Mixotrophy of *Platanthera minor*, an orchid associated with ectomycorrhiza-forming Ceratobasidiaceae fungi

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Summary

• We investigated the fungal symbionts and carbon nutrition of a Japanese forest photosynthetic orchid, *Platanthera minor*, whose ecology suggests a mixotrophic syndrome, that is, a mycorrhizal association with ectomycorrhiza (ECM)-forming fungi and partial exploitation of fungal carbon.
• We performed molecular identification of symbionts by PCR amplifications of the fungal ribosomal DNA on hyphal coils extracted from *P. minor* roots. We tested for a ¹³C and ¹⁵N enrichment characteristic of mixotrophic plants. We also tested the ectomycorrhizal abilities of orchid symbionts using a new protocol of direct inoculation of hyphal coils onto roots of *Pinus densiflora* seedlings.
• In phylogenetic analyses, most isolated fungi were close to ECM-forming Ceratobasidiaceae clades previously detected from a few fully heterotrophic orchids or environmental ectomycorrhiza surveys. The direct inoculation of fungal coils of these fungi resulted in ectomycorrhiza formation on *P. densiflora* seedlings. Stable isotope analyses indicated mixotrophic nutrition of *P. minor*, with fungal carbon contributing from 50% to 65%.
• This is the first evidence of photosynthetic orchids associated with ectomycorrhizal Ceratobasidiaceae taxa, confirming the evolution of mixotrophy in the Orchideae orchid tribe, and of ectomycorrhizal abilities in the Ceratobasidiaceae. Our new ectomycorrhiza formation technique may enhance the study of unculturable orchid mycorrhizal fungi.

Introduction

The Orchidaceae is one of the largest plant families, which is estimated to contain >20000 species (Dixon et al., 2003). One of the distinctive features of this family is the production of a large number of minute seeds with only minimal reserves of nutrients (Arditti & Ghani, 2000). Because of this feature, orchids depend upon mycorrhizal fungi for the carbon resources necessary for germination and subsequent growth into the so-called protocorm stage (Rasmussen, 1995). In the majority of chlorophyllous orchids, photosynthesis at adulthood allows autotrophy (Cameron et al., 2006; Smith & Read, 2008), whereas achlorophyllous mycoheterotrophic orchids found in forests depend on the carbon supplied by mycorrhizal fungi throughout their lifetimes (McKendrick et al., 2000; Smith & Read, 2008). It was recently that several chlorophyllous orchids were found to obtain carbon not only from photosynthesis, but also from associated mycorrhizal fungi at 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) and has been reported in *Cephalanthera* and *Epipactis* spp. of the tribe Neottieae (Bidartondo et al., 2004; Julou et al., 2005), and in *Cymbidium* spp. of the tribe Cymbidieae (Motomura et al., 2010). In mycoheterotrophic and mixotrophic orchids, ectomycorrhiza (ECM)-forming fungi, such as Sebacinales, Russulaceae and Thelephoraceae, are most often identified as mycobionts, suggesting that the photosynthates of nearby trees likely represent the final carbon sources. The natural abundance of ¹³C and ¹⁵N isotopes, presented as δ¹³C and δ¹⁵N, can be used to estimate nutritional sources in food chains. The fruit bodies of ECM fungi generally show relatively high values of δ¹³C and δ¹⁵N compared with green plant tissues (Trudell et al., 2004). Mycoheterotrophic plants associated with ECM fungi exhibit similarly high values of δ¹³C and δ¹⁵N, strongly indicating that these fungi serve as the carbon and nitrogen sources for these plants (Gebauer & Meyer, 2003). In mixotrophic orchids, the values of δ¹³C are typically intermediate between those of autotrophic plants and mycoheterotrophic plants or ECM fruit bodies (Bidartondo et al., 2004; Julou et al., 2005; Motomura et al., 2010). By comparison of the
\(\delta^{13}C\) content of mixotrophic plants with that of autotrophic and mycoheterotrophic reference plants, the contribution of fungal source (heterotrophy) to carbon nutrition can be quantified.

