cultivation, seedlings of *C. falcata* were excavated for three times to measure the survival rate, fresh weights and

total root length. In order to maintain mycoheterotrophic orchid growth for 30 months, we excavated the seedlings on beginning of spring, March in 2007, 2008 and 2009. Host leafless tree has no ability to provide carbon for orchid via mycobionts in winter, from October to March, and the fungal network should be reestablished in spring with beginning of host tree growth. The shoot height was measured after 20 months (in May in 2008) from the co-planting. One-way ANOVA was applied to evaluate the differences of seedling fresh weight and root length among the sampling time for each fungal treatment, and mean values were then compared by Tukey' test.

Molecular identification of the mycorrhizal fungi

From cultured fungi, DNA was extracted by PrepMan Ultra Reagent (Applied Biosystems, Tokyo, Japan). The reagent was also applied for DNA extraction from three ectomycorrhizal root tips formed on each *Q. serrata* seedlings after 3 months from the fungal inoculation, and ca. 20 fungal coils isolated from roots of the cultivated *C. falcata* seedlings after 30 months from the co-planting. The fungal DNA of internal transcribed spacer region of the nuclear ribosomal RNA gene (ITS rDNA) was amplified from the extracted DNA with a primer pair ITS1F/ITS4 (Gardes and Bruns 1993) by polymerase chain reactions (PCR). The reaction mixture contained 1 μl of the extracted DNA solution, 0.75 units of Taq polymerase, 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate, and 3 μl of the supplied PCR buffer in 30 μl of the total amount. The PCR program performed on the 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 35 cycles at 94°C for 20 s, 55°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 10 min. All PCR

products were cloned using pGEM-T Easy Vector System I (Promega, Tokyo, Japan), and plasmid DNAs were extracted from the cloned products using MagExtractor Plasmid (TOYOBO). The plasmid inserts were sequenced using the dideoxysequencing method with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) by Genetic Analyser 3130 (Applied Biosystems, Tokyo, Japan). The obtained DNA sequence data were subjected to BLAST searches (Altschul et al. 1997), and similar sequences were downloaded. Multiple sequence alignments of the sequence data were carried out using CLUSTAL W version 1.83 (Thompson et al. 1994), in which gaps were treated as missing data. Phylogenetic analyses were conducted by the maximum likelihood (ML) method with additional Neighbor-joining (NJ) method (Saitou and Nei 1987) using MEGA 5 (Tamura et al. 2011). The best-fit ML trees were inferred under GTR+GAMMA model which was estimated with MrModeltest 2.2 (Nylander 2004) in PAUP* 4.0b10 (Swofford 2002). To check statistical support for the tree topology obtained, the bootstrap option was used under the automatically assigned, GTA+CAT model, setting the number of replications to 1,000.

Results

Isolation and identification of the mycorrhizal fungi of *C. falcata*

Eight fungal isolates were obtained from root samples of *C. falcata* (Table 1). The colonies of the fungal isolates is colored with dark brown, and clamp connections were observed at the septa of the hyphae. Full-length sequences of ITS1, 5.8S and ITS2 were obtained for all fungal isolates. The DNA sequences were deposited to the DDBJ database with accession numbers of

AB697128-AB697135 (Table. 1). The ML analysis showed that the obtained fungi were divided into three phylotypes (CT1-3) in the Thelephoraceae (Fig. 1). Five isolates in CT1 clade supported by statistical value of ML analysis (MLBS) and NJ analysis (NJBS), 95/99, showed high sequence similarity, 99.7-99.2%, one another and formed the same clade with *Tomentella* sp. (JF273546) collected in China and ectomycorrhizal fungus of *Castanopsis fagesii* Franch (GQ240904) detected in China. One isolate K1-2 formed a CT2 clade supported by MLBS and NJBS, 100/99, with a mycorrhizal fungus of *C. falcata* (AB259135) detected in the same habitat in Mizuho by Yamato and Iwase (2008). Two isolates in CT3 clade supported by MLBS and NJBS, 99/93, were close to the mycorrhizal fungi of *C. austinae* obtained by Taylor and Bruns (1997).

