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Stable isotope signatures illuminate diverse nutritional strategies in rhizoctonias and their orchid partners

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Summary

Understanding the nutritional ecology of orchids, particularly those in symbiosis with rhizoctonias, presents a complex challenge. This complexity arises partly from the absence of macroscopic fruit bodies in rhizoctonias, which impedes the acquisition of their stable isotope data. In this study, we investigated the fungal associations and isotopic signatures in the pelotons of *Stigmatodactylus sikokianus* (associated with non-ectomycorrhizal (non-ECM) rhizoctonias) and *Chamaegastrodia shikokiana* (associated with ECM rhizoctonias). Our research reveals elevated levels of ^{13}C enrichment in *S. sikokianus* plants and their pelotons, similar to those found in fully mycoheterotrophic orchids and their mycobionts. Interestingly, *C. shikokiana* plants and their pelotons exhibited even higher levels of ^{13}C and ^{15}N enrichment than many other fully mycoheterotrophic species. Our findings imply that both ECM and saprotrophic mycobionts, including certain rhizoctonias, can fulfill the carbon needs of highly mycoheterotrophic orchids. This finding also indicates that ^{13}C enrichment can be an indicator of mycoheterotrophy in at least some rhizoctonia-associated orchids, despite the typically low ^{13}C enrichment in non-ECM rhizoctonias. Our demonstration of partial mycoheterotrophy in *S. sikokianus* suggests a broader prevalence of this nutritional strategy among orchids, given that almost all orchids are associated with non-ECM rhizoctonias.

Keywords: metabarcoding, mycoheterotrophy, mycorrhiza, nutritional mode, Orchidaceae, peloton, rhizoctonia, stable isotopes

Introduction

All orchids are highly dependent on mycorrhizal fungi for carbon during seed germination and seedling establishment (a phenomenon called 'mycoheterotrophy'), owing to their minute seeds and lack of reserves (Merckx, 2013; Jacquemyn & Merckx, 2019). Orchid mycorrhizal fungi primarily belong to the polyphyletic group called rhizoctonias, which includes various fungi from the families Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae (Dearnaley *et al.*, 2012). These fungi play diverse and non-exclusive ecological roles, mainly as saprotrophs, facultative plant pathogens, and root endophytes (Dearnaley *et al.*, 2012; Selosse & Martos, 2014). Additionally, various non-rhizoctonia fungal taxa, typically characterized by saprotrophic or ectomycorrhizal (ECM) lifestyles, have been secondarily recruited by terrestrial orchids as mycorrhizal partners (Wang *et al.*, 2021; Selosse *et al.*, 2022).

It is widely accepted that orchids rely on mycorrhizal fungi during their chlorophyll-deficient juvenile stages, yet it has been traditionally believed that most mature orchid plants achieve complete autotrophy (Cameron *et al.*, 2008; Field *et al.*, 2015). However, a significant paradigm shift in our understanding of plant nutrition is the discovery that some green orchid species not only undergo photosynthesis but also acquire additional carbon resources from mycorrhizal fungi (partial mycoheterotrophy or mixotrophy) (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Selosse & Roy, 2009; Hynson *et al.*, 2013).

Partial mycoheterotrophy in orchids has primarily been demonstrated using stable isotopes, primarily ^{13}C and ^{15}N (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005), and more recently, ^{2}H and ^{18}O (Gebauer *et al.*, 2016). The core principle of isotope analysis is based on the heterotrophic nature of fungi, resulting

in significant enrichments in ^{2}H, ^{13}C, and/or ^{15}N relative to their substrates and co-occurring autotrophic plants (Gebauer & Dietrich, 1993; Gleixner *et al.*, 1993; Ziegler, 1995). Consequently, the isotopic composition of mycoheterotrophic plants mirrors or even exceeds the elevated $\delta^{13}C$ and $\delta^{15}N$ values found in their associated fungi (Schiebold *et al.*, 2017). This correlation arises from mycoheterotrophic plants acquiring fungal material through processes such as hyphal lysis or direct transfer across intact membranes between the fungus and the plant (Bougoure *et al.*, 2014; Kuga *et al.*, 2014).

Following pioneering studies (Gebauer & Meyer, 2003; Trudell *et al.*, 2003), numerous investigations have examined orchid trophic modes through stable isotope analysis (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Tedersoo *et al.*, 2007; Motomura *et al.*, 2010; Gebauer *et al.*, 2016; Suetsugu *et al.*, 2020b, 2021b; Suetsugu & Matsubayashi, 2021). For instance, Bidartondo et al. (2004) revealed that orchids primarily associating with rhizoctonias showed no significant ^{13}C enrichment, while orchids forming symbiotic relationships with ECM fungi or saprotrophic non-rhizoctonia fungi displayed notable enrichments in both ^{13}C and ^{15}N. These results suggested that mycoheterotrophic evolution in orchids often involves a transition in mycorrhizal fungal partners, shifting from rhizoctonias to ECM fungi or saprotrophic non-rhizoctonia fungi (Bidartondo *et al.*, 2004; Ogura-Tsujita *et al.*, 2012, 2021; Yagame *et al.*, 2016). Interestingly, a few specific rhizoctonia clades have also been secondarily recruited by fully mycoheterotrophic orchids as mycorrhizal partners, likely because these rhizoctonia clades can also function as ECM fungi on trees (Yagame *et al.*, 2008; Bougoure *et al.*, 2010; Pecoraro *et al.*, 2020).

On the other hand, orchids associated with non-ECM rhizoctonias (hereafter rhizoctonia-associated orchids) are likely less predisposed to develop mycoheterotrophic growth at the adult stage (Selosse & Roy, 2009; Hynson *et al.*, 2013; Jacquemyn &

Merckx, 2019). However, most previous studies estimated fungal-derived carbon in rhizoctonia-associated orchids using linear two-source isotope mixing models, with a ^{13}C enrichment factor derived from fully mycoheterotrophic orchids that exploit ECM fungi as a reference (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Liebel *et al.*, 2010). Consequently, the infrequent detection of significant carbon gain in these mixing models may partially be due to the lower-than-expected ^{13}C enrichment of rhizoctonias, considering fully mycoheterotrophic protocorms (non-photosynthetic underground seedlings; Dearnaley *et al.*, 2016) of some rhizoctonia-associated orchids are less enriched in ^{13}C compared to those associated with ECM fungi (Stöckel *et al.*, 2014; Schweiger *et al.*, 2018). Another approach to assessing the degree of partial mycoheterotrophy involves analyzing the isotopic composition of fruit bodies, which serve as their mycorrhizal partners (Trudell *et al.*, 2003; Julou *et al.*, 2005; Ogura-Tsujita *et al.*, 2009; Yagame *et al.*, 2012; Lee *et al.*, 2015). However, this method faces challenges when dealing with rhizoctonias, as these fungi do not produce discernible fruit bodies (Zahn *et al.*, 2023).