*Platanthera* is a photosynthetic and mycoheterotrophic orchid genus of the tribe Orchideae consisting of 150 species (Karasawa, 2003) that are found in various environments, from grasslands to forest understories. Most mycobionts of *Platanthera* species identified are members of the teleomorphic fungal families Ceratobasidiaceae and Tulasnellaceae (Currach et al., 1990; Zelmer & Currah, 1995; Zelmer et al., 1996; Sharma et al., 2003; Bidartondo et al., 2004), which belong to the anamorphic, polyphyletic form-genus Rhizoctonia. The association with Rhizoctonia-type fungi is a common feature of many photosynthetic orchids (Rasmussen, 2002; Dearnaley, 2007). Although their ecology has not been well studied, these fungi are generally considered as saprobes or plant pathogens and several species may have endophytic abilities (Dearnaley et al., 2012). However, ECM-forming Ceratobasidiaceae fungi have been isolated from two mycoheterotrophic orchid species, *Rhizanthella gardneri* R.S. Rogers (Bougoure et al., 2009) and *Chamaegastrodia sikokiana* Makino & F. Maek. (Yagame et al., 2008). Despite this, few Ceratobasidiaceae have been detected from the numerous ECM community analyses performed to date (these few exceptions include Rosling et al., 2003, AF477004; Collier & Bidartondo, 2009).

*Platanthera minor* is a common terrestrial orchid found in various forest types in the southern part of Japan, China, and Taiwan (Hashimoto et al., 1991). We have noted that the canopy trees of the habitats of this orchid invariably form ECM associations. *Platanthera minor* habitats are often deeply shaded and, as some studies suggested mixotrophy for several European *Platanthera* species (Gebauer & Meyer, 2003; Tedersoo et al., 2007), we also suspected that mixotrophic nutrition occurs at the adult stage in *P. minor*.

Here, we investigated the identities of mycobionts and mycorrhizal specificity of *P. minor* in various habitats. Furthermore, the ECM-forming ability of the mycobionts was evaluated in pot cultures. We also examined the mixotrophy of *P. minor* by determining its natural abundance in \(^{13}C\) and \(^{15}N\) compared with those of surrounding photosynthetic plants and ECM fruit bodies.

**Materials and Methods**

**Sample collection and fungal coil isolation**

To identify mycobionts of *P. minor* (Miq.) Rchb. 41 individual plants were collected from nine sampling sites in Japan (Tables 1, 2). The plant samples were placed in individual plastic bags and kept cool until processed, within 2 d after sampling. From each individual collected, two to three brown-pigmented roots (indicating fungal colonization) were selected for isolation of fungal coils (intracellular fungal hyphae; Table 2). The roots were first washed with tap water, and then root sections were observed by differential interference microscopy to confirm fungal colonization. Colonized roots were cut into pieces c. 5 mm long and the surface of the root fragments was sterilized by immersion in 70% ethanol for 30 s, followed by immersion in sodium hypochlorite solution with 1% available chlorine for 30 s. The root fragments were placed in a mortar with 1 ml sterilized distilled water and crushed using a sterilized pestle to disperse fungal coils. The coils were transferred into a vial and rinsed five times with sterilized distilled water.

**Molecular identification of mycobionts of *P. minor***

DNA was extracted from a pool of c. 20 fungal coils per root fragment according to Kristiansen et al. (2001), with modification to use PrepMan Ultra Reagent (Applied Biosystems, Tokyo, Japan). Two to three roots fragments were used per plant collected. The primer pair ITS1F/ITS4 was used to amplify the internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene (nrDNA; Gardes & Bruns, 1993). The PCR 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 Ex Taq buffer in a total of 30 µl. The PCR was performed using the Program Temp Control System PC-818S (Astec, Fukuoka, Japan) with an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 20 s, 55°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 10 min. As the ITS1F–ITS4 primer pair might not amplify most Tulasnellaceae, which are common mycobionts of several *Platanthera* species (Currach et al., 1990; Bidartondo et al., 2004), an additional PCR was performed with the primer pair ITS1-OF–ITS4-OF (Taylor & McCormick, 2008) for all samples, using the same PCR program.

In addition to the ITS analysis, one DNA sample was arbitrarily selected from each sampling site for partial amplification of the large subunit (LSU) nrDNA sequence. This PCR was performed with the primer pair NL1–NL4 (O’Donnell, 1993) and the following program: an initial denaturation step at 94°C for 1 min, followed by 35 cycles at 94°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 pGEM-T Easy Vector System I (Promega, Tokyo, Japan) and three colonies with DNA inserts were arbitrarily selected from each cloning for sequencing with the dideoxy sequencing method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a Genetic Analyzer 3130 (Applied Biosystems). In instances when the three sequences obtained from one sample of fungal coils were > 99.5% identical, they were treated as a single consensus sequence.