Ectomycorrhiza formation

Among the eight isolates, two isolates K1-1 and S1-1 grew well on the medium, and were used as inoculums for the tripartite symbiosis. Three months after the fungal inoculation, ectomycorrhizas with fungal sheath and Hartig net were formed on roots of *Q. serrata* (Fig. 2a, b). Morphological differences of the formed ectomycorrhizas were not confirmed between two isolates. For some randomly selected ectomycorrhizal root tips, fungal identities with inoculated fungi were confirmed based on their ITS rDNA sequences (data not shown).

Growth of *C. falcata* under the tripartite symbiosis

After 60 days from the seed sowing, the germination rate was 51.1±8.7% (mean±SE). After five

month from the seed sowing, the rooting rate was 5.6±2.3%. Eight months after the transfer to the culture bottles, seedlings with root elongation (Fig. 3a, e) were applied for co-planting with ECM seedlins of *Q. serrata*.

Survivals of *C. falcata* seedlings were checked 6, 18, and 30 months after the co-planting (Table 2). Though 80% seedlings were survived after 6 months even in control (without ECM seedlings), it was decreased to 20% after 18 month, and no seedlings were survived after 30 month. Meanwhile, 73.3 and 86.7% of the seedlings were still survived after 30 months for those with ECM seedlings by K1-1 and S1-1, respectively.

No shoots were found in non-fungal control, while shoot growth was found in four individuals with K1-1 inoculation and in two with S1-1 (Table 3,4) (Fig. 3d). One shoot growth from each shooting seedling was found twice during the 30 months cultivation. The mean height of the second shoot in K1-1 and S1-1 measured after 20 month from the co-planting were 7.7 and 6.4 cm, respectively. The seedlings were divided into two groups, with and without shoot, for each fungal inoculation, and the fresh weight and total root length were evaluated, respectively. Though the fresh weight and the total root length were much decreased in non-fungal control, significant increases were found for those with ECM seedlings even in those without shoots (Table 3, 4).

Pictures of an excavated seedling with no shoot in symbiosis with K1-1 were shown after 6 and 18 months from the co-planting, in which root growth was confirmed (Fig. 3b, c). Brownish root pigmentations on the roots suggested the fungal colonization. The seedling with shoot in symbiosis with K1-1 was also shown after 6 and 18 month from co-planting in Fig. 3f, g.

After 30 months from the co-planting, the mycobionts were isolated form five *C. falcata* seedlings in each inoculated condition. The isolated fungal coil is shown in Fig. 3h. The fungal ITS sequences obtained from the isolated fungal coils were 100% identical to that of the inoculated fungi. (data not shown).

Discussion

All the mycorrhizal fungi isolated from roots of *C. falcata* were close to *Tomentella* or *Thelephora* in Thelephoraceae in the phylogenetic analysis. Though fungal cultures have been obtained by monosporous culture or from ectomycorrhizal root tips for Thelephoraceae fungi (Biggs 1938; Marx et al. 1970; Finlay et al. 1992), isolation from orchid roots can be another useful method to obtain cultures of this fungal group. Not only the Thelephoraceae, but also *Russula* and Sebacinaceae fungi have been detected in roots of *C. falcata* (Yamato and Iwase 2008; Matsuda et al, 2009), but no fungi in these groups were not isolated in this study. The low number of the isolated fungi can be the reason for the bias on Thelephoraceae, while the difficulties of fungal isolations depending on fungal taxa may also be the reason. Bidartondo and Read (2008) identified diverse ECM fungi in Thelephoraceae, Cortinariaceae, and Sebacinaceae from adult plants of *C. damasonium* and *C. longifolia*, while only some Thelephoraceae fungi were detected from seedlings of these species. Functional significance of the symbioses with diverse fungi may be revealed by the tripartite symbiotic culture method developed for *C. falcata* in this study.