To address these challenges, recent studies have measured the stable isotope ratios of fungal pelotons (intracellular hyphae within orchid roots) extracted from orchid roots (Gomes *et al.*, 2023; Zahn *et al.*, 2023). By comparing this data with the isotopic compositions of orchid and reference plant tissues, researchers have revealed that pelotons extracted from rhizoctonia-associated orchids in the genera *Anoectochilus*, *Epipactis*, *Ophrys*, and *Orchis* exhibit notably lower ^{13}C enrichments (ranging from 1.4–2.4‰) than pelotons isolated from *Epipactis* orchids associated with ECM fungi (ranging from 4.3–6.8‰) (Gomes *et al.*, 2023; Zahn *et al.*, 2023). Consequently, they have concluded that the meager ^{13}C enrichment observed in rhizoctonias constrains the

115 applicability of ^{13}C enrichment as an indicator of mycoheterotrophy in rhizoctonia-
116 associated orchids.

117 However, it is regrettable that data regarding the isotopic compositions of pelotons
118 in rhizoctonia-associated orchids are confined to species exhibiting non-significant ^{13}C
119 enrichment in their leaf materials. We acknowledge the existence of variations in ^{13}C and
120 ^{15}N abundance among rhizoctonia-associated orchids, likely attributable to the diverse
121 ecological niches occupied by rhizoctonias (Selosse & Martos, 2014; Suetsugu *et al.*,
122 2019, 2021a). This assertion is substantiated by the tendency of endophytic associations
123 to result in decreased ^{13}C levels (Halbwachs *et al.*, 2013; Selosse & Martos, 2014), along
124 with the fact that endophytism has been observed at least for some rhizoctonias (Veldre
125 *et al.*, 2013).

126 It is noteworthy that some rhizoctonia-associated orchids exhibit a higher degree
127 of ^{13}C enrichment compared to autotrophic reference plants. For example, albino
128 *Platanthera hondoensis* (+8.9 ± 0.4‰), albino *Goodyera velutina* (+8.8 ± 0.9‰), albino
129 *Cypripedium debile* (+9.8 ± 0.5‰), and *Stigmatodactylus sikokianus* with extremely
130 reduced leaves (+10.6 ± 1.4‰), manifest ^{13}C enrichment similar to that observed in
131 orchids associated with litter-decaying non-rhizoctonia fungi (Suetsugu *et al.*, 2019,
132 2021a,c; Suetsugu & Matsubayashi, 2022). The isotopic data from fungal pelotons in
133 these species would have undoubtedly facilitated a more lucid interpretation of the
134 nutritional mode of rhizoctonias and rhizoctonia-associated orchids.

135 In this study, we focused on two Japanese orchids, *Stigmatodactylus sikokianus* and
136 *Chamaegastrodia shikokiana* (Fig. 1–2). Notably, despite *S. sikokianus* having
137 specialized associations with non-ECM rhizoctonias and possessing chlorophyll on its
138 stem and small leaves (Suetsugu *et al.*, 2021a), the ^{13}C enrichment of *S. sikokianus*

surpasses even the mean ^{13}C enrichment of mycoheterotrophic orchids exploiting ECM fungi (+8.0‰) and litter-decaying fungi (+8.7‰) (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009; Hynson *et al.*, 2013; Lee *et al.*, 2015; Suetsugu *et al.*, 2020a; Hynson *et al.*, 2016). On the other hand, *C. shikokiana* is a fully mycoheterotrophic species associated with ECM-forming Ceratobasidiaceae (Yagame *et al.*, 2008). Given the extraordinary ^{13}C and ^{15}N enrichments observed in a partially mycoheterotrophic *A. nipponica* associated with the same ECM-forming Ceratobasidiaceae clade (+7.2 and +21.8‰, respectively) (Suetsugu & Matsubayashi, 2021), the values of *C. shikokiana* likely exceed the mean reported values for mycoheterotrophic orchids exploiting ECM fungi (+8.0‰ and +11.5‰) (Hynson *et al.*, 2016).

Given that orchid leaf isotope compositions probably mirror those of their mycobionts (Schiebold *et al.*, 2017), we hypothesized that rhizoctonia pelotons from *S. sikokianus* show ^{13}C and ^{15}N enrichment similar to mycoheterotrophic orchids associated with saprotrophic non-rhizoctonia fungi. We also expected rhizoctonia pelotons from *C. shikokiana* to display ^{13}C and ^{15}N enrichment similar to pelotons from mycoheterotrophic orchids associated with other ECM fungi. Additionally, we fortunately found two other (nearly) fully mycoheterotrophic orchids, *Cephalanthera subaphylla* and *Gastrodia nipponica*, associated with ECM or SAP non-rhizoctonia fungi, respectively (Kinoshita *et al.*, 2016; Sakamoto *et al.*, 2016; Suetsugu *et al.*, 2020a), near the *S. sikokianus* plants. The isotopic compositions of *C. subaphylla* and *G. nipponica* pelotons were utilized to explore whether rhizoctonias demonstrate distinct isotopic compositions within the same fungal guilds (i.e., ECM or SAP fungi).

Materials and Methods

Sampling localities

Our fieldwork for *S. sikokianus* was conducted in Iya, Tanabe City, Wakayama Prefecture, Japan, on 25 August 2023. The study site is a warm-temperate forest dominated by *Cryptomeria japonica*. In the year of our study, the site contained over 100 flowering individuals of *S. sikokianus*. Other prevalent species on the forest floor included *Wisteriopsis japonica*, *Quercus glauca*, *Ficus sarmentosa*, and *Hylodesmum podocarpum* subsp. *oxyphyllum* var. *japonicum*.

Our fieldwork for *C. shikokiana* took place in Nogawa, Uwajima City, Wakayama Prefecture, Japan, on 25 August 2023. The study area is characterized by a warm-temperate forest dominated by *Castanopsis sieboldii* subsp. *sieboldii*. That year, the site hosted over 50 *C. shikokiana* flowering plants. Several other plant species, such as *Eurya japonica*, *Trachelospermum asiaticum*, and *Myrsine seguinii* were also commonly found in the forest understory.

We established four 1-meter by 1-meter quadrats containing flowering individuals of *S. sikokianus* or *C. shikokiana* at Iya and Nogawa, respectively. From these quadrats, we collected the aboveground parts and mycorrhizal samples for subsequent peloton extraction and stable isotopic analysis. For *C. shikokiana*, a single specimen per quadrat was sampled; however, due to the diminutive size of *S. sikokianus*, which rendered a single individual insufficient for comprehensive analysis, three specimens per quadrat were collected and pooled. We also gathered leaves from at least three co-occurring autotrophic reference species within each quadrat for isotopic analysis. To minimize the influence of environmental variables, such as the isotopic composition of atmospheric CO_2, microscale light conditions, and soil type (Gebauer & Schulze, 1991), we collected

leaves from reference plants growing at the same height as the target orchid species.