The sequence data obtained were subjected to BLAST searches (Altschul et al., 1997) to determine their taxonomic positions. Multiple sequence alignments of the sequence data were performed using CLUSTAL W version 1.83 (Thompson et al., 1994), with gaps being treated as missing data. For all of the ITS sequences obtained, neighbor joining (NJ) (Saitou & Nei, 1987) was first conducted as a preliminary analysis using MEGA version 4 (Tamura et al., 2007) with bootstrap analysis of 1000 replications (Felsenstein, 1985). For the NJ analysis, evolutionary distances were estimated using \(\gamma\)-distributed rates. In the
numerous fresh fungal coils obtained from this sample were used. One of the P. minor samples collected from the Shimoina site had a large tuberous root, c. 10 cm long and 8 mm in diameter. Numerous fresh fungal coils obtained from this sample were used as inoculum to examine their ECM-forming ability on Pinus densiflora Siebold & Zucc., the sole ECM tree species at Shimoina. Nonmycorrhizal P. densiflora seedlings were grown on 500 ml of autoclaved volcanic soil for 4 months. A portion of the pot soil was carefully removed to expose part of the root system, and a 3 × 3 cm square sheet of a nonwoven textile with 50 μm pore size was placed under the exposed fine roots. The roots were inoculated with > 1000 fungal coils introduced by sterilized pipette, and a second square sheet was placed to cover the inoculated site of the root system, which was then covered with the previously removed soil. The inoculated seedlings were grown in a glasshouse at 18–25°C in the day and 15–20°C at night for 4 months. To prevent excessive drying and washing away of the fungal coils from inoculated sites, dishes containing sterilized water were placed under the pots. After checking ECM formation, the identity of the fungi associated with ECM root tips was determined using the molecular method described above for ITS.

### Isotope analysis

We examined the degree of heterotrophy of P. minor at Shimoina, Sayama and Kitamorokata. At each site, six leaves of P. minor located on the stem at a height of c. 10 cm were collected from different individuals. As references of autotrophic plants, leaves of Smilax china L. (Smilacaceae) and Clethra barbinervis Siebold & Zucc. (Clethraceae) at Shimoina and Sayama, and of S. china and Dryopteris erythrosora (Eatr.) Kuntze (Dryopteridaceae) at Kitamorokata were also collected, at the same distance from the soil as for P. minor. As no chlorophyllous mycoheterotrophic plant was available, we collected some ECM fungi as reference heterotrophic carbon sources: two fruit bodies of Russula sp. (Russulaceae) at Shimoina, three fruit bodies of Amanita volvata (Peck) Lloyd (Amanitaceae) and two fruit bodies of Thelephora sp. (Thelephoraceae) at Sayama, and four fruit bodies of Xerocomus sp. (Boletaceae) at Kitamorokata. The ITS sequences of these fungal species were obtained after PCR amplification and sequencing, as described earlier, and deposited in the DDBJ database with the respective accession numbers JF723271, JF723273, JF723272, and JF723274.

The samples collected were dried at 60°C for 4 d and then ground with 1.1-mm tungsten carbide balls (Biospec Products, Inc., Bartlesville, OK, USA) in 1.5-ml tubes using a Retch MM301 grinder (Retch GmbH and Co., Haan, Germany). The

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**Table 1** Sampling sites and dates of Platanthera minor with the mycorrhizal trees present in the habitats

