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ORIGINAL ARTICLE

Partial and full mycoheterotrophy in green and albino phenotypes of the slipper orchid *Cypripedium debile*

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Abstract

Most green orchids form mycorrhizal associations with rhizoctonia fungi, a polyphyletic group including Serendipitaceae, Ceratobasidiaceae, and Tulasnellaceae. Although accumulating evidence indicated that partial mycoheterotrophy occurs in such so-called rhizoctonia-associated orchids, it remains unclear how much nutrition rhizoctonia-associated orchids obtain via mycoheterotrophic relationships. We investigated the physiological ecology of green and albino individuals of a rhizoctonia-associated orchid *Cypripedium debile*, by using molecular barcoding of the mycobionts and stable isotope (^{13}C and ^{15} N) analysis. Molecular barcoding of the mycobionts indicated that the green and albino individuals harbored *Tulasnella* spp., which formed a clade with the previously reported *C. debile* mycobionts. In addition, stable isotope analysis showed that both phenotypes were significantly enriched in ^{13}C but not in ^{15} N. Therefore, green and albino individuals were recognized as partial and full mycoheterotrophs, respectively. The green variants were estimated to obtain 42.5 ± 8.2% of their C from fungal sources, using the ^{13}C enrichment factor of albino individuals as a mycoheterotrophic endpoint. The proportion of fungal-derived C in green *C. debile* was higher than that reported in other rhizoctonia-associated orchids. The high fungal dependence may facilitate the emergence of albino mutants. Our study provides the first evidence of partial mycoheterotrophy in the subfamily Cypripedioideae. Partial mycoheterotrophy may be more general than previously recognized in the family Orchidaceae.

Keywords DNA barcoding · ^{13}C natural abundance · ^{15}N natural abundance · Mixotrophy · Mycoheterotrophy · Orchidaceae · Saprotrophic fungi · *Tulasnella*

Introduction

The evolution of full mycoheterotrophs, which have lost their photosynthetic ability, is one of the most exciting and challenging topics in plant evolution (Merckx 2013). Full mycoheterotrophy has been observed in a wide range of plant taxa and is estimated to have evolved independently approximately 50 times (Merckx 2013). Fully mycoheterotrophic taxa are more common in the Orchidaceae than in other families—more than 1% of all orchid species have entirely lost their photosynthetic ability (Bidartondo 2005). All the orchids depend on fungal partners at early seedling stage, and this initial mycoheterotrophy might have led to the evolution of life-long mycoheterotrophy in orchids (Leake 1994). In fact, many green orchids have been shown to utilize C resources from mycorrhizal partners during their adult stage based on high ^{13}C and ^{15} N abundances reflecting isotopic signature of their fungal symbionts (Gebauer and Meyer 2003; Selosse et al. 2004; Selosse and Roy 2009; Yagame et al. 2012; Hynson et al. 2013; Bellino et al. 2014; Suetsugu et al. 2017, 2021; Suetsugu and Matsubayashi 2021). The nutritional mode combining autotrophy and mycoheterotrophy in the adult stage is called partial mycoheterotrophy or mixotrophy (Gebauer and Meyer 2003; Selosse and Roy 2009).

Many green orchids are associated with the rhizoctonia group in basidiomycetes, a polyphyletic taxon encompassing

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Serendipitaceae, Ceratobasidiaceae, and Tulasnellaceae (Dearnaley et al. 2012). These rhizoctonia fungi are generally considered saprotrophs, endophytes, or plant pathogens, although a few very specific rhizoctonia clades are ectomycorrhizal (ECM) fungi on trees (Dearnaley et al. 2012). In contrast, many studies have shown that most fully mycoheterotrophic orchids associate with ECM fungi or non-rhizoctonia saprotrophic fungi (Martos et al. 2009; Ogura-Tsujita et al. 2009; Selosse and Roy 2009; Hynson et al. 2013; Lee et al. 2015; Suetsugu et al. 2020). It is interesting to note that some ECM-forming rhizoctonias have been isolated from fully mycoheterotrophic orchids (Selosse et al. 2002; Yagame et al. 2008; Bougoure et al. 2009), whereas these specific lineages are rarely mycorrhizal partners of green orchids (Dearnaley et al. 2012). Intriguingly, putatively initially mycoheterotrophic *Cymbidium* species are dependent on the non-ECM Tulasnellaceae (Ogura-Tsujita et al. 2012). In contrast, the partially mycoheterotrophic *Cymbidium* species associate with both Tulasnellaceae and several ECM families, and the two nearly fully mycoheterotrophic *Cymbidium* species exhibit specialized interactions with the ECM *Sebacina* (Ogura-Tsujita et al. 2012; Suetsugu et al. 2018). Therefore, it is likely that several orchid lineages have increased their fungal dependence at the adult stage, via their mycorrhizal switch to ECM fungi (Selosse and Roy 2009; Hynson et al. 2013).

Notably, recent studies have also suggested that partial mycoheterotrophy occurs in many non-ECM rhizoctonia-associated orchids (hereafter rhizoctonia-associated orchids), based on the low ^{13}C enrichment and somewhat higher ^{15}N and ^{2}H enrichment (Selosse and Martos 2014; Gebauer et al. 2016; Schweiger et al. 2018). Moreover, molecular identification of mycobionts has shown that some rhizoctonia-associated orchids are also associated with ECM fungi, whereas rhizoctonias are still the main mycobionts (Liebel et al. 2015; Suetsugu et al. 2019). Therefore, a distinction between rhizoctonia-associated and ECM-associated orchid species may not be strict (Jacquemyn et al., 2017; Jacquemyn and Merckx 2019). However, the extreme rarity of albino mutants in rhizoctonia-associated orchids suggests the insufficient capacity of rhizoctonia to support full mycoheterotrophy at the adult stage (Selosse and Martos 2014; Suetsugu et al. 2019). Schweiger et al. (2018) have also shown that the proportional C gains from fungal sources in rhizoctonia-associated orchids are in the lower range (approximately 20%). Therefore, it seems that non-ECM rhizoctonia fungi are less suited than ECM fungi to fulfill the substantial C demand for fully mycoheterotrophic adult orchids.