During our sampling process, we serendipitously discovered *S. sikokianus* protocorms near the four individual *S. sikokianus* specimens. These protocorms, which were found around the respective *S. sikokianus* specimens, were subsequently combined and used for both mycobiont molecular identification and isotopic analysis.

We further sampled plants from other mycoheterotrophic orchids associated with ECM or SAP non-rhizoctonia fungi (*Cephalanthera subaphylla* and *Gastrodia nipponica*) in a quadrat with *S. sikokianus* for molecular identification of mycobionts and isotopic analysis (Fig. S1).

Peloton isolation

The rhizomes and roots of the orchid species investigated were excised and examined to ascertain the presence of mycorrhizal colonization, utilizing a light microscope. Notably, *S. sikokianus* and *C. shikokiana* consistently displayed mycorrhizal formation within the cortical cells of their rhizomes (Fig. 1–2). In contrast, mycorrhizal colonization was observed only in the dark-colored roots of *C. subaphylla* and *G. nipponica* (Fig. S2–3).

We extracted pelotons from the rhizomes and roots, following the methodology of Zahn et al. (2023) with a slight modification. In brief, we made longitudinal sections of the mycorrhizal underground parts using a razor blade. Subsequently, we subjected them to sonication in deionized water for 15 minutes to release the pelotons. The solution containing the extracted pelotons was carefully transferred to another centrifuge tube using a pipette. This process was repeated iteratively, with deionized water consistently added to the root fragments until the solution became clear.

The purification process involved a series of stacked stainless-steel sieves with

mesh sizes of 250, 106, and 53 micrometers (Sanpo, Tokyo, Japan) to effectively separate the pelotons from any remaining plant residues. To ensure the highest peloton purity based on mesh size, the sieve surfaces were thoroughly rinsed with deionized water multiple times to remove potential contaminants. Notably, the majority of pelotons were retained by the 106-micrometer sieve. Subsequently, these retained pelotons were carefully pipetted into Eppendorf tubes and centrifuged for 10 seconds. The supernatant, devoid of pelotons, was removed, leaving behind the peloton pellets. The isolated pelotons were utilized for both molecular identification and isotopic analysis, except for *S. sikokianus* protocorms, which were too diminutive for peloton extraction. For these, bulk samples were used for molecular identification and isotopic analysis.

Molecular identification of mycobionts

Although the mycobionts of *S. sikokianus* and *C. shikokiana* are known to have specialized interactions with non-ECM rhizoctonias and ECM rhizoctonias, respectively (Yagame *et al.*, 2008; Yagame & Yamato, 2008; Suetsugu *et al.*, 2021a), we performed molecular identification to confirm the consistency with prior findings.

The mycorrhizal fungi of the ITS region were amplified using the primer set ITS86F/ITS4, a set renowned for its efficiency in studying fungal communities associated with orchid roots (Waud *et al.*, 2014). These primers were attached with 3–6-mer Ns and Illumina sequencing primers in a procedure delineated in Suetsugu *et al.* (2021a). We employed the Q5 High-Fidelity DNA Polymerase kit for the PCR, with the following conditions: an initial denaturation at 98°C for 40 seconds, followed by 35 cycles of 98°C for 5 seconds, 58°C for 10 seconds, and 72°C for 20 seconds, and a final extension for 10 minutes at 72°C. To infuse the Illumina P5/P7 adapter sequences and sample-centric

indices, an additional PCR was executed (Syed *et al.*, 2009; Suetsugu & Matsubayashi, 2021). This process involved an initial denaturation at 98 °C for 40 s, 12 cycles of 5 s at 98 °C, 10 s at 65 °C, and 20 s at 72 °C, culminating in a 10-minute extension at 72 °C. The pooled library was then sequenced on the Illumina MiSeq platform, utilizing the MiSeq Reagent Micro Kit v2 (300 cycles). The sequence data have been deposited in the DDBJ Sequence Read Archive under the BioProject accession number PRJDB8982.

After sequencing, bioinformatic analysis was conducted using Claident v0.9.2020.12.06 (Tanabe & Toju, 2013), as outlined by Suetsugu & Okada (2021). Our methodology encompassed primer region excision, discarding low-quality reads, and denoising erroneous sequences using DADA2 (Callahan *et al.*, 2016) implemented in Claident. Subsequently, the sequences potentially arising from PCR chimeras or index-hopping were eradicated using the clremovechimev and clremovecontam commands in Claident (Esling *et al.*, 2015; Nilsson *et al.*, 2019). The filtered sequences were then grouped into operational taxonomic units (OTUs) with a similarity threshold of 97% via VSEARCH v2.8.0 (Rognes *et al.*, 2016) implemented in the clclassseqv command. The dominant sequence from each OTU served as the representative sequence for that cluster. Taxonomic assignment of the OTUs was performed based on the query-centric auto-*k*-nearest-neighbor (QCauto) and the lowest common ancestor (LCA) algorithms (Huson *et al.*, 2007), using the "overall_genus" reference database in Claident. Only those OTUs assigned to potentially orchid mycorrhizal fungi (Dearnaley *et al.*, 2012; Wang *et al.*, 2021) were retained for further analysis.

Both *S. sikokianus* and *C. shikokiana* specimens predominantly exhibited colonization by Serendipitaceae and Ceratobasidiaceae fungi, respectively. Therefore, we conducted phylogenetic analyses to further investigate these relationships. Specifically,

the OTUs identified as mycobionts of *S. sikokianus* and *C. shikokiana* were subjected to a BLAST query against the International Nucleotide Sequence Database Collaboration (INSDC) for comparative purposes (Altschul *et al.*, 1997). Subsequently, we retrieved several sequences that were closely related phylogenetically, along with other representative sequences. These sequences were aligned using ClustalW, and a maximum-likelihood phylogenetic tree was constructed using MEGA X (Kumar *et al.*, 2018) with a GTR + I + G model and 1,000 bootstrap replicates (ln L = −5518.53 and −3492.03, respectively).