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Latitude/longitude</th>
<th>Altitude (m)</th>
<th>Sampling date</th>
<th>Ectomycorrhizal trees in the habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitamorokata</td>
<td>31°40′35″ N, 131°09′52″ E</td>
<td>600</td>
<td>26 May 2009</td>
<td>Castanopsis sieboldii</td>
</tr>
<tr>
<td>Maniwa</td>
<td>35°17′25″ N, 133°35′09″ E</td>
<td>640</td>
<td>11 June 2009</td>
<td>Quercus serrata, Pinus densiflora</td>
</tr>
<tr>
<td>Kanazawa</td>
<td>36°3′56″ N, 136°39′45″ E</td>
<td>170</td>
<td>14 June 2009</td>
<td>Abies firma</td>
</tr>
<tr>
<td>Kiso</td>
<td>35°3′99″ N, 137°36′08″ E</td>
<td>650</td>
<td>27 June 2009</td>
<td>Q. serrata</td>
</tr>
<tr>
<td>Shimoina</td>
<td>35°3′44″ N, 137°51′41″ E</td>
<td>800</td>
<td>27 June 2009</td>
<td>P. densiflora</td>
</tr>
<tr>
<td>Sayama</td>
<td>35°3′9′10″ N, 139°25′39″ E</td>
<td>93</td>
<td>7 July 2009</td>
<td>P. densiflora</td>
</tr>
<tr>
<td>Kamogawa</td>
<td>35°1′07″ N, 140°06′14″ E</td>
<td>204</td>
<td>8 July 2009</td>
<td>A. firma</td>
</tr>
<tr>
<td>Naka</td>
<td>33°51′10″ N, 134°19′18″ E</td>
<td>800</td>
<td>24 August 2009</td>
<td>P. densiflora</td>
</tr>
<tr>
<td>Hatto</td>
<td>35°2′50′00″ N, 134°26′01″ E</td>
<td>810</td>
<td>16 September 2009</td>
<td>Fagus crenata</td>
</tr>
</tbody>
</table>

**Ectomycorrhiza formation in pot culture**

One of the P. minor samples collected from the Shimoina site had a large tuberous root, c. 10 cm long and 8 mm in diameter. Numerous fresh fungal coils obtained from this sample were used as inoculum to examine their ECM-forming ability on Pinus densiflora Siebold & Zucc., the sole ECM tree species at Shimoina. Nonmycorrhizal P. densiflora seedlings were grown on 500 ml of autoclaved volcanic soil for 4 months. A portion of the pot soil was carefully removed to expose part of the root system, and a 3 × 3 cm square sheet of a nonwoven textile with 50 μm pore size was placed under the exposed fine roots. The roots were inoculated with > 1000 fungal coils introduced by sterilized pipette, and a second square sheet was placed to cover the inoculated site of the root system, which was then covered with the previously removed soil. The inoculated seedlings were grown in a glasshouse at 18–25°C in the day and 15–20°C at night for 4 months. To prevent excessive drying and washing away of the fungal coils from inoculated sites, dishes containing sterilized water were placed under the pots. After checking ECM formation, the identity of the fungi associated with ECM root tips was determined using the molecular method described above for ITS.
amounts of carbon and nitrogen stable isotopes in each sample were measured as described by Tedersoo et al. (2007). The relative abundances of stable isotopes are presented as $\delta^{13}C$ and $\delta^{15}N$ using the equation: 

$$\delta^{13}C = R_{\text{sample}} / R_{\text{standard}} - 1 \times 1000:\%$$

where $R$ is the molar ratio of the stable isotopes, either $^{15}N/^{14}N$ or $^{13}C/^{12}C$. Pee Dee belemnite and atmospheric $N_2$ were used as the standards of carbon and nitrogen, respectively. The $\delta^{13}C$ and $\delta^{15}N$ values were tested for normality using a $\chi^2$ test for goodness of fit and for homogeneity of variances using the Bartlett test. After testing the isotope-abundance datasets for normal distributions and homogeneity of variances, one-way ANOVA was applied to evaluate the differences in $\delta^{15}N$ and $\delta^{13}C$ among $P. \text{minor}$, autotrophic plants and ECM fruit bodies at each sampling site, and mean values were then compared by the Bonferroni-corrected pairwise $t$-test. The degree of carbon heterotrophy in $P. \text{minor}$ was calculated using a linear two-source isotopic mixing model according to the equation:

$$x = (\delta^{13}C_{\text{MX}} - \delta^{13}C_{\text{ECM}}) / (\delta^{13}C_{\text{R}} - \delta^{13}C_{\text{ECM}}) \times 100:\%,$$

as described in Gebauer & Meyer (2003), where $\delta^{13}C_{\text{R}}, \delta^{13}C_{\text{ECM}}$ and $\delta^{13}C_{\text{MX}}$ are the mean values of autotrophic plants, ECM fruit bodies (used here as proxy for values in mycoheterotrophic plants; see the Discussion) and putatively mixotrophic plant species, respectively.