Two fungal isolate, S1-1 and K1-1 formed ectomycorrhiza on *Q. serrata*. To our knowledge, this

is the first study to achieve the ECM formation by inoculation of Thelephoraceae fungi isolated from orchid in pot culture condition. Co-plantation of the ECM seedling of *Q. serrata* and the aseptic seedlings of *C. falcata* induced the growth of *C. falcata*, even in those with no shoot. This result clearly showed actual mycoheterotrophic growth of this orchid in the condition, which suggested that the photosynthates of *Q. serrata* were supplied to *C. falcata* through the mycorrhizal fungi. Yamato and Iwase (2008) planted aseptic seedlings of *C. falcata* into a natural habitat, in which the growth to flowering was found after 4 years. Meanwhile, no flowering was found for the *C. falcata* with 30 months cultivation in this study. Because the growth was continued during the cultivation, longer cultivation may lead to a growth to flowering in the pot culture condition.

Because the number of seedlings with shoot growth was low, effect of shoot on the plant growth was not statistically evaluated. Further study is therefore required to evaluate the contribution of photosynthesis on the seedling growth, which could confirm the mixotrophy from the viewpoint of actual growth.

Many mixotrophic orchids are known as endangered species. This kind of tripartite symbiosis in pot culture condition can be applied for cultivation of excavated orchids, which would be useful for conservation of orchid individuals obtained from wild habitats.

Acknowledgement

This study was supported by a R and D fund from The General Environmental Technos Co. Ltd. and the Global COE Program "Advanced utilization of fungus/mushroom resources for sustainable society in harmony with nature" from the ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Abadie JC, Puttsepp U, Gebauer G, Faccio AN, Bonfante P, Selosse MA (2006) *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and nonphotosynthetic individuals. Can J Bot 84:1462-1477

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Biggs R (1938) Cultural studies in The Thelephoraceae and related fungi. Mycologia 30: 64-78

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Pro R Soc B 271: 1799-1806

Bidartondo MI, Read DJ (2008) Fungal specificity bottlenecks during orchid germination and development. Mol Ecol 17: 3707-3716

Bougoure JJ, Brundrett M, Grierson PF (2009) Identity and specificity of the fungi forming mycorrhizas with the rare myco-heterotrophic orchid *Rhizanthella gardneri*. Mycological Research 113: 1097-1106

Bougoure JJ, Brundrett MC, Grierson PF (2010) Carbon and nitrogen supply to the underground orchid, *Rhizantella gernderi*. New Phytol 186: 947-956

Dearnaley, JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17: 475-486

Finlay RD, Frostegård Å, Sonnerfeldt AM (1992) Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Cougl. Ex Loud. New Phytol 120: 105-115

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:133-118

Gebauer G, Meyer M (2003) ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. New Phytol 160: 209-223

Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse MA (2005) Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. New Phytol 166: 639-653

Kano K (1968) Acceleration of the germination of so-called hard-to-germination orchid seeds. Am Orchid Soc Bull 37:690-698

Kitamura S, Murata G, Koyama T (1964) Colored illustrations of herbaceous plants of Japan (Monocotyledoneae), vol III. Hoikusha, Osaka

Leake JR (2004) Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. Curr Opin Plant Biol 7: 422-428.

Marx DH, Bryan WC, Grand LF (1970) Colonization, isolation, and cultural descriptions of *Thelephora terrestris* and other ectomycorrhizal fungi of shortleaf pine seedlings grown in fumigated soil. Can J Bot 48: 207-211

Matsuda Y, Amiya A, Ito S (2009) Colonization patterns of mycorrhizal fungi associated with two rare orchids, *Cephalanthera falcata* and *C.erecta.* Ecol Res 24: 1023-1031

McKendrick SL, Leake JR, Read DJ (2000) Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal Salix repens and Vetula pendula to the prchid Corallorhiza tririda through shared hyphal connections. New Phytol 145: 539-548

Motomura H, Selosse MA, Martos F, Kagawa A, Yukawa T (2010) Mycoheterotrophy evolved from mixotrophic ancestors: evidence in *Cymbidium* (Orchidaceae). Ann Bot 106: 573-581

Nylander JAA (2004) MrModeltest v2.2 program distributed by the author. Evolutionary Biology Centre, Uppasala University.