$\delta^{13}C$ and $\delta^{15}N$ analysis

The natural abundance of ^{13}C and ^{15}N isotopes in the orchid species investigated, their pelotons, and co-occurring autotrophic reference plants was measured using a continuous-flow isotope-ratio mass spectrometer connected to an elemental analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), as described by Suetsugu and Matsubayashi (2021). The stable isotope ratios were calculated as follows:

$$\delta^{13}C \text{ or } \delta^{15}N\ [‰] = (R_{sample}/R_{standard} - 1) \times 1{,}000,$$

where R_{sample} represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of each sample, and $R_{standard}$ represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of Vienna PeeDee belemnite or atmospheric N_2, respectively. The C and N isotope ratios were calibrated using the following laboratory standards: CERKU-01 (DL-alanine, $\delta^{13}C$ = −25.36‰, $\delta^{15}N$ = −2.89‰), CERKU-02 (L-alanine, $\delta^{13}C$ = −19.04‰, $\delta^{15}N$ = 22.71‰), and CERKU-05 (L-threonine, $\delta^{13}C$ = − 9.45‰, $\delta^{15}N$ = −2.88‰) (Tayasu *et al.*, 2011). The analytical standard deviations (SD) of these

283 standards were 0.07‰ ($\delta^{13}C$, n = 18) and 0.09‰ ($\delta^{15}N$, n = 18) for DL-alanine, 0.04‰
284 ($\delta^{13}C$, n = 5) and 0.08‰ ($\delta^{15}N$, n = 12) for L-alanine, and 0.03‰ ($\delta^{13}C$, n = 12) for L-
285 threonine.

286 To cancel out location effects and facilitate comparisons with previous studies, we
287 also converted δ values into site-independent enrichment factors (ε) using the equation: ε
288 = $\delta_S - \delta_{REF}$. In this equation, δ_S represents the $\delta^{13}C$ or $\delta^{15}N$ value of a sample from the
289 orchid species under investigation or their pelotons, while δ_{REF} denotes the average value
290 of all autotrophic reference plants located within a specific sampling plot (Preiss &
291 Gebauer, 2008). We compared the $\delta^{13}C$, $\delta^{15}N$, $\varepsilon^{13}C$, and $\varepsilon^{15}N$ values among different
292 groups using a linear mixed model, fitting 'identity' as a fixed term and 'plot' as a random
293 term. Subsequently, a post hoc Tukey–Kramer test was utilized to determine if the values
294 of target orchid samples and autotrophic reference samples significantly differ. The R
295 statistical software (R Core Team, 2023) was used for all analyses, with the lme4 package
296 (Bates *et al.*, 2015) employed for the LMM and the multcomp package (Hothorn *et al.*,
297 2008) for the Tukey-Kramer test.
298
299 **Results**
300
301 **Molecular identification of mycobionts**
302 Community profiling using metabarcoding techniques confirmed a specialized
303 association between *S. sikokianus* and a specific Serendipitaceae OTU (Sebacinales group
304 B) (4,943 reads [100% of total reads] in flowering plants and 2,084 reads [100% of total
305 reads] in protocorms) (Fig. 3, Table S1). Phylogenetic examination further revealed that
306 the mycobionts of *S. sikokianus* formed a well-supported clade with the mycobiont

307 previously identified in *S. sikokianus* (Yagame & Yamato, 2008; Suetsugu *et al.*, 2021a).
308 These mycobionts also aligned in a highly supported clade with mycobionts identified in
309 several green orchids and the *Serendipita vermifera* species complex (Deshmukh *et al.*,
310 2006). In parallel, *G. nipponica* and *C. subphylla* are primarily associated with two OTUs
311 from the litter-decomposing genus *Mycena* (29,417 reads [99.6% of total reads]) and two
312 OTUs from the ectomycorrhizal genus *Tomentella* (10,371 reads [99.9% of total reads]),
313 respectively.
314 Additionally, community profiling using the metabarcoding approach also revealed
315 that *C. shikokiana* consistently associates with a single Ceratobasidiaceae OTU (Fig. 4,
316 Table S1). All four individuals were dominated by a single OTU ascribed to the genus
317 *Ceratobasidium* (28,565 reads [99.7% of total reads]). Phylogenetic assessment indicated
318 that the OTU found in *C. shikokiana* formed a robust monophyletic cluster with the
319 mycobiont previously detected in *C. shikokiana* and with those from a fully
320 mycoheterotrophic orchid *Rhizanthella gardneri*, and two partially mycoheterotrophic
321 orchids, *Platanthera minor* and *Apostasia nipponica* (Yagame *et al.*, 2008, 2012;
322 Bougoure *et al.*, 2009; Suetsugu & Matsubayashi, 2021). Furthermore, phylogenetic
323 analysis indicated that the OTU is an ectomycorrhizal fungus belonging to the EcM 1
324 clade sensu Veldre *et al.* (2013).
325
326 **$\delta^{13}C$ and $\delta^{15}N$ analysis**
327 The $\delta^{13}C$ values of not only *S. sikokianus* protocorms (−23.4 ± 0.1‰; mean ± SD) but
328 also *S. sikokianus* aboveground parts (−24.2 ± 0.1‰) were significantly higher than those
329 observed in autotrophic reference plants (−35.5 ± 1.4‰; $P < 0.001$; Fig. 5, Table S2).
330 Notably, the $\delta^{13}C$ values of fungal pelotons from *S. sikokianus* (−25.1 ± 0.8‰) were also

significantly higher than those of autotrophic reference plants ($P < 0.001$). There was no statistically significant difference in $\delta^{13}C$ values among the protocorms, the aboveground parts, and the pelotons of *S. sikokianus* ($P > 0.3$ in all combinations). Using the mean value of enrichment factors in the *S. sikokianus* protocorm ($\varepsilon^{13}C = 12.1‰ \pm 0.3‰$) or a fully mycoheterotrophic orchid *G. nipponica* ($\varepsilon^{13}C = 12.2‰$) as fully mycoheterotrophic endpoints, the fungal-derived carbon in *S. sikokianus* aboveground parts was estimated to be 93.5 ± 1.9% and 92.8 ± 1.9% of the total carbon, respectively.

On the other hand, the $\delta^{15}N$ values of both *S. sikokianus* protocorms (−1.4 ± 1.5‰) and *S. sikokianus* aboveground parts (0.5 ± 0.5‰) were not significantly higher than those observed in autotrophic reference plants (−1.3 ± 1.4‰; $P > 0.1$ in all combinations). Fungal pelotons from *S. sikokianus* (−1.6 ± 0.7‰) also did not show enriched ^{15}N levels compared to those from autotrophic reference plants ($P = 0.99$).

The $\delta^{13}C$ values of *C. shikokiana* flowers (−25.1 ± 0.6‰) and *C. shikokiana* stems (−25.9 ± 0.2‰) were significantly higher than those observed in autotrophic reference plants (−33.4 ± 1.0‰; $P < 0.001$; Fig. 5, Table S3). The $\delta^{13}C$ values of fungal pelotons from *C. shikokiana* (−26.4 ± 0.2‰) were also notably higher than those of the autotrophic reference plants ($P < 0.001$). There was no statistically significant difference in $\delta^{13}C$ values among the flowers, the stems, and the pelotons of *C. shikokiana* ($P > 0.05$ in all combinations).