We note that most previous studies have estimated the proportion of fungal-derived C in rhizoctonia-associated orchids, with linear two-source isotope mixing models that have used fully mycoheterotrophic ECM-exploiting species as the upper endpoint (Gebauer and Meyer 2003; Bidartondo et al. 2004; Liebel et al. 2010). However, Stöckel et al. (2014) showed the significantly lower ^{13}C and ^{15}N enrichments in the protocorms of rhizoctonia-associated orchids than those of orchids associated with ECM fungi. Consequently, even though the mixing model calculations rarely detected a significant C gain (Gebauer and Meyer 2003; Bidartondo et al. 2004; Hynson et al. 2009; Liebel et al. 2010, 2015), the proportion of C gain by the orchids from fungal associations can be underestimated, owing to the lower-than-expected ^{13}C enrichment of rhizoctonia (Stöckel et al. 2014). To accurately determine the proportion of C obtained from mycorrhizal fungi by adult rhizoctonia-associated orchids, Schweiger et al. (2018) used the isotopic composition of the initially mycoheterotrophic protocorms as the proxy of fully mycoheterotrophic plants. However, given that protocorms are composed of both plant and mycorrhizal fungi, they should have intermediate isotope ratios between fully mycoheterotrophs and fungi; therefore, using protocorms as upper limit of mixing model may generate estimation errors (Johansson et al. 2015).

As an alternative method for determining the nutritional mode of the rhizoctonia-associated orchids, a comparison of isotope ratios of green and achlorophyllous (hereafter referred to as albino) individuals within a single species must be useful. Indeed, the existence of both phenotypes enables the comparison of their physiological characteristics within a shared genetic background (Selosse et al. 2004; Julou et al. 2005; Selosse and Roy 2009; Suetsugu et al. 2017). Suetsugu et al. (2019) investigated the nutritional modes of green and albino individuals of *Goodyera velutina*, an orchid species mainly associated with *Ceratobasidium* spp., by measuring the ^{13}C and ^{15}N abundances. The ^{13}C and ^{15}N enrichment of albino individuals were similar to those of mycoheterotrophic orchids that exploit litter-decomposing fungi (Suetsugu et al. 2019). Therefore, Suetsugu et al. (2019) concluded that albino *G. velutina* phenotype is a full mycoheterotroph for which C is derived from rhizoctonias that likely behave as litter-decomposing fungi. However, unfortunately, because green individuals of *G. velutina* are depleted in ^{13}C relative to those of the co-occurring autotrophic plants, Suetsugu et al. (2019) could not quantify the proportion of C derived from fungi by green individuals.

For this study, we have found a *Cypripedium debile* population that includes both green and chlorophyll-deficient (putative albino) individuals. Given that it was reported that *C. debile* is primarily associated with *Tulasnella* species (Shefferson et al. 2007) that belongs to non-ECM clades (Tedersoo et al. 2010), the population may provide the opportunity to understand the ^{13}C- and ^{15}N-enrichment patterns of green and albino individuals of a non-ECM rhizoctonia-associated orchid. It is also noteworthy that *C. debile* belongs to the subfamily Cypripedioideae where partial mycoheterotrophy was

hitherto not demonstrated, whereas *G. velutina* belongs to the subfamily Orchidoideae. In addition, *Tulasnella* species can use ammonium but not nitrate as inorganic N forms, whereas *Ceratobasidium* species can use both ammonium and nitrate (Fochi et al. 2017; Nurfadilah et al. 2013). These phylogenetic and physiological differences, in both the plant and fungal taxa, may influence the plant-fungal interactions during the exchange of C and N, thereby producing interspecific variations in ^{13}C and ^{15} N enrichment (Hynson et al. 2016).

Accordingly, we investigated the physiological ecology of green and putative albino *C. debile* individuals. Specifically, we aimed to investigate (i) whether the chlorophyll-deficient phenotype is incapable of photosynthesis (i.e., the phenotype can be functionally categorized as albino), (ii) whether green and albino individuals are associated with similar mycobionts, (iii) whether they show distinct ^{13}C and ^{15} N profiles, (iv) whether the isotopic profiles of albino *C. debile* are similar to those of the mycoheterotrophic orchids that exploit either saprotrophic fungi or ECM fungi, and (v) whether the degree of mycoheterotrophic nutrition provided to green *C. debile* is similar to that of the partially mycoheterotrophic orchids that exploit either rhizoctonia fungi or ECM fungi.

Material and methods

Study species and site

Cypripedium debile is a widespread and common species in China, Taiwan, and Japan (Cribb 1997). It can be distinguished from other *Cypripedium* species by its paired heart-shaped leaves, which are borne well above the soil surface, and by its pendent inflorescence, which bears small, green, dark-purple-veined flowers (Cribb 1997).

The study was conducted in Oshino Village, Minamitsuru County, Yamanashi Prefecture, Japan (35.47 N, 138.86 E), a cool temperate area. The population of *C. debile* that was investigated included > 100 green and < 10 putative albino individuals (Fig. 1). The green and putative albino plants were at least more than 50 cm apart. Therefore, it is not likely that putative albino phenotypes were connected via a rhizome to green phenotypes that were responsible for photosynthesis for the entire plant. In addition, we observed some putative albino individuals emerged in two consecutive years from 2016 to 2017, demonstrating that they survived for at least 2 years. Therefore, it is highly possible that these albino individuals obtained at least some C from its mycobionts and not from underground reserves from the previous year. The site is covered with conifer plantations dominated by *Cryptomeria japonica* (L.f.) D.Don and *Chamaecyparis obtusa* (Sieb. et Zucc.) Endl., with sparsely distributed *Zelkova serrata* (Thunb.) Makino and *Pinus densiflora* Siebold & Zucc. Since *P. densiflora*, the only common ECM tree in the site, often did not grow anywhere near the sampling plots, ectomycorrhizas were rarely observed near the orchids. All samples were collected on 9 June 2017.