Rasmussen HN (2002) Recent development in the study of orchid mycorrhiza 244: 149-163

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Swofford DL (2002) PAUP 4.0b10: phylogenetic analysis using parasimony. Sinauer Associates, Sunderland.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumer S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28: 2731-2739

Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two non photosynthetic orchids. Proc Natl Acad Sci USA 94:4510-4515

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequencing weighting, position sequence gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680

Van Waes JM, Debergh PC (1986) Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. Physiol Plant 66: 435-442

Warcup JH (1985) *Rhizanthella gardneri* (Orchidaceae), its *Rhizoctonia* endophyte and close association with *Melaleuca uncinata* (Myrtaceae) in Western Australia. New Phytol 99: 273-280

Yagame T, Orihara T, Selosse MA, Yamato M, Iwase K (2012) Mixotrophy of *Platanthera minor*, an orchid associated with ectomycorrhiza-forming Ceratobasidiaceae fungi. New Phytol 193: 178-187

Yamato M, Iwase K (2008) Introduction of asymbiotically propagated seedlings of *Cephalanthera falcata* (Orchidaceae) into natural habitat and investigation of colonized mycorrhizal fungi. Ecol Res 23:329-337

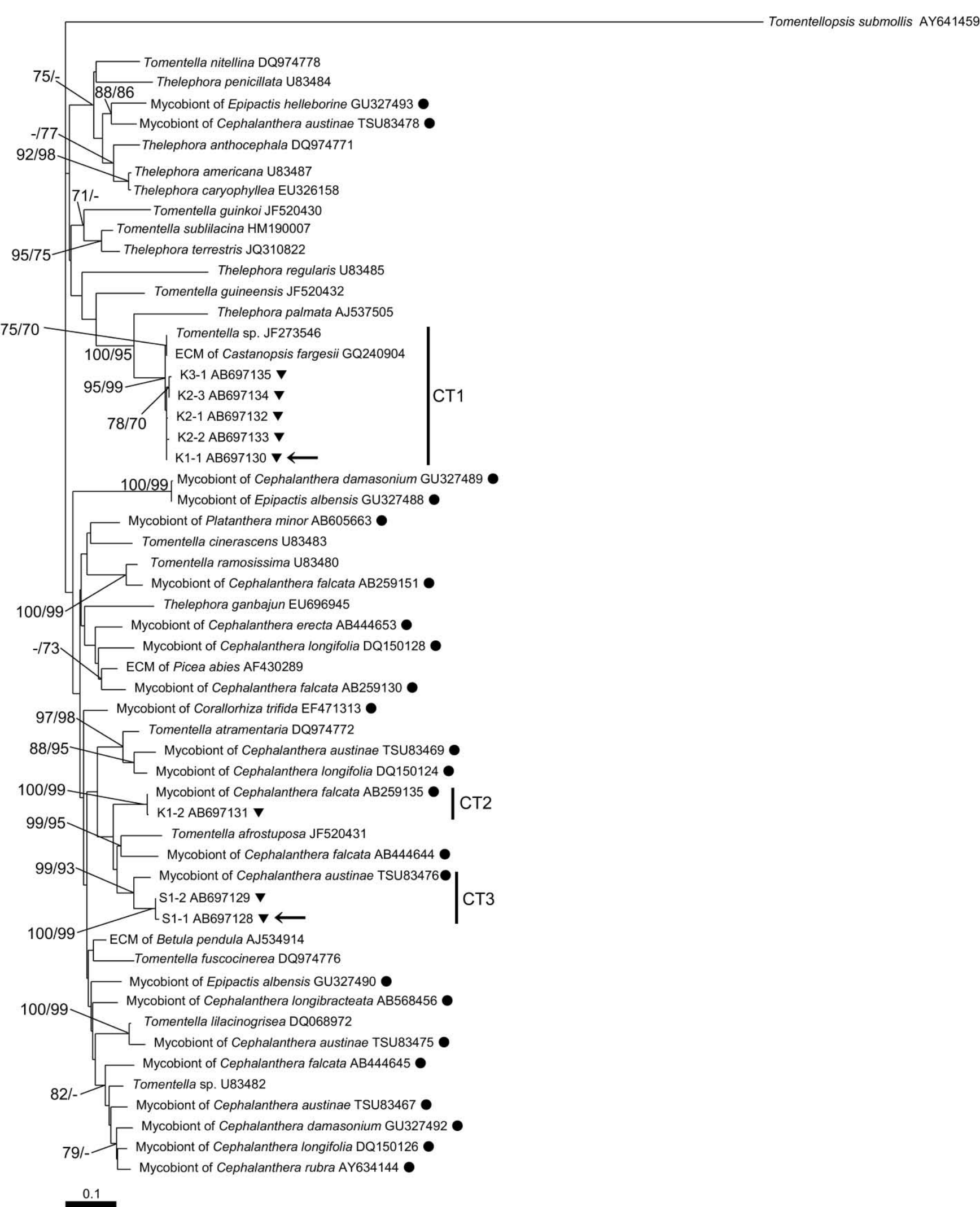
Figure legends

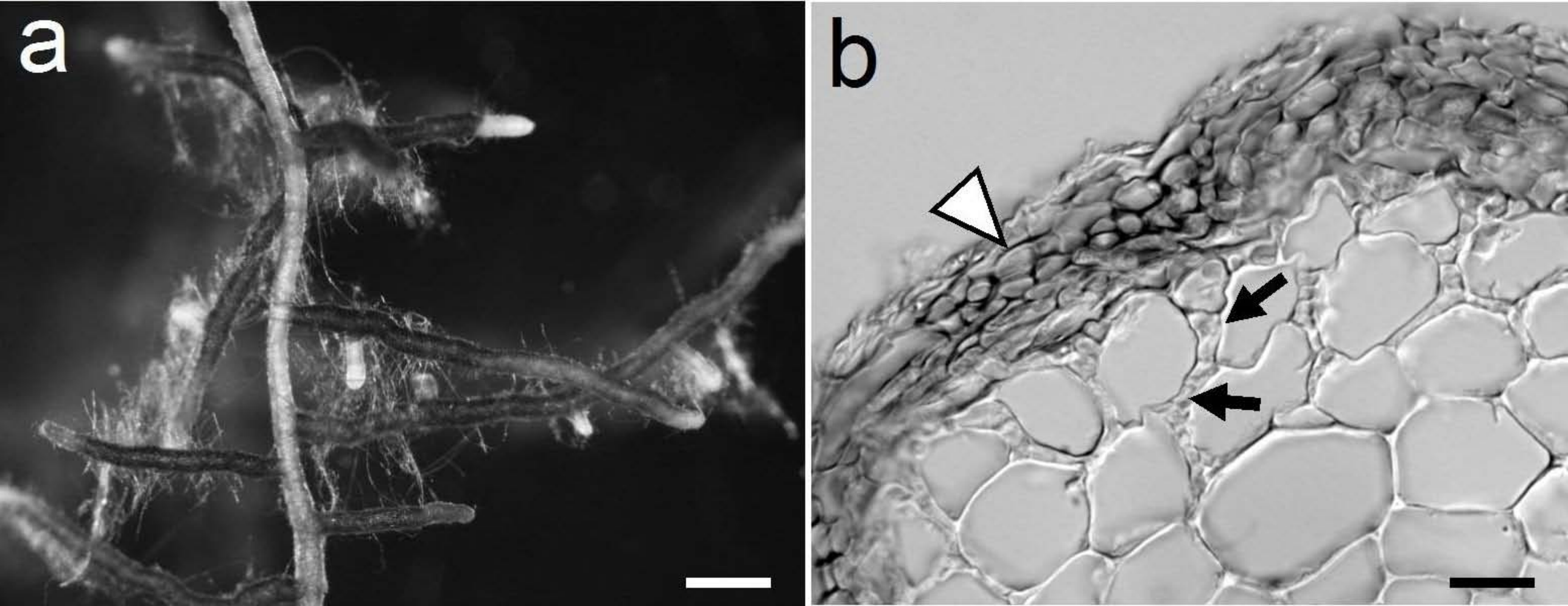
Fig. 1: The ML tree (In *L*= -6,848.01) based on the sequences of ITS rDNA of *Tomentella* and *Thelephora* fungi in Thelephoraceae obtained from roots of *Cephalanthera falcata* and Genbank database. The tree is rooted to *Tomentellopsis submollis* (Thelephoraceae). The black triangles and circles indicate fungal isolates of *Cephalanthera falcata* and mycobionts of *Cephalanthera* species, respectively. Allows show fungal isolates used for tripartite symbiotic culture. Bootstrap values with 1,000 replication in ML analysis (MLBS) and NJ analysis (NJBS) are indicating as MLBS/NJBS at branches of nodes. For BS values, those over 70% are only shown. A scale is shown to infer evolutionary distances.