Additionally, the $\delta^{15}N$ values of *C. shikokiana* flowers (11.4 ± 0.9‰) and *C. shikokiana* stems (8.9 ± 1.6‰) were significantly higher than those observed in autotrophic reference plants (−3.6 ± 0.9‰; $P < 0.001$). The $\delta^{15}N$ values of fungal pelotons from *C. shikokiana* (7.4 ± 0.6‰) were also significantly elevated compared to those of autotrophic reference plants ($P < 0.001$).

Discussion

^{13}C enrichment in non-ECM rhizoctonia pelotons can resemble that in typical SAP-type pelotons

Community profiling using metabarcoding confirmed that the pelotons of *S. sikokianus* at our study site predominantly consist of an OTU belonging to the family Serendipitaceae (Yagame & Yamato, 2008; Suetsugu *et al.*, 2021a). Furthermore, these mycobionts formed a strongly supported clade with mycobionts identified in several green orchids. Thus, we can conclude that the fungal associations of *S. sikokianus* align with the typical fungal partnerships observed within the family Orchidaceae.

Despite (i) its typical orchid mycorrhizal associations and (ii) the general trend of low ^{13}C enrichment in fungal pelotons of many rhizoctonia-associated orchids, we expected a high ^{13}C enrichment in pelotons extracted from *S. sikokianus*. Our expectation was based on the observation that the leaf material of *S. sikokianus* exhibited a ^{13}C enrichment level similar to that of fully mycoheterotrophic orchids associated with saprotrophic non-rhizoctonia fungi (Suetsugu *et al.*, 2021a). As hypothesized, the ^{13}C enrichment in pelotons extracted from *S. sikokianus* (10.4 ± 0.7‰) closely resembled the ε^{13}C values of pelotons isolated from *G. nipponica* associated with saprotrophic non-rhizoctonia fungi (12.2‰). Interestingly, the ^{13}C enrichment is even higher than that in pelotons from ECM-associated orchids *Epipactis atrorubens* (4.3 ± 1.3‰) and *E. leptochila* (6.8 ± 0.7‰) (Zahn *et al.*, 2023). The observed differences could be attributed to the fact that δ^{13}C values in ectomycorrhizal fungi are approximately 3‰ lower

379 compared to those in saprotrophic fungi (Hobbie *et al.*, 1999; Högberg *et al.*, 1999; Kohzu
380 *et al.*, 1999).

381 Based on the ^{13}C enrichment of pelotons, which exhibit values similar to those
382 found in other fully mycoheterotrophic orchids, we can dismiss the idea that the
383 significant ^{13}C enrichment in the aboveground tissues of *S. sikokianus* is due to a specific
384 physiology unrelated to mycoheterotrophy. Consequently, the notably high ^{13}C
385 enrichment observed in the plant bodies strongly suggests a high degree of
386 mycoheterotrophic nutrition, even in rhizoctonia-associated orchids.

387 Intriguingly, previous research has demonstrated that the transit time of carbon
388 from the atmosphere to *S. sikokianus* is approximately 4.0 ± 0.8 years based on
389 radiocarbon approaches (Suetsugu *et al.*, 2021a). This indicates that its mycobionts are
390 mainly litter-decaying, rather than endophytic, as the latter would result in much younger
391 carbon ages. The notably high ^{13}C enrichment of pelotons also supports that the
392 mycobiont of *S. sikokianus* has escaped the typical (and poorly understood, probably
393 saprotrophic ± endophytic) ecological niche of other rhizoctonias, given that an
394 endophytic lifestyle would decrease $\delta^{13}C$ (Halbwachs *et al.*, 2013; Selosse & Martos,
395 2014). Moreover, the similarity in $\delta^{13}C$ and $\delta^{15}N$ of the rhizoctonia-associated *S.*
396 *sikokianus* and the saprotrophic non-rhizoctonia mycorrhizal *G. nipponica*, along with
397 their respective pelotons, suggests that the fungal hosts of these two orchids utilize similar
398 substrates (i.e., decaying litter).

399 Overall, considering that (i) the high ^{13}C enrichments in *S. sikokianus* and some
400 albino mutants in the genera *Cypripedium*, *Goodyera*, and *Platanthera* resemble those
401 found in mycoheterotrophic orchids relying on saprotrophic fungi, (ii) saprotrophic non-
402 rhizoctonia fungi can meet the nutrient requirements of fully mycoheterotrophic orchids

(e.g., Ogura-Tsujita et al., 2009; Suetsugu et al., 2020), and (iii) the ^{13}C enrichment of *S. sikokianus* pelotons indicates that the fungi primarily behave as litter-decaying fungi, it is plausible that saprotrophic fungi, including rhizoctonia ones, effectively support the development of (nearly) fully mycoheterotrophic orchids during the adult stage. In contrast, rhizoctonias with the usual ecological niche may not efficiently support mycoheterotrophic growth in adulthood. This is also suggested by (i) the absence of significant ^{13}C enrichment in the leaves of rhizoctonia-associated orchids in the genera *Anoectochilus*, *Epipactis*, *Ophrys*, and *Orchis* despite a small but significant enrichment in their pelotons (Gomes *et al.*, 2023; Zahn *et al.*, 2023), and (ii) the lack of fully mycoheterotrophic orchids associated with putatively endophytic rhizoctonias (Suetsugu *et al.*, 2019, 2021a,c; Suetsugu & Matsubayashi, 2022).

^{13}C and ^{15}N enrichment in ECM rhizoctonia pelotons can surpass that in ECM basidiomycetous pelotons previously reported

Community profiling using metabarcoding techniques confirmed that the pelotons of *C. shikokiana* in our study site consistently consist of a Ceratobasidiaceae OTU closely related to the mycobionts of conspecific species (Yagame *et al.*, 2008), as well as other mycoheterotrophic orchids (Bougoure *et al.*, 2009; Yukawa *et al.*, 2009; Yagame *et al.*, 2012; Pecoraro *et al.*, 2020; Suetsugu & Matsubayashi, 2021) and ECM trees (Yagame *et al.*, 2008, 2012; Bougoure *et al.*, 2010).

We observed a high ^{13}C enrichment in the aboveground parts of *C. shikokiana* (8.2 ± 0.6‰ in flowers and 7.5 ± 0.7‰ in stems), which corresponds to the relatively high ^{13}C enrichment of pelotons extracted from *C. shikokiana* (6.9 ± 0.6‰). By comparing the

427 stable isotope data from *C. shikokiana* pelotons with published data from fungal fruit
428 bodies or pelotons composed of ECM fungi (Schiebold *et al.*, 2017; Zahn *et al.*, 2023),
429 we found that the observed high ^{13}C enrichment in *C. shikokiana* associated with ECM-
430 forming rhizoctonias is consistent with typical ^{13}C isotopic compositions within the same
431 ECM fungal guilds.