Chlorophyll analysis

Because some of the leaf veins of putative albino individuals were slightly green, the chlorophyll fluorescence and concentrations of four green and four putative albino individuals were measured to confirm the fully heterotrophic status. To measure chlorophyll fluorescence, the samples were dark-adapted for 15 min before measuring their steady-state

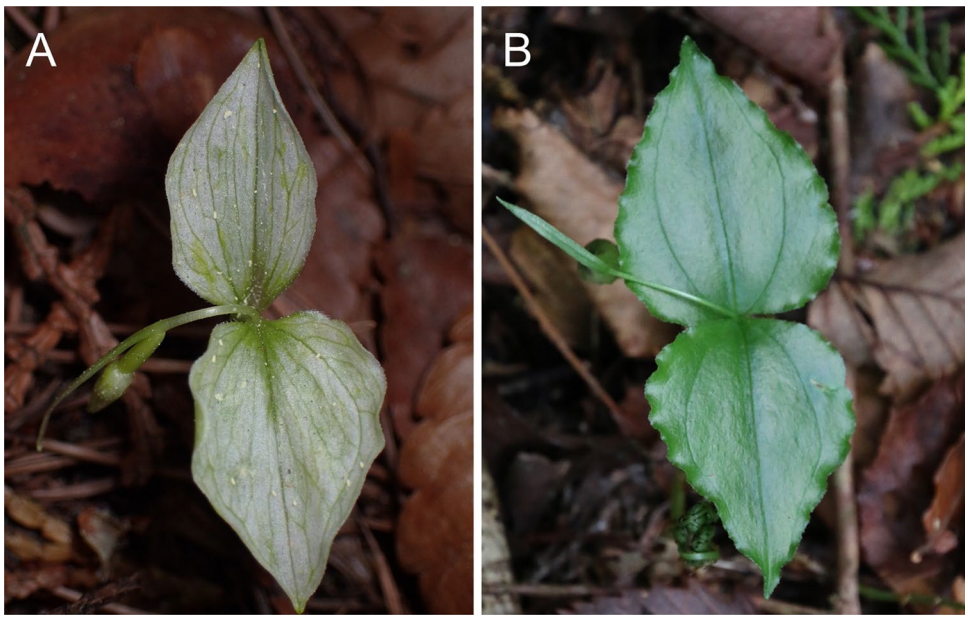

Fig. 1 Phenotypes of the *Cypripedium debile* individuals in the studied population. **A** Chlorophyll-deficient individual. **B** Green individual

photosystem II quantum yield (QY) values using the FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). These QY values were defined as the ratios of actual fluorescence yield (F_v) to maximum fluorescence (F_m). Chlorophyll concentrations (*Chl*) were calculated from the Soil Plant Analysis Development (SPAD) values, using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Osaka, Japan), according to the following equation (Monje and Bugbee 1992): $Chl = 1.034 + (0.308 \times \mathrm{SPAD}) + (0.110 \times \mathrm{SPAD}^2)$ (mg m^{-2}).

Differences in the QY and Chl values between green and albino *C. debile* individuals were compared using Student's *t*-tests.

Molecular identification of mycobionts

Root systems were harvested from four green (G1–G4) and four albino (A1–A4) *C. debile* individuals. Light microscopy was used to select orchid roots with fungal pelotons, and three fragments of about 0.5 cm each were collected from each of the three roots (nine root fragments in total) from each plant and preserved in 99.5% ethanol until subsequent DNA extractions.

Total DNA was extracted from the mycorrhizal root fragments using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Primers ITS1-OF and ITS4-OF (Taylor and McCormick 2008) were used to amplify the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) of the mycobionts by polymerase chain reaction (PCR). The 15-μL PCR mixtures contained 1 μL of extracted DNA (1 ng μL^{-1}), 0.375 U of Takara Ex Taq Hot Start Version (Takara Bio, Otsu, Japan), 0.25 μM of each primer, 200 μM of each dNTP, and 1.5 μL of the supplied PCR buffer. The PCR was performed using the GeneAtlas G02 thermal cycler (Astec Co., Ltd., Fukuoka, Japan) under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles of 20 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and a final elongation step at 72 °C for 10 min.

All the PCR products were cloned using the pGEM-T Easy Vector System I (Promega, Madison, WI, USA), and eight colonies with DNA inserts were arbitrarily selected from each sample for sequencing at a commercial sequencing facility (Takara Bio, Otsu, Japan). The obtained sequences were queried against the International Nucleotide Sequence Database Collaboration (INSDC) database using the basic local alignment search tool (BLAST) (Altschul et al. 1997), and then separated into three taxa (*Tulasnella*, Ceratobasidiaceae, and Sebacinales). The Ceratobasidiaceae and *Tulasnella* sequences from Veldre et al. (2013) and Suetsugu et al. (2019) were downloaded. Sebacinales sequences identified as Sebacinaceae that is mainly ECM, or Serendipitaceae that is mainly endophytic or saprotrophic (Weiß et al. 2016), were also downloaded. The downloaded sequences of each fungal taxon were then subjected to maximum-likelihood (ML) analysis in MEGA7 (Kumar et al. 2016), and phylogenetic trees were constructed using FigTree ver. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Stable isotope analysis

Because mycoheterotrophic plants exploiting ECM fungi usually have a higher relative ^{15}N abundance than those exploiting wood- or litter-decaying saprotrophic fungi (Ogura-Tsujita et al. 2009), stable isotope ratios can be useful tools for estimating the nutritional source of mycoheterotrophic plants.

Here, we conducted a stable C and N isotope analysis to estimate whether albino and green individuals mainly exploit saprotrophic fungi or ECM fungi. First, we set six quadrats of 1 × 1 m around green and albino individuals of *C. debile*. We sampled the leaves of green and albino *C. debile* individuals and sampled the leaves of the understory plant species within each quadrat as reference plants. The leaves of reference plants were collected at the same height of the focal *C. debile* leaves to limit the influence of environmental factors, such as atmospheric CO_2 isotope composition, photosynthetic recycling of CO_2 produced by forest soil respiration, microscale light climate (which affects C isotope values), and soil type (which affects N isotope values) (Gebauer and Schulze 1991). This criterion led us to sample *Comanthosphace japonica* (Miq.) S.Moore, *Dioscorea nipponica* Makino, and *Viola tokubuchiana* Makino var. *takedana* (Makino) F.Maek., as autotrophic reference species, since no other plants were present in more than two quadrats. We also collected another non-ECM rhizoctonia-associated green orchid, *Neottia makinoana* (Ohwi) Szlach. (Yagame et al. 2016) for which the nutritional mode was unknown in all the quadrats (Table S1).