Fig. 2: Ectomycorrhizas on *Quercus serrata* formed by K1-1, a fungal isolate from *Cephalanthera falcata*. (a) External appearance of the ectomycorrhizas. *Bar*= 1 mm, (b) A cross-section of the ectomycorrhizas showing the Hartig net (arrows) and sheath (arrowhead). *Bar*= 20μm.

Fig. 3: *Cephalanthera falcata* seedlings with no shoot growth (a-c) and with shoot growth (d-g) in symbiosis with K1-1 excavated from pot culture, and extracted fungal coil from cultured seedling. (a) The aseptic seedling. *Bar*= 1 cm (b) The seedling after 6 months showing brownish pigmentation (arrowheads). *Bar*= 1 cm (c) The seedling after 18 month. *Bar*= 1 cm (d) Shoot growth of *Cepalanthera falcata* seedling after 7 month under tripartite symbiotic condition. Shoot (arrow). *Bar*= 5 cm (e) The aseptic seedling. *Bar*= 1 cm (f) The seedling after 6 months showing brownish

pigmentation (arrowheads). *Bar*= 1 cm (g) The seedling with shoot after 18 month. *Bar*= 1 cm (h) Fungal coil extracted from root of *Cepalanthera falcata* seedling cultured tripartite symbiosis. *Bar*= 50 μm


Tomentellopsis submollis AY641459
Tomentella nitellina DQ974778
Thelephora penicillata U83484
Mycobiont of Epipactis helleborine GU327493
Mycobiont of Cephalanthera austinae TSU83478
Thelephora anthocephala DQ974771
Thelephora americana U83487
Thelephora caryophyllea EU326158
Tomentella guinkoi JF520430
Tomentella sublilacina HM190007
Thelephora terrestris JQ310822
Thelephora regularis U83485
Tomentella guineensis JF520432
Thelephora palmata AJ537505
Tomentella sp. JF273546
ECM of Castanopsis fargesii GQ240904
K3-1 AB697135
K2-3 AB697134
K2-1 AB697132
K2-2 AB697133
K1-1 AB697130
CT1
Mycobiont of Cephalanthera damasonium GU327489
Mycobiont of Epipactis albensis GU327488
Mycobiont of Platanthera minor AB605663
Tomentella cinerascens U83483
Tomentella ramosissima U83480
Mycobiont of Cephalanthera falcata AB259151
Thelephora ganbajun EU696945
Mycobiont of Cephalanthera erecta AB444653
Mycobiont of Cephalanthera longifolia DQ150128
ECM of Picea abies AF430289
Mycobiont of Cephalanthera falcata AB259130
Mycobiont of Corallorhiza trifida EF471313
Tomentella atramentaria DQ974772
Mycobiont of Cephalanthera austinae TSU83469
Mycobiont of Cephalanthera longifolia DQ150124
Mycobiont of Cephalanthera falcata AB259135
K1-2 AB697131
CT2
Tomentella afrostuposa JF520431
Mycobiont of Cephalanthera falcata AB444644
Mycobiont of Cephalanthera austinae TSU83476
S1-2 AB697129
S1-1 AB697128
CT3
ECM of Betula pendula AJ534914
Tomentella fuscocinerea DQ974776
Mycobiont of Epipactis albensis GU327490
Mycobiont of Cephalanthera longibracteata AB568456
Tomentella lilacinogrisea DQ068972
Mycobiont of Cephalanthera austinae TSU83475
Mycobiont of Cephalanthera falcata AB444645
Tomentella sp. U83482
Mycobiont of Cephalanthera austinae TSU83467
Mycobiont of Cephalanthera damasonium GU327492
Mycobiont of Cephalanthera longifolia DQ150126
Mycobiont of Cephalanthera rubra AY634144
75/-
88/86
-/77
92/98
71/-
95/75
75/70
100/95
95/99
78/70
100/99
100/99
-/73
97/98
88/95
100/99
99/95
99/93
100/99
100/99
82/-
79/-
0.1