432 Remarkably, pelotons extracted from *C. shikokiana* exhibit significantly higher ^{15}N
433 enrichment (11.1 ± 0.3‰) compared to the fruit bodies of ECM Basidiomycota (5.2 ±
434 4.0‰) or ECM-type basidiomycetous pelotons (4.3 ± 1.1‰) reported previously
435 (Schiebold *et al.*, 2017; Zahn *et al.*, 2023). While ECM fungi generally display higher
436 ^{15}N enrichment than saprotrophic fungi (Mayor *et al.*, 2009), there are variations within
437 ectomycorrhizal fungi, probably due to the substrates they utilize (Gebauer & Taylor,
438 1999; Schiebold *et al.*, 2017). Previous studies have demonstrated a positive correlation
439 between ^{15}N enrichment and fungal soil depth exploitation or the recalcitrance of soil
440 organic matter (Natelhoffer & Fry, 1988; Gebauer & Schulze, 1991; Taylor *et al.*, 1997).
441 Thus, ECM-forming Ceratobasidiaceae fungi probably access compounds in deep soil
442 layers or recalcitrant soil organic matter.

443 The significant ^{15}N enrichment in pelotons impacts the extraordinarily high ^{15}N
444 enrichment in *C. shikokiana* itself (15.1 ± 0.8‰ in flowers and 12.6 ± 2.1‰ in stems).
445 Additionally, the average ^{15}N enrichment in *C. shikokiana* flowers compared to its
446 pelotons is 4.0 ± 0.6‰. This value even exceeds the typical increase of 2.2–3.4‰ in $\delta^{15}N$
447 observed in consumers relative to their diet in traditional food chain dynamics (Minagawa
448 & Wada, 1984; Zanden & Rasmussen, 2001; McCutchan Jr *et al.*, 2003). Leaves of some
449 ECM-exploiting orchids, including *Epipactis atrorubens*, *E. leptochila*, and
450 *Cephalanthera subaphylla*, also possess ^{15}N levels surpassing the usual increment of 2.2–

3.4‰ in $\delta^{15}N$ relative to the hyphae of ECM-associated orchids (Zahn *et al.*, 2023). In contrast, *G. nipponica* and *S. sikokianus*, associated with SAP fungi, show negligible or slightly elevated ^{15}N levels relative to the hyphae of these fungi. Therefore, while mycoheterotrophic plants associated with ECM fungi are believed to have higher $\delta^{15}N$ values than those associated with SAP fungi due to differing isotopic compositions of their mycobionts (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009; Hynson *et al.*, 2013; Lee *et al.*, 2015; Suetsugu *et al.*, 2020a), the variance in nutritional transport mechanisms between ECM and SAP fungi-associated mycoheterotrophs might also contribute to their distinct $\delta^{15}N$ values.

Furthermore, mycorrhizal morphology might influence the extent or pattern of ^{15}N enrichment from pelotons to orchid tissues. Given that fungal proteins and amino acids have approximately 10‰ higher $\delta^{15}N$ compared to fungal chitin (Taylor *et al.*, 1997; Hobbie & Colpaert, 2003), it has been proposed that the smaller ^{15}N enrichments in some orchid pelotons are attributed to the preferential incorporation of ^{15}N-enriched protein-derived hyphal cell content into the plant, while ^{15}N-depleted fungal cell wall chitin-N largely remains in the extracted pelotons (Zahn *et al.*, 2023). However, in species such as *G. nipponica* and *S. sikokianus*, where fungal hyphae are limited to a few cell layers and do not form extensive peloton clusters, the extracted pelotons likely reflect the original fungal isotopic composition more accurately. Investigating how this differential ^{15}N enrichment varies from pelotons to above-ground orchid tissues across different species remains an important area for future research.

Conclusion

Hydrogen isotopes have become a powerful tool for identifying mycoheterotrophic

nutrition and suggesting the widespread occurrence of partial mycoheterotrophy in rhizoctonia-associated orchids (Gebauer *et al.*, 2016; Fay *et al.*, 2018; Schiebold *et al.*, 2018; Gebauer & Clemens, 2021; Yagi *et al.*, 2024). Notably, Yagi et al. (2024) indicated that the heterogeneity of exchangeable hydrogens does not significantly impact the estimation of partial mycoheterotrophy. Nonetheless, the net carbon flow from fungus to plant in these orchids remains debated (Lallemand *et al.*, 2018; Jacquemyn *et al.*, 2021).

In contrast, our study clearly shows that *S. sikokianus* primarily derives its carbon from non-ECM rhizoctonias, elucidating the pattern of ^{13}C enrichment in both pelotons and leaves, which closely resembles that of other fully mycoheterotrophic orchids. Additionally, the ^{13}C enrichment of ECM rhizoctonia pelotons from *C. shikokiana* matches that of pelotons from fully mycoheterotrophic orchids associated with ECM non-rhizoctonia fungi. Our study underscores the diversity of the ^{13}C and ^{15}N enrichment patterns in rhizoctonias and rhizoctonia-associated orchids, highlighting the challenge of making generalized conclusions. This diversity can be attributed to several factors: (i) rhizoctonias encompass a phylogenetically heterogeneous group comprising Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae, (ii) these families exhibit a wide range of trophic modes, including saprotrophs, facultative plant pathogens, root endophytes, and even ectomycorrhizal fungi, and (iii) variances in plant physiological attributes, particularly the assimilation mechanisms for specific nutrients (Dearnaley *et al.*, 2012; Veldre *et al.*, 2013; Selosse & Martos, 2014; Jacquemyn *et al.*, 2021). Given that rhizoctonias are the most common fungal group involved in orchid mycorrhizas, it is crucial to understand the diversity of these interactions. Extracting pelotons from orchid roots offers a promising approach to resolve the controversy surrounding the nutritional modes of rhizoctonia-associated orchids, which could be applied more broadly to study

their nutritional modes.

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Author contributions

KS conceptualized and designed the study, collected materials, and wrote the initial draft of the manuscript. KS and JM conducted the laboratory experiments. KS, HO, and JM performed the analyses. HO and JM contributed to the improvement of the manuscript and approved the final version.

Competing interests

We declare we have no competing interests.

Data Availability Statement

The sequence data have been deposited in the DDBJ Sequence Read Archive (accession no. PRJDB8982). Additional supporting information is available online in the Supporting

Information section at the end of the article.