The collected leaves were dried at 60 °C for 4 d and then ground using scissors and an agate mortar. The abundances of the stable ^{13}C and ^{15}N isotopes and C and N concentrations were measured at the Research Institute for Humanity and Nature (Kyoto, Japan) using a Delta plus XP mass spectrometer connected to a Flash EA 1112 elemental analyzer via the Conflo III interface (Thermo Fisher Scientific, Massachusetts, USA). The relative abundances of the stable isotopes were calculated as $\delta^{15}\mathrm{N}$ or $\delta^{13}\mathrm{C} = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000$ (‰), where R_{sample} represents the ^{13}C/^{12}C or ^{15}N/^{14}N ratio of the sample, and R_{standard} represents the ^{13}C/^{12}C ratio of Vienna Pee Dee Belemnite, or the ^{15}N/^{14}N ratio of atmospheric N_2. The C and N isotope ratios were calibrated using two laboratory standards: DL-alanine ($\delta^{13}\mathrm{C} = -25.36$‰, $\delta^{15}\mathrm{N} = -2.89$‰), L-alanine ($\delta^{13}\mathrm{C} = -19.04$‰, $\delta^{15}\mathrm{N} = 22.71$‰) and glycine ($\delta^{13}\mathrm{C} = -34.92$‰, $\delta^{15}\mathrm{N} = 2.18$‰) (Tayasu et al. 2011). The analytical standard deviations (SDs) were 0.02‰ (δ^{13}C,

$n = 8$) and 0.08‰ (δ^{15}N, $n = 4$) for DL-alanine, and 0.03‰ (δ^{13}C, $n = 5$) and 0.01‰ (δ^{15}N, $n = 4$) for glycine. The total N concentrations of the leaf samples were calculated using the sample weights and the CO_2 and N_2 gas volumes of the laboratory standards (Tayasu et al. 2011).

After confirming (using Bartlett's test) that the δ^{13}C and δ^{15}N datasets were normally distributed, the differences in the δ^{13}C values and N concentrations of green and albino *C. debile* individuals, and those of the autotrophic reference plants, were analyzed using one-way ANOVA, followed by Fisher's multiple comparisons tests. Because the δ^{15}N values were not normally distributed, a Kruskal–Wallis nonparametric test and a sequential Bonferroni-corrected Mann–Whitney *U* test were used. In addition, enrichment factors (ε) sensu Preiss and Gebauer (2008) were calculated from the δ values of each plant group based on $\varepsilon = \delta_S - \delta_{REF}$, where δ_S represents the δ^{13}C or δ^{15}N value of either green or albino *C. debile* variant and δ_{REF} represents the mean value of all autotrophic reference plants from a specific sampling plot. The proportion of C derived from fungi (% C_{df}) in the leaf tissues of green *C. debile* specimens was calculated using the linear two-source mixing model: % $C_{df} = (\varepsilon GC/\varepsilon AC) \times 100$, where εGC represents the enrichment factor of green *C. debile* individuals and εAC represents the mean enrichment factor of albino *C. debile* individuals, which was used as the endpoint.

Results

Chlorophyll fluorescence and concentration

The mean chlorophyll concentration of putative albino leaves was 1.5 ± 0.3 (mean ± SD) mg m^{-2}, whereas that of green leaves was 149.7 ± 20.6 mg m^{-2}. Thus, the mean chlorophyll concentrations of green leaves were approximately 100 times greater than those of albino leaves. The QY values of green individuals (0.77 ± 0.04) fell within the range of the values reported for healthy plants, thereby confirming that green individuals retained a functional PS_{II} apparatus (Maxwell and Johnson 2000). In contrast, the QY values of albino individuals (0.07 ± 0.04) were considerably lower than those of green individuals ($P < 0.001$) and nearly zero, suggesting that the chlorophyll-deficient phenotype is incapable of performing photosynthesis and can be regarded as a functional albino variant.

Molecular identification of mycobionts

Ten fungal ITS sequences were obtained from two albino and three green individuals, and these included four Tulasnellaceae sequences, two Ceratobasidiaceae sequences, and one Sebacinales sequence (Table 1), although ITS sequences of *C. debile* itself have dominantly been amplified.

For the Tulasnellaceae phylogenetic analysis, the partial 5.8S rDNA region was used because of the high divergence at the ITS1 and ITS2 regions. The ML model using the Kimura 2-parameter (K2) and gamma distribution (+G) was selected as the best-fit model (lnL = −411.3176843). Four fungal sequences, two from green and two from albino individuals, formed a clade with the *C. debile* mycobionts (Bidartondo et al. 2003), and they were separated from the ECM Tulasnellaceae clade (Shefferson et al. 2007) (Fig. 2).

For the Ceratobasidiaceae phylogenetic analysis, the ML model using Tamura 3-parameter + G was selected as the best-fit model (lnL = −4627.608028). The nutritional mode of the Ceratobasidiaceae fungi detected in a green individual belonged to non-ECM fungal clade (Veldre et al. 2013) (Fig. 3).

For the Sebacinales phylogenetic analysis, the partial 5.8S rDNA region was once again used, because of

Table 1 Fungal ITS rDNA sequences obtained from roots of green and albino *Cypripedium debile* with their top hit sequences agaist the INSDC database.

Plant type	Plant no.	Sequence no.	Top hit against the INSDC database			
			Accession no.	Query cover (%)	Ident (%)	Taxon
Green	G2	G2OF-4	MH730164	88	98	Ceratobasidiaceae sp.
		G2OF-5	MH730164	89	98	Ceratobasidiaceae sp.
		G2OF-6	DQ925504	74	97	Uncultured Tulasnellaceae
		G2OF-7	JX043209	77	85	Uncultured fungus
	G3	G3OF-5	HQ154376	95	98	Uncultured *Sebacina*
	G4	G4OF-1	HM230883	90	90	Uncultured Leotiomycetes
		G4OF-2	DQ925506	72	97	Uncultured Tulasnellaceae
Albino	A2	A2OF-1	DQ925504	73	97	Uncultured Tulasnellaceae
		A2OF-8	HG936817	88	99	Uncultured *Neonectoria*
	A3	A3OF-8	DQ925506	74	98	Uncultured Tulasnellaceae