### Results

**Identification of $P. \text{minor}$ mycobionts**

Roots of $P. \text{minor}$ were typically thick and short (> 5 mm diameter, < 10 cm long) (Fig. 1a). In cross-sections of pigmented roots, most cortical cells contained sparse fungal coils with thick, brownish, septate hyphae of c. 5–8 $\mu$m diameter (Fig. 1b). Fungal ITS were successfully amplified using the primer pair ITS1F/ITS4 from all pools of fungal coils isolated (104 root samples of 41 individual plants; Table 2). In total, 123 fungal ITS rDNA sequences were obtained, among which 95% (117/123) were identified as members of Ceratobasidiaceae. These 117 sequences were separated into five phylotypes, I1 to I5, according to the divisions in the phylogenetic tree (Fig. 2), and selected representative sequences of each phylotype were deposited in the DDBJ database under the accession numbers AB604952 and AB605635–AB605649 (Table 2). Bayesian, NJ and MP phylogenetic analyses further conducted on the representative Ceratobasidiaceae sequences confirmed the division of the five phylotypes (Fig. 2). Among the phylotypes identified, I1 and I2 formed a highly supported clade with mycobionts of the mycoheterotrophic orchids $R. \text{gardneri}$ and $C. \text{sikokiana}$, which are known to have ECM-forming ability (Yagame et al., 2008; Bougoure et al., 2009). Phylotype I3 fungi were closely related to mycobionts of a few autotrophic and mixotrophic orchid species, including $\text{Pterygodium alatum}$ Sw. (FJ788721; Waterman et al., 2011), $\text{Hexalectris brevicaulis}$ L.O.Williams (HQ667792; Kennedy et al., 2011) and $\text{Limodorum abortivum}$ (L.) Sw (HM117643; Paduano et al., 2011; Fig. 2). No Ceratobasidiaceae sequences in the GenBank database were closely related to phylotype I4 fungi. Phylotype I5 fungi were related to a few fungi detected from the ECM root tips of $\text{Pinus banksiana}$ Lindl. &
Gord. (HQ285361), Tsuga heterophylla (Raf.) Sarg. (DQ481981; Wright et al., 2009) and Lithocarpus densiflorus (Thunb.) Nakai (DQ273373; Bergemann & Garbelotto, 2006; Fig. 2).

Phylotypes I1 and I2 were predominantly detected among the *P. minor* samples, with c. 90% of the sequences identified as Ceratobasidiaceae belonging to these two groups (Table 2). Notably, however, all fungi detected from the *Fagus crenata* forest at the Hatto site were from phylotype I4. The primer pair ITS1–ITS4–OF amplified fungal sequences identical to those amplified by ITS1F–ITS4, and no Tulasnellaceae ITS sequences were detected from any of the samples examined.

For analysis of the LSU nrDNA region of Ceratobasidiaceae mycobionts, a total of nine sequences was obtained (DDBJ database accession numbers AB605650–AB605658; Table 2), which could be divided into three phylotypes: L1, L2 and L4 (Fig. 2). Based on the sequences obtained from the ITS and LSU, phylotypes L1, L2 and L4 corresponded to ITS phylotypes I1, I2 and I4.

Fig. 1 Morphological characteristics of mycorrhizas in *Platanthera minor*. (a) Underground organs of *P. minor*, showing roots (arrows) and a stem (arrowhead). Bar, 2 cm. (b) A root cortical cell of *P. minor* showing a fungal coil. Bar, 50 μm.

Fig. 2 Bayesian 50% majority-rule consensus topology based on the nuclear (a) internal transcribed spacer (ITS) and (b) large subunit (LSU) nrDNA sequences of the Ceratobasidiaceae mycobionts, including *Platanthera minor*, obtained from 7602 to 8642 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. The likelihoods (In L) of the best states for cold chains of the two runs were −10 039.76 and −10 047.51 for the ITS, and −2 263.81 and −2 269.66 for LSU sequences. Maximum likelihood (ML) analysis of the identical dataset resulted in one ML tree (In L = −6744.617326) for the ITS and one ML tree (In L = −2239.574146) for LSU sequences. Only BS values over 70% are shown. Closed and open circles indicate *P. minor* mycobionts (named by sampling site) and those from other orchids, respectively. Pathogenic and putatively ectomycorrhizal fungi are indicated by squares and triangles, respectively.
I4, respectively. The close relationships between phylotypes L1 and L2, and their associations with mycobionts of *Rhizanthella* were confirmed in the LSU analysis. During the PCR amplification of the LSU rDNA region, the primer pair NL1–NL4 also amplified several sequences of *P. minor* itself (DDBJ database accession number AB623232), likely because of the presence of plant DNA in the fungal coil pools.