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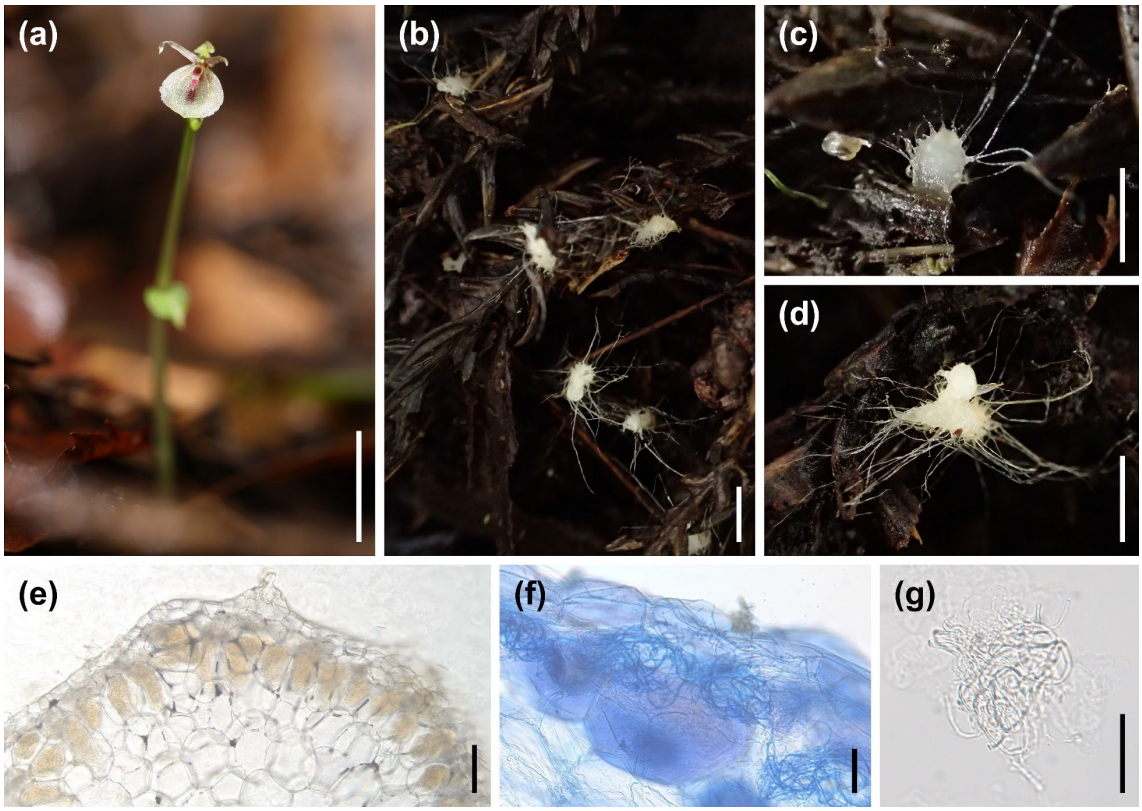
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777

778 **Figure legends**



779

780 **Fig. 1.** *Stigmatodactylus sikokianus* and its mycorrhizal interaction. (a) Flowering plant.
781 (b) Protocorms. (c) Initial protocorm. (d) Developed protocorm. (e) Cross-section of the
782 rhizome. (f) Close-up of rhizome cells with undegenerated and degenerated fungal coils
783 stained with trypan blue. (g) Fungal peloton released from a rhizome cell. Scale bars: 5
784 mm (a–b), 1 mm (c), 3 mm (d), 300 μm (e), and 50 μm (f–g).

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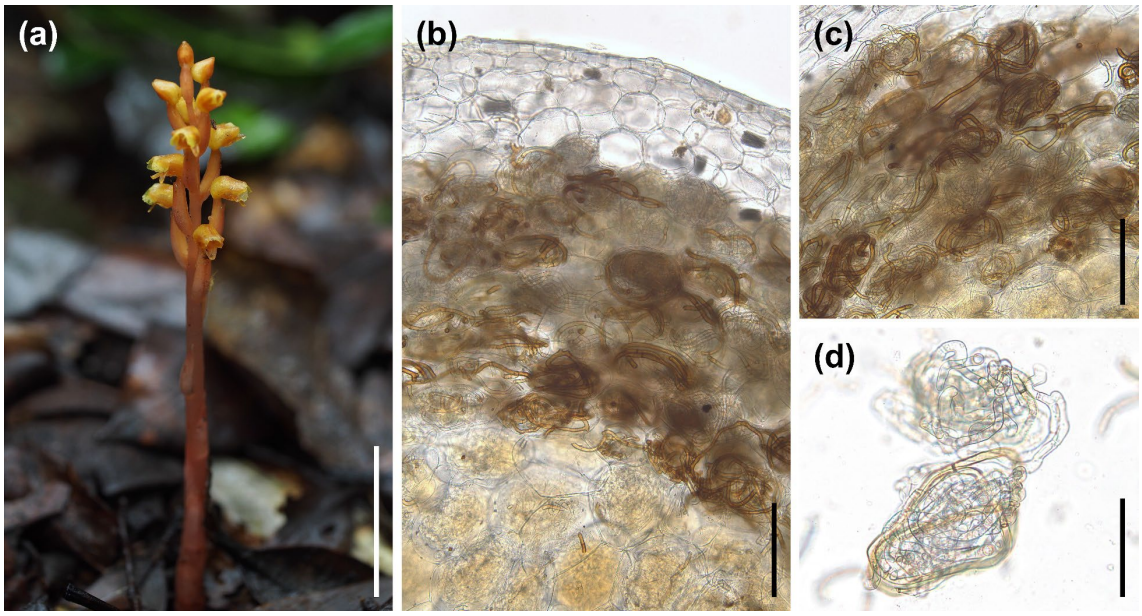
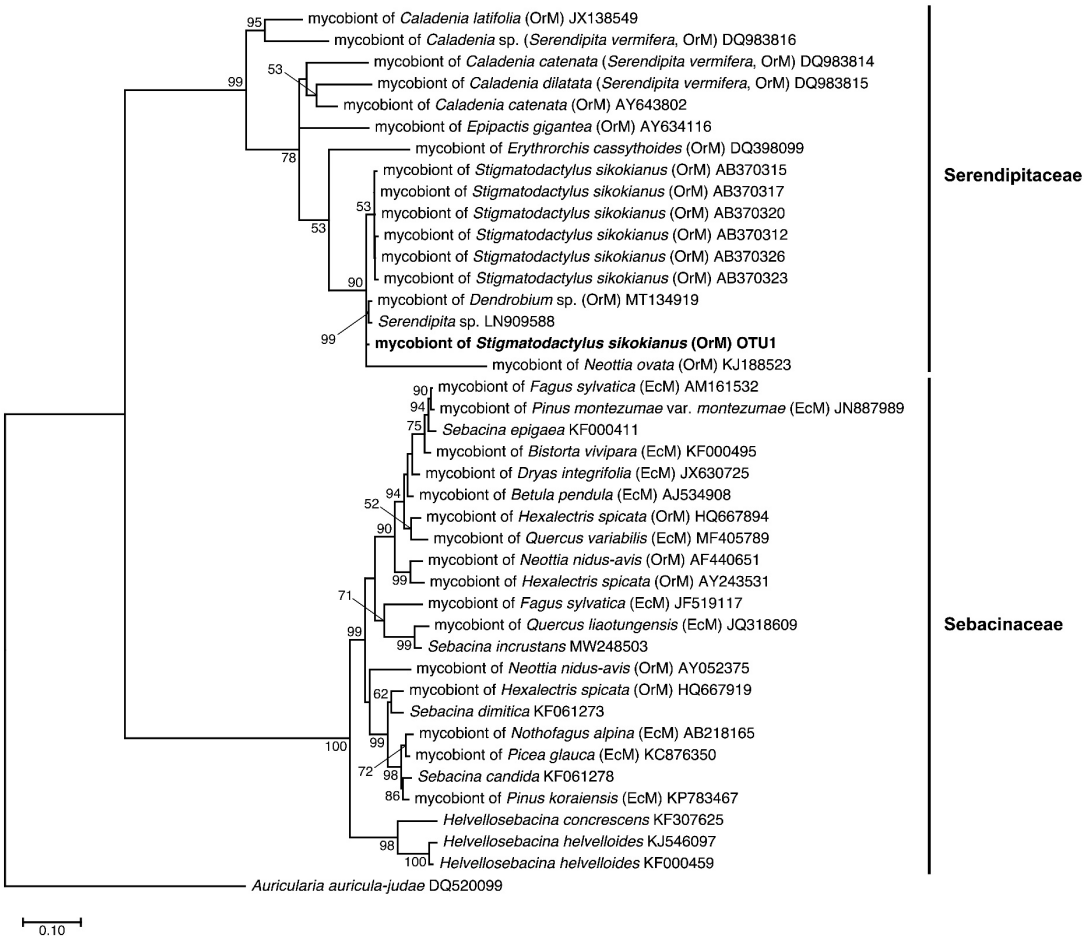