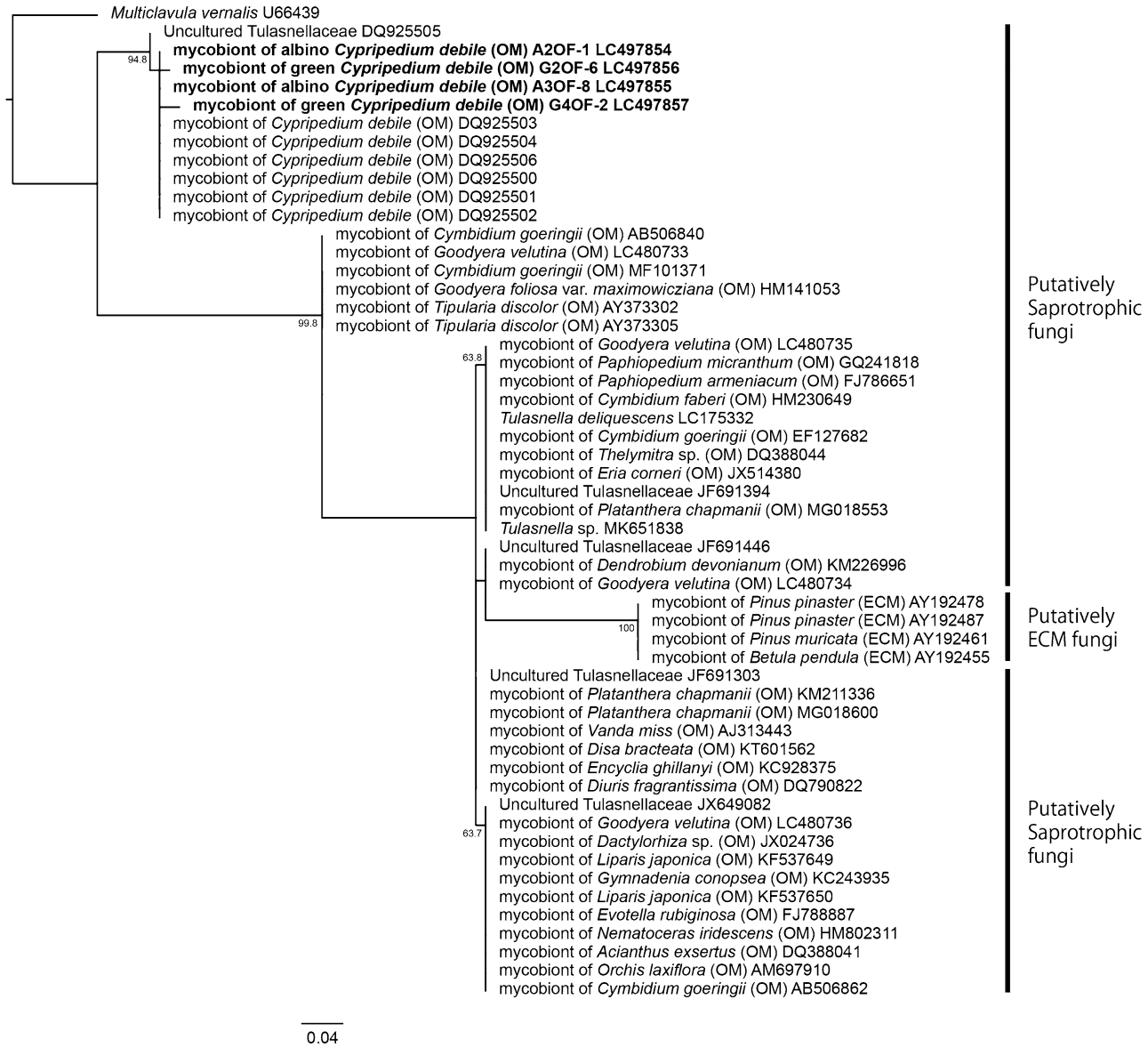

Fig. 2 Maximum-likelihood phylogenetic tree based on partial 5.8S rDNA sequences (119–120 bp) of Tulasnellaceae fungi obtained from *Cypripedium debile* roots and the International Nucleotide Sequence Database Collaboration (INSDC) database. INSDC accession numbers are given for all sequences. The tree was rooted using *Multiclavula vernalis* (Accession U66439). Bootstrap values (BS; 1000 replications) are shown at each node (only BS > 60% are shown). The scale bar indicates the number of substitutions per site

the high divergence at the ITS1 and ITS2 regions. The ML model using K2 was selected as the best-fit model (lnL = −360.686777), and the fungal sequence obtained from one green individual was grouped within Sebacinaceae that is mainly ECM (Weiß et al. 2016) (Fig. 4).

Stable isotope analysis

The δ^{13}C values of albino individuals (−24.5 ± 0.3‰; mean ± SD) were significantly higher ($P < 0.001$) than those of green individuals (−30.0 ± 0.8‰), autotrophic reference plants (−33.8 ± 0.9‰), and another rhizoctonia-associated orchid, *N. makinoana* (−35.0 ± 0.6‰; Fig. 5; Supplementary Information Table S1). In addition, the δ^{13}C values of green individuals were significantly higher than those of the two autotrophic plants and *N. makinoana* ($P < 0.001$). The δ^{13}C values of *N. makinoana* were significantly lower than those of both the autotrophic reference plants ($P < 0.01$).

In contrast, there was no significant difference among the δ^{15}N values of albino *C. debile* individuals (−3.9 ± 0.3‰), green *C. debile* individuals (−4.7 ± 0.5‰), or reference autotrophic plants (−4.2 ± 0.8‰). However, the δ^{15}N values