Six fungi in Thelephoraceae, Sebacinales, Russulaceae, Clavariaceae and Ascomycota were also detected by ITS analysis in *P. minor* and Ascomycota were also detected by ITS analysis in *P. minor* from the *Abies firma* forests at the Kamogawa and Kanazawa sites (Fig. 3; DDBJ database accession numbers AB605659–AB605664; Table 3).

**Ectomycorrhiza formation**

Six *P. densiflora* seedlings were used for the fungal inoculation. Four months after the inoculation, ECM root tips with dichotomous branching were found on the inoculated site of one seedling (Fig. 4a). Typical fungal sheaths and Hartig nets were observed in the ECM (Fig. 4b). The fungal ITS sequence obtained from the ECM was 100% identical to that of the inoculated fungi, which was identified as belonging to the phylotype I1 (DDBJ database accession number AB604952).

**Carbon and nitrogen isotopic abundances in *P. minor***

Finally, we examined the relative abundances of the stable 13C and 15N isotopes in samples from the Shimoina, Sayama, and Kitamorokata sites. At all three sites investigated, the δ13C values of *P. minor* were intermediate between those of the autotrophic plants and ECM fruit bodies examined, with significant differences detected (*P* < 0.01; Fig. 5). The δ15N values of *P. minor* were significantly higher than those of autotrophic plants and close to those of ECM-forming fruit bodies at all three sites (Fig. 5). The carbon to nitrogen elemental ratio values of *P. minor* were significantly lower than those of other plants and fungi at Kitamorokata (*P* < 0.01), but did not significantly differ at Shimoina or Sayama (same trends in the total percentage of nitrogen; data not shown). Based on the mixing-model calculation using δ13C values, the ratio of carbon acquired through mycoheterotrophy in *P. minor* was estimated to be 50.7 ± 1.15% at Sayama, 61.6 ± 11.13% at Kitamorokata, and 65.5 ± 0.92% at Shimoina (mean ± SE; see the Discussion section for limits of this results).

**Discussion**

*P. minor* is specifically associated with Ceratobasidiaceae

Nearly all *P. minor* mycobionts were identified as Ceratobasidiaceae, a fungal family detected or isolated from many photosynthetic orchids (Rasmussen, 1995, 2002; Dearnaley, 2007; Dearnaley et al., 2012). Ceratobasidiaceae occur in several *Platanthera* species, together with more frequent Tulasnellaceae species (occasionally under the anamorphic name ‘Epulorhiza’; Currach et al., 1990; Zelmer & Currah, 1995; Zelmer et al., 1996; Sharma et al., 2003). Zettler (2005) induced seed germination and seedling growth to the green leaf stage in *Platanthera leucophaea* (Nutt.) Lindley in an *in vitro* symbiotic culture with a Ceratobasidiaceae isolated from a *P. leucophaea* seedling.

In rare *P. minor* samples, ECM and saprobic fungi were also detected at the Kamogawa and Kanazawa sites. However, it is unclear whether this reflects a lower specificity in these *P. minor* populations and whether these fungi provide any nutrients. More evidence is needed to clarify the plasticity of *P. minor* for their mycobionts and the functional roles of these fungi, if any.

These data suggest a high specificity in *P. minor* symbiotic relationships. Such specificity has already been reported in some mixotrophic orchids (Bidartondo et al., 2004; Girlanda et al., 2006), but not all are specific (see review in Dearnaley et al., 2012). The phylogenetic analyses showed that a majority of *P. minor* Ceratobasidiaceae were in phylotypes I1 and I2 for the ITS region, corresponding to phylotypes L1 and L2 for the LSU region, which formed a highly supported clade with ECM-forming Ceratobasidiaceae isolated from the mycoheterotrophic orchids *R. gardneri* and *C. sikokiana* (see later). The mycobionts in phylotype I3 formed a clade with orchid mycobionts of the mixotrophic *L. abortivum* (HM117643; Paduano et al., 2011) and the mycoheterotrophic *H. brevicaulis* (HQ667792; Kennedy et al., 2011) (Fig. 2). Our results suggest that multiple lineages within the Ceratobasidiaceae associate with *P. minor* and other mycoheterotrophic orchids, although a more precise phylogeny of this family deserves further attention.
Ectomycorrhizal abilities in Ceratobasidiaceae