Fig. 2. *Chamaegastrodia shikokiana* and its mycorrhizal interaction. (a) Flowering plant. (b) Cross-section of the rhizome. (c) Close-up of rhizome cells with undegenerated fungal coils. (d) Fungal pelotons released from rhizome cells. Scale bars: 30 mm (a), 500 μm (b), 300 μm (c), and 100 μm (d).



802

803 **Fig. 3.** Maximum-likelihood phylogenetic tree of partial ITS rDNA sequences from
804 *Stigmatodactylus sikokianus*-associated fungus and their related taxa. Bold text indicates
805 the sequence obtained in the present study. Other sequences, with their respective
806 accession numbers, were obtained from the INSDC database. Node values indicate
807 bootstrap support (1000 replicates) of ≥50%. The scale bar indicates the number of
808 substitutions per site. OM, orchid mycorrhizal; ECM, ectomycorrhizal.

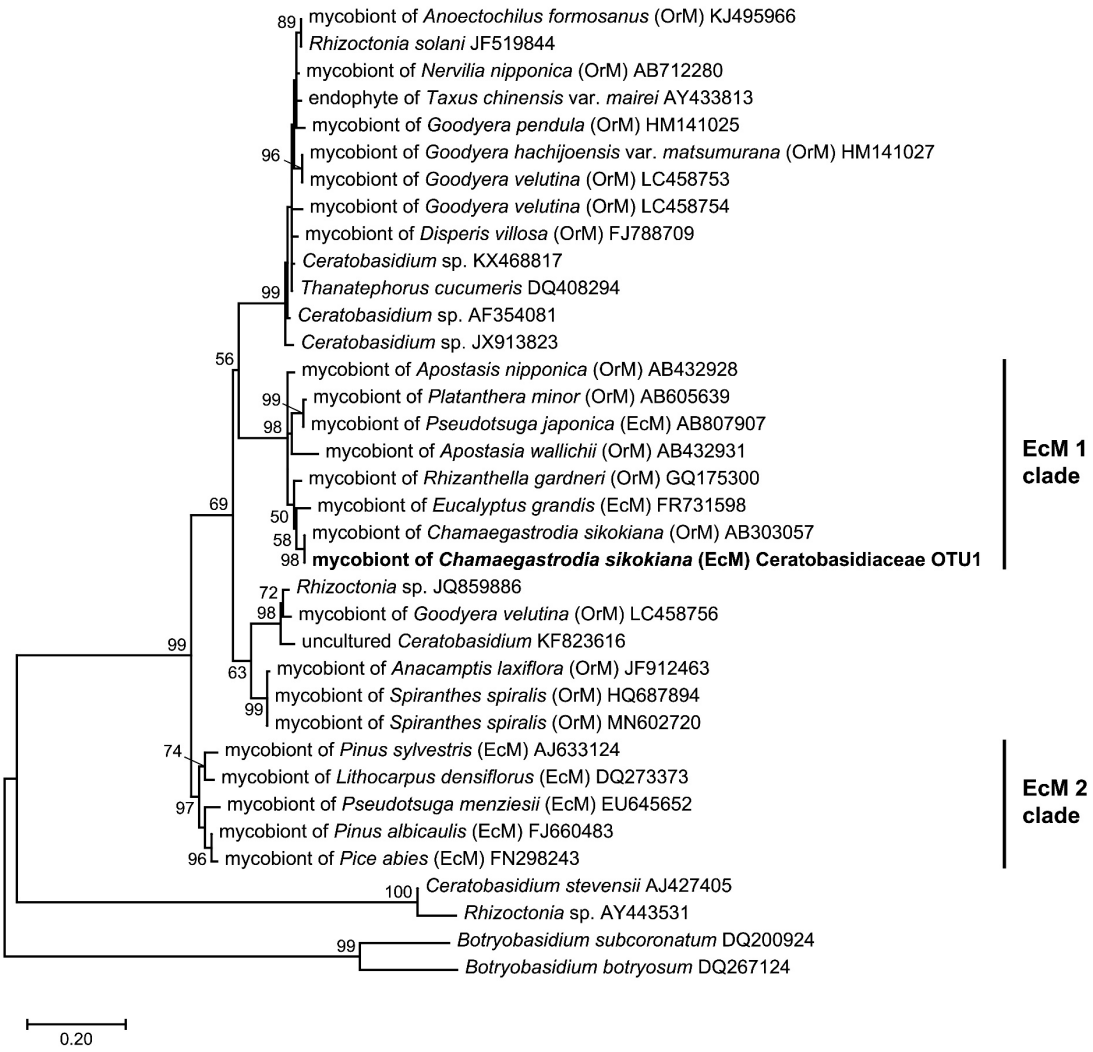