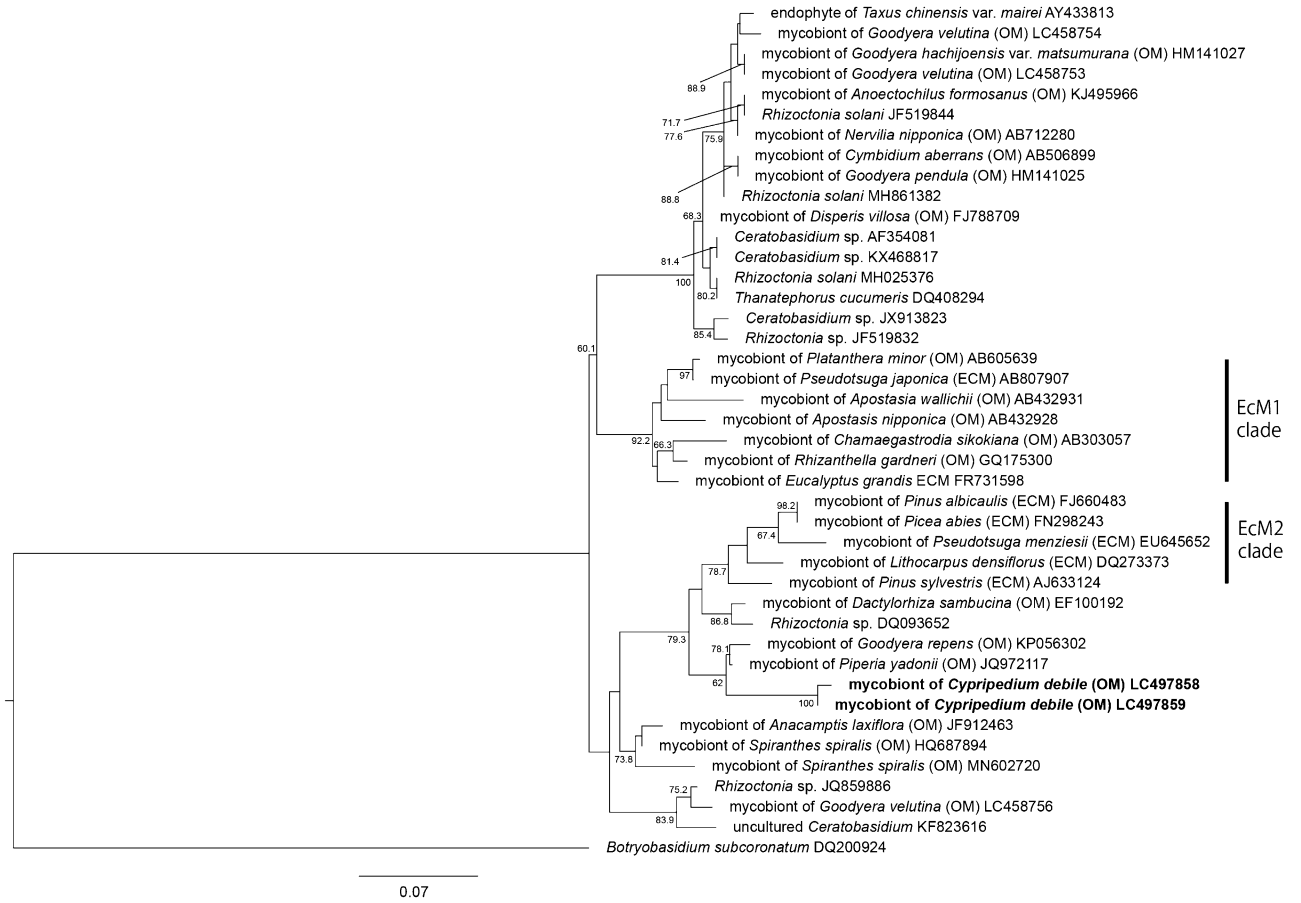

Fig. 3 Maximum-likelihood phylogenetic tree based on partial ITS rDNA sequences (503–557 bp) of Ceratobasidiaceae fungi obtained from *Cypripedium debile* roots and the International Nucleotide Sequence Database Collaboration (INSDC) database. INSDC accession numbers are given for all sequences. The tree was rooted using *Botryobasidium subcoronatum* (Accession DQ200924). Bootstrap values (BS; 1000 replications) are shown at each node (only BS > 60% are shown). The scale bar indicates the number of substitutions per site

of *N. makinoana* (−2.9 ± 0.8‰) were significantly higher than those of green *C. debile* individuals ($P < 0.05$; Fig. 5). The ^{13}C and ^{15} N enrichment factors of albino individuals were 9.8 ± 0.5‰ and 0.5 ± 0.8‰, respectively, whereas the ^{13}C and ^{15} N enrichment factors of green individuals were 4.2 ± 0.9‰ and −0.4 ± 1.1‰, respectively. Using the mean ^{13}C enrichment factors of albino *C. debile* individuals as the fully mycoheterotrophic endpoint, the fungal-derived C proportion in green *C. debile* individuals was calculated to be 42.5 ± 8.2%.

The total N concentrations of the leaves from albino *C. debile* individuals (4.0 ± 0.4 mmol g^{-1}) were significantly higher than those of the leaves from green *C. debile* individuals (2.2 ± 0.2 mmol g^{-1}), *N. makinoana* (2.7 ± 0.4 mmol g^{-1}), and reference autotrophs (2.3 ± 0.2 mmol g^{-1}; $P < 0.001$). In contrast, there were no significant differences in the total N concentrations of green *C. debile* individuals and reference autotrophic plants ($P = 0.64$).

Discussion

The chlorophyll fluorescence and concentration data are consistent with albino *C. debile* individuals being fully mycoheterotrophic. Specifically, the leaves of albino individuals contained approximately 100 times less chlorophyll than those of green individuals, and the fluorescence measurements indicated that a functional PSII apparatus was present in green individuals but virtually absent from albino individuals. Furthermore, both the total chlorophyll concentrations (1.5 ± 0.3 mg m^{-2}) and QY values (0.07 ± 0.04) of albino individuals were similar to those reported for albino individuals of other species (Abadie et al. 2006; Julou et al. 2005). For example, the total chlorophyll concentrations and QY values of *Cephalanthera damasonium* (Mill.) Druce albinos are 1.6 ± 0.1 mg m^{-2} and 0.05 ± 0.04, respectively (Julou et al. 2005; Stöckel et al. 2011). Therefore, it is unlikely that photosynthesis