Ceratobasidiaceae are generally recognized as saprobic or parasitic (e.g. Rasmussen, 1995; Smith & Read, 2008). For example, the fungus involved in the culture of P. leucophaea mentioned earlier (Zettler, 2005) appears to have saprobic abilities, in the sense that no other plant was required for its culture. However, ECM-forming ability has also been observed in some Ceratobasidiaceae. For example, Warcup (1985) isolated a mycobiont from R. gardneri, a subterranean mycoheterotrophic orchid that grows near Melaleuca uncinata R. Br. (Myrtaceae) shrubs in South Western Australia. The fungal isolate, morphologically identified as Thanatephorus gardneri (Ceratobasidiaceae), formed typical ectomycorrhiza on M. uncinata and other ECM plants. Furthermore, seed germination and growth until flowering of R. gardneri were observed in association with ectomycorrhiza of the fungal isolate on M. uncinata in pot culture. Bougoure et al. (2009) also isolated mycorrhizal fungi from the mycoheterotrophic R. gardneri and Rhizanthella slateri (Rupp) M.A. Clem. & P.J. Cribb from Central Eastern Australia. The isolates were Ceratobasidiaceae based on ITS sequences and showed ECM-forming abilities on Melaleuca roots. Ectomycorrhiza-forming Ceratobasidiaceae also occur in Japan. The mycoheterotrophic orchid C. sikokiana associates with Ceratobasidiaceae, based on ITS sequences (Yagame et al., 2008), and one isolate formed ECM on seedlings of A. firma Siebold & Zucc. after inoculation of the cultured mycelium. Interestingly, the mycobionts of R. gardneri and C. sikokiana are closely related and cluster with our phylotypes I1 and I2 (Fig. 2, Bougoure et al., 2009), further supporting the ECM-forming abilities of the Ceratobasidiaceae found in this study. Phylotype I5 sequence was also closely related to fungal sequences detected from ECM root tips (Wright et al., 2009).

A recent meta-analysis by Tedersoo et al. (2010) suggested that ECM symbiosis evolved at least five times in Cantharellales, including twice in the Ceratobasidiaceae. As Ceratobasidiaceae are generally recognized as nonECM fungi, their detection in ectomycorrhiza may have been considered as contamination by saprobic or endophytic fungi in many previous studies – but see Rosling et al. (2003), Wright et al. (2009) and Collier & Bidartondo (2009) for detection of ECM colonized by Ceratobasidiaceae. Unfortunately, Ceratobasidiaceae do not form conspicuous fruit bodies that would confirm the ECM vs saprobic status of the various clades of this family by assessment of their $^{13}$C and $^{15}$N abundance (Trudell et al., 2004).

In the phylogenetic analyses, mycobionts of Apostasia species (Orchidaceae) also clustered with phylotype I1, and the fungal coils of P. minor exhibited sparse and thick hyphae similar to those of Apostasia cf. wallichii reported by Yukawa et al. (2009). Considering the distribution of P. minor in East Asia, R. gardneri in South Western Australia, C. sikokiana in East and South Asia and Apostasia species from East Asia to the Oceanic islands, ECM-forming Ceratobasidiaceae may be distributed in these regions at least.

We developed a new technique to form ECM by direct inoculation of fungal coils isolated from orchid mycorrhizas onto seedling roots. This technique, which confirmed the ECM-forming ability of P. minor and R. gardneri in the laboratory, is described below.

### Table 3

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<th>Sampling site</th>
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*May belong to Dothideomycetes.

**Putative ecology: ECM, ectomycorrhizal; SS, soil saprobe.

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*May belong to Dothideomycetes.
capability of *P. minor* mycobionts, represents a promising method to study uncultivable orchid mycorrhizal fungi, and to further test ECM-forming abilities in other orchid mycorrhizal Ceratobasidiaceae. In addition, the resulting ECM seedlings can be used for the cultivation of mycoheterotrophic and mixotrophic orchids requiring tripartite symbioses for growth. In the future, such *ex situ* tripartite symbioses could be used for physiological studies, as well as for propagation of endangered orchids.