a
b

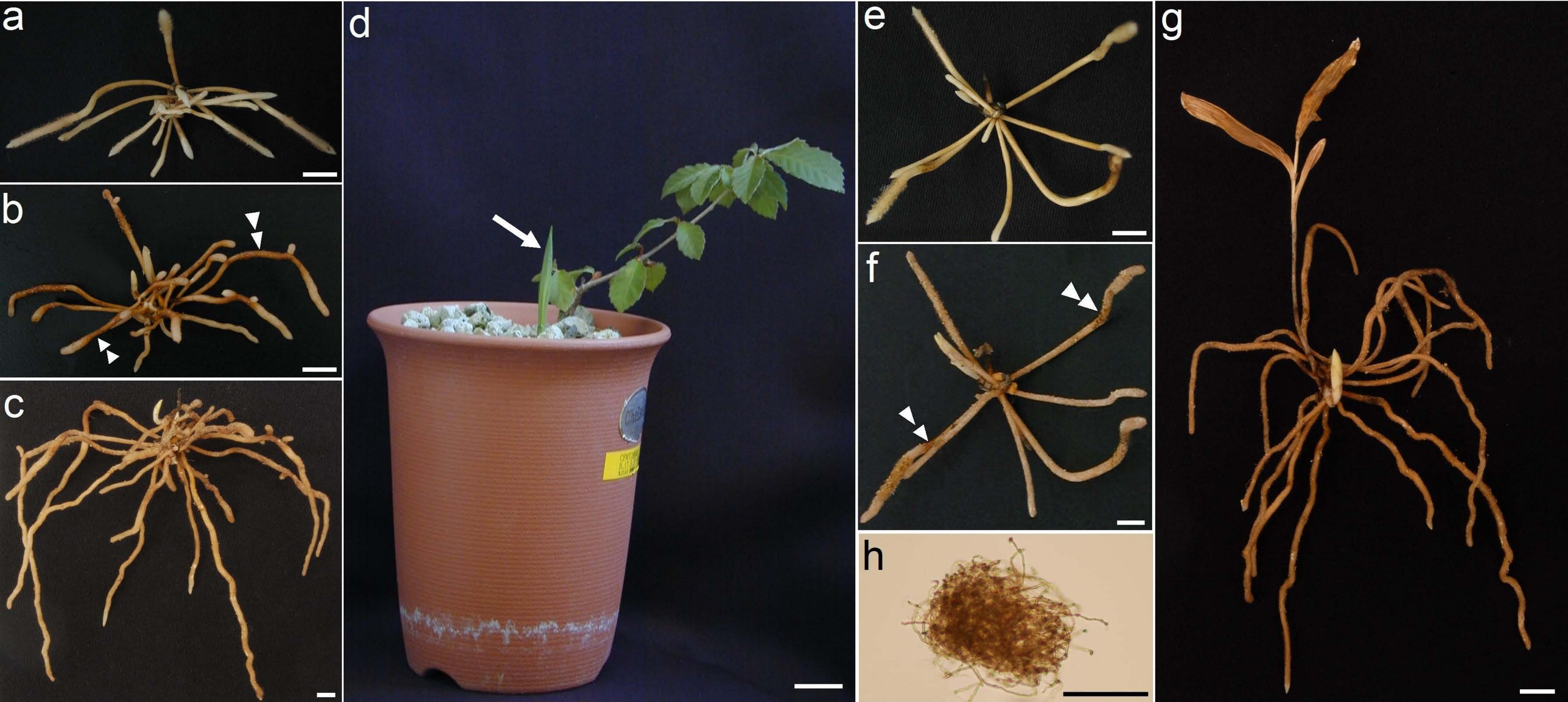

a
b
c
d
e
f
g
h

Table 1. Fungal isolates of *Cephalanthera falcata.*

Fungal isolates[a]	Sampling date	Sampling site	DDBJ accession No.[b]
S1-1	13-Jul-04	Sayama	AB697128
S1-2	13-Jul-04	Sayama	AB697129
K1-1	15-May-05	Mizuho	AB697130
K1-2	15-May-05	Mizuho	AB697131
K2-1	15-May-05	Mizuho	AB697132
K2-2	15-May-05	Mizuho	AB697133
K2-3	15-May-05	Mizuho	AB697134
K3-1	15-May-05	Mizuho	AB697135

[a]Isolate numbers consist of plant numbers and isolate numbers

[b]DNA sequences of ITS rDNA

Table 2. Survival rates (%) of *Cephalanthera falcata* seedlings in tripartite symbioses with Thelephoraceae fungi (K1-1, S1-1) and *Quercus serrata*.

Months[a]	Fungal treatments		
	Control	K1-1	S1-1
6	80	100	100
18	20	86.7	93.3
30	0	73.3	86.7

[a]Cultivation period of 13 month old aseptic seedlings under tripartite symbiotic condition.

Table 3. Fresh weights (g) of seedlings of *Cephalanthera falcata* in tripartite symbioses with Thelephoraceae fungi (K1-1, S1-1) and *Quercus serrata.*

Months[a]	Fungal treatments				
	Control	K1-1		S1-1	
	N (15)	N (11)	S (4)	N (13)	S (2)
0	0.82[a]	0.73[a]	0.78[a]	0.78[a]	0.73
6	0.43[b]	0.94[a]	1.01[a]	0.80[a]	0.82
18	0.02[c]	1.64[ab]	2.08[ab]	1.20[ab]	1.91