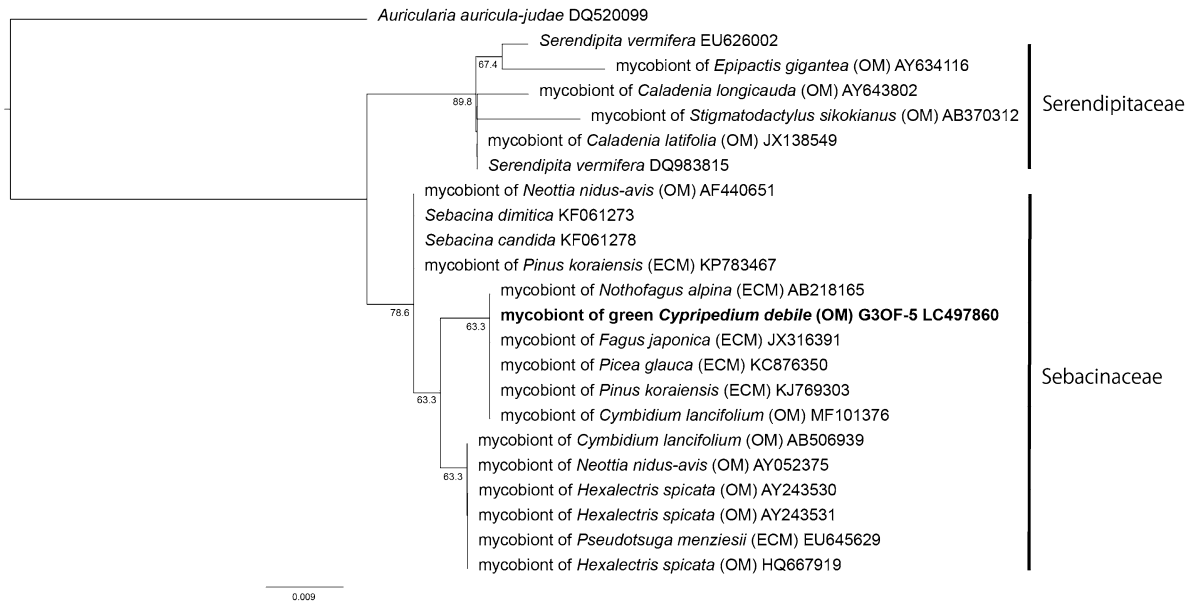

Fig. 4 Maximum-likelihood phylogenetic tree based on 5.8S rDNA sequences (167–169 bp) of Sebacinales fungi obtained from *Cypripedium debile* roots and from the International Nucleotide Sequence Database Collaboration (INSDC) database. INSDC accession numbers are given for all sequences. The tree was rooted using *Auricularia auricula-judae* (Accession DQ520099). Bootstrap values (BS; 1000 replications) are shown at each node (only BS > 60% are shown). The scale bar indicates the number of substitutions per site

contributes to the C budget of the chlorophyll-deficient *C. debile* individuals.

Molecular barcoding of the mycobionts indicated that both green and albino individuals were associated with *Tulasnella* spp. that are closely related to the previously reported *C. debile* mycobionts (Shefferson et al. 2007). Even though some members of Tulasnellaceae are known to form ECM associations (Tedersoo et al. 2010), our ML phylogenetic analyses revealed that *Tulasnella* species associated with *C. debile* does not belong to an ECM clade (Bidartondo et al. 2003). Therefore, based on the available evidence, the *Tulasnella* species associated with the *C. debile* roots are likely to be saprotrophs, endophytes, or plant pathogens (Selosse and Martos 2014). In addition, although (i) a green individual was associated with *Ceratobasidium* fungi and (ii) ECM status has evolved twice (EcM 1 clade and EcM 2 clade) within the Ceratobasidiaceae (Veldre et al. 2013), they were not included into these ECM fungal clades. Therefore, the present study indicates that both *C. debile* phenotypes harbored typical orchid mycorrhizal fungi (i.e., non-ECM

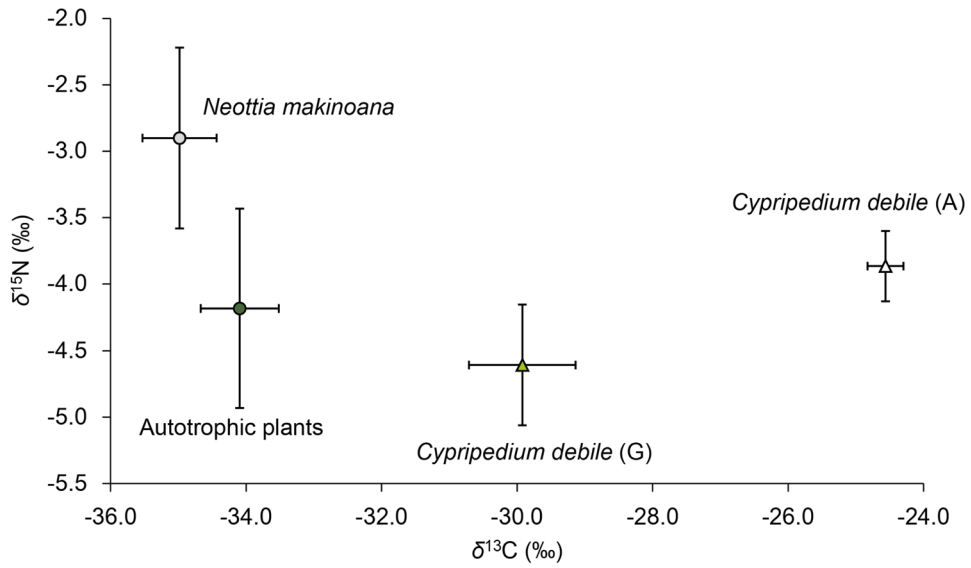

Fig. 5 Mean (± standard deviation) of $\delta^{13}C$ and $\delta^{15}N$ values in the leaves of green and albino individuals of *Cypripedium debile*, its neighboring autotrophic plants, and another rhizoctonia-associated orchid, *Neottia makinoana*. A: albino. G: green

rhizoctonia). However, it should be noted that one green individual was associated with a *Sebacina* sp. that belongs to an ECM clade (Riess et al. 2013; Tedersoo et al. 2014). Therefore, our data suggest that *C. debile* can also associate with some ECM rhizoctonias. Interestingly, the other ECM genus *Russula* has also been noted as occasional mycorrhizal partners of some *Cypripedium* species (Shefferson et al. 2007). Considering that (i) most partially mycoheterotrophic orchids have been found to form relationships with ECM fungi (Selosse and Roy 2009), and (ii) the number of mycobionts detected in our fungal DNA analysis was very limited, *C. debile* may be somewhat dependent on ECM rhizoctonias as well as other ECM fungi that remained undetected in the present study. Future detailed molecular analyses using high-throughput sequencing techniques with additional sampling efforts could provide a more comprehensive picture of *Cypripedium* mycorrhizal associations.