Mycoheterotrophy in the genus *Plantanthera*

The δ13C values of *P. minor* samples were significantly above those of autotrophic plants, indicating that this orchid is mixotrophic (Gebauer & Meyer, 2003; Selosse & Roy, 2009). It is known that δ15N values are high in ECM fungi (Gebauer & Taylor, 1999; Trudell et al., 2004) and mixotrophic orchids associating with ECM fungi (Gebauer & Meyer, 2003; Julou et al., 2005): thus, the high δ13C values in *P. minor* also support its mixotrophy. This nutritional mode likely allows the growth of *P. minor* in shaded forest habitats. Based on δ13C values, the dependency on fungal carbon supply was estimated to exceed 50%. Unfortunately, as no other mycoheterotrophic plants were available at the study sites, we used ECM fruit bodies as a proxy for δ13C values of mycoheterotrophic carbon, which may have biased our calculations for two reasons. First, there is considerable variability in ECM δ13C values at a given site (Trudell et al., 2004), so that the available species may not approximate well to Ceratobasidiaceae δ13C values. Second, the ECM-forming ability of the Ceratobasidiaceae reported does not preclude some saprobic use of soil carbon, which would entail higher δ13C values (Trudell et al., 2004). Accordingly, mycoheterotrophic orchids associated with saprobic fungi are enriched in 13C (Martos et al., 2009), so that the dependency on fungal carbon may have been overestimated. However, because no fungal growth was found for the isolated fungal coils on Modified Melin Norkrans (MMN) medium plates (Marx, 1969; data not shown), we suspected a low saprobic ability.

To date, isotopic data are only available for two closely related European *Plantanthera* species (Hapeman & Inoue, 1997; Bateman et al., 2009) and display contrasting results. In one study, *P. chlorantha* was associated with fungi that were not necessarily ECM, and was autotrophic (Bidartondo et al., 2004), whereas in two studies the dependency on fungal carbon in *P. bifolia* ranged from 11% (δ13C values did not significantly differ from autotrophs; Gebauer & Meyer, 2003) to 60% (δ13C values significantly differed from autotrophs; Tedersoo et al., 2007). As the phylogenetic position of *P. minor* may be quite distant from *P. bifolia* (Hapeman & Inoue, 1997), a shift to mixotrophy may have occurred at least twice in *Plantanthera*. Moreover, one *Plantanthera* species from Borneo, namely *P. saprophytica* J.J. Sm., is fully mycoheterotrophic, although its exact phylogenetic placement and fungal partners remain unknown (Wood & Cribb, 1994).

**Fig. 4** Morphological characteristics of ectomycorrhizas in *Pinus densiflora*. (a) External appearance of ectomycorrhizas formed on the roots of *P. densiflora* by *Platanthera minor* mycobionts. Bar, 500 μm. (b) A cross-sectional image of the ectomycorrhizas on *P. densiflora* formed by *P. minor* mycobionts showing the Hartig net (arrows) and sheath (arrowhead). Bar, 20 μm.

**Fig. 5** δ15N and δ13C values of *Platanthera minor* plants, autotrophic plants and ectomycorrhizal (ECM) fruit bodies collected at the (a) Shimoina, (b) Sayama and (c) Kitamorokata sites; bars indicate standard errors. Pm, *P. minor*; cl, autotrophic *Clethra barbinervis*; sm, autotrophic *Smilax china*; dr, autotrophic *Dryopteris erythrosora*; ru, ECM *Russula* sp.; th, ECM *Thelephora* sp.; am, ECM *Amanita volvata*; xe, ECM *Xerocomus* sp.
The confirmation of mixotrophy in *Platanthera* spp., together with the strong link between *P. minor* and ECM-forming fungi, further demonstrates that this mixotrophic syndrome evolved repeatedly in terrestrial orchid tribes from temperate forests. Mycoheterotrophic germination likely predisposes to the emergence of a mixotrophic or mycoheterotrophic mode of growth, but these strategies are often associated with a shift to ECM fungal partners. This evolution is somewhat paradoxical, as nonECM Rhizoctonia-type fungi successfully support mycoheterotrophic germination up to the protocorm stage in most orchid species (Rasmussen, 1995). It is especially relevant that when mixoheterotrophic and mycoheterotrophic orchids associate with fungi from Rhizoctonia-type clades, they always target ECM subsclades, such as in Ceratobasidiaceae (this study), as well as in Sebacinales (Selosse et al., 2002). The reasons for the inability of nonECM Rhizoctonia-type to support the mycoheterotrophic growth of orchids beyond the protocorm stage, such as insufficient carbon availability, warrant further study.

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**References**