30	0.00[c]	2.21[b]	2.54[b]	1.51[b]	2.11

N, *C. falcata* seedlings with no shoot

S, *C. falcata* seedlings with shoot

The seedling numbers examined are shown in parentheses.

Means with the same letter in each fungal treatment with division by shoot existence are not significantly different among the observation times (P <0.05).

[a]Cultivation period of 13 month old aseptic seedlings under tripartite symbiotic con

Table 4. Total root lengths (cm) of seedlings of *Cephalanthera falcata* in tripartite symbioses with Thelephoraceae fungi (K1-1, S1-1) and *Quercus serrata*.

Months[a]	Fungal treatments				
	Control	K1-1		S1-1	
	N (15)	N (11)	S (4)	N (13)	S (2)
0	26.1[a]	22.1[a]	20.5[a]	25.3[a]	28.5
6	11.2[b]	27.1[a]	27.4[a]	25.9[a]	27.2
18	0.91[c]	68.5[ab]	73.7[ab]	29.9[ab]	36.4
30	0.00[c]	82.9[b]	96.0[b]	55.0[b]	60.1

N, *C. falcata* seedlings with no shoot

S, *C. falcata* seedlings with shoot

The seedling numbers examined are shown in parentheses.

Means with the same letter in each fungal treatment with division by shoot existence are not significantly different among the observation times (P <0.05).

[a]Cultivation period of 13 month old aseptic seedlings under tripartite symbiotic cond