Despite somewhat inconclusive evidence obtained with molecular barcoding, stable isotopic analysis strongly suggests that the albino variants mainly depend on non-ECM fungi. The ^{15}N enrichment factor of albino individuals (0.5 ± 0.8‰) was much lower than the average one of the mycoheterotrophic orchids known to exploit ECM fungi (11.5‰; Hynson et al. 2016). Mycoheterotrophic plants that exploit ECM fungi usually have higher relative ^{15}N abundances than mycoheterotrophs exploiting saprotrophic fungi (Martos et al. 2009; Ogura-Tsujita et al. 2009; Lee et al. 2015; Suetsugu et al. 2019, 2020), reflecting the traits of their mycobionts (Mayor et al. 2009). Therefore, saprotrophic fungi, rather than ECM fungi, are most likely the main C sources of albino individuals. In addition, the ^{13}C enrichment factor of albino *C. debile* individuals (9.8 ± 0.5‰) was significantly higher than the mean enrichment factor of mycoheterotrophic orchids that exploit ECM fungi (8.0‰) (Hynson et al. 2016). Given that ECM fungi are usually less enriched in ^{13}C than saprotrophic fungi (Mayor et al. 2009), the high ^{13}C enrichment of albino individuals probably provides additional support for the exploitation of saprotrophic fungi.

Isotopic evidence also indicates that green *C. debile* individuals were partial mycoheterotrophs. The green *C. debile* individuals had significantly higher δ^{13}C values than the co-occurring autotrophic plants ($\varepsilon^{13}C = 4.2 \pm 0.9$‰), whereas there were no significant differences in the δ^{15}N values of green individuals and co-occurring autotrophic plants ($\varepsilon^{15}N = -0.4 \pm 1.1$‰). The green *C. debile* individuals were estimated to obtain 42.5 ± 8.2% of their total C from fungal partners, using albino individuals as a fully mycoheterotrophic endpoint. Based on a meta-analysis of the published enrichment factors of partially and fully mycoheterotrophic orchids, Hynson et al. (2016) estimated that, on average, 39.6% of the C gained by partially mycoheterotrophic orchids associated with ECM fungi is mycorrhizal-derived. Therefore, although previous studies have suggested that rhizoctonia fungi are less suited than ECM fungi to meet the substantial C demands of adult orchids (Lallemand et al. 2018; Schweiger et al. 2018), the estimated C gain from mycorrhizal partner in *C. debile* is well within the range reported for other partially mycoheterotrophic orchids and pyroloids that exploit ECM fungi (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Gonneau et al. 2014; Suetsugu et al. 2017; Matsuda et al. 2020; Shutoh et al. 2020).

It is interesting to note that (i) green *C. debile* individuals have relatively high fungal-C dependence and (ii) as suspected by the emergence of albinos, partial mycoheterotrophy in *C. debile* is probably significant, compensating for a low C gain from photosynthesis. These characteristics probably facilitate the emergence of albino mutants in *C. debile*. The high ^{13}C enrichment and the high C dependence may infer that *C. debile* has somewhat shifted to ECM partners, especially given that an ECM rhizoctonia (*Sebacina* sp.) was detected in a *C. debile* individual. However, we still consider that *C. debile* individuals obtain most C from non-ECM rhizoctonias, although to a lesser degree, *C. debile* probably can exploit some ECM fungi. This is because (i) green and albino *C. debile* individuals show much lower ^{15}N enrichment (-0.4 ± 1.1‰ and 0.5 ± 0.8‰) than partially and fully mycoheterotrophic orchids exploiting ECM fungi (9.6‰ and 11.5‰ in average; Hynson et al. 2016) and (ii) previous studies have shown that *C. debile* as well as other *Cypripedium* species are consistently associated with non-ECM Tulasnellaceae (Shefferson et al. 2007, 2019). Taken together, molecular and isotopic evidence indicates that albino *C. debile* individuals are full mycoheterotrophs, which obtain some C from non-ECM rhizoctonia fungi and that green *C. debile* individuals are partial mycoheterotrophs, which receive approximately 40% of their C from similar mycobionts.

We considered that the great differences in ^{13}C and ^{15}N abundance among so-called rhizoctonia-associated orchids is related to diverse ecological niches of non-ECM rhizoctonia fungi (Selosse and Martos 2014; Suetsugu et al. 2019), given that endophytism results in depleted ^{13}C levels (Halbwachs et al. 2013), and the transition from saprotrophy to endophytism has often occurred in rhizoctonias (Veldre et al. 2013). In fact, although the ^{13}C enrichment is absent or low in most rhizoctonia-associated orchids, similar high ^{13}C enrichments have been found in the protocorms of some rhizoctonia-associated orchids (7.7 ± 1.2‰; Schweiger et al. 2018) and albino variants of *Goodyera velutina* (8.8 ± 0.9‰; Suetsugu et al. 2019). These discrepancies in ^{13}C abundances could be ascribed to their nutritional modes. Given that (i) the ^{13}C enrichments of *Goodyera velutina* and *C. debile* albinos are similar to those of mycoheterotrophic orchids exploiting saprotrophic fungi (e.g., 10.2 ± 1.3‰ in *Gastrodia confusa*; Ogura-Tsujita et al. 2009) and (ii) saprotrophic

non-rhizoctonia fungi can support the nutrient demand of fully mycoheterotrophic orchids (e.g., Ogura-Tsujita et al. 2009; Suetsugu et al. 2020), we considered that *Tulasnella* associated with *C. debile* is likely to be a saprotroph. We even speculated that not endophytic but saprotrophic rhizoctonias might efficiently support mycoheterotrophic growth at the adult stage. The daring hypothesis warrants further investigation, but the ecology of rhizoctonia species is difficult to unveil, since rhizoctonias are not easy to sample for isotopic measurements due to their microscopic habit. Further studies such as radiocarbon approaches (e.g., Suetsugu et al. 2020) will be needed to characterize the physiological ecology of rhizoctonia-associated orchids.

In conclusion, both isotopic and molecular data suggest that green and albino *C. debile* individuals are partial and full mycoheterotrophs that obtain at least some C from non-ECM rhizoctonia fungi. Although partial mycoheterotrophy has been demonstrated in three subfamilies of the Orchidaceae (Suetsugu and Matsubayashi 2021), we provide the first evidence of partial mycoheterotrophy in Cypripedioideae. Partial mycoheterotrophy may be more general than previously recognized in orchids.

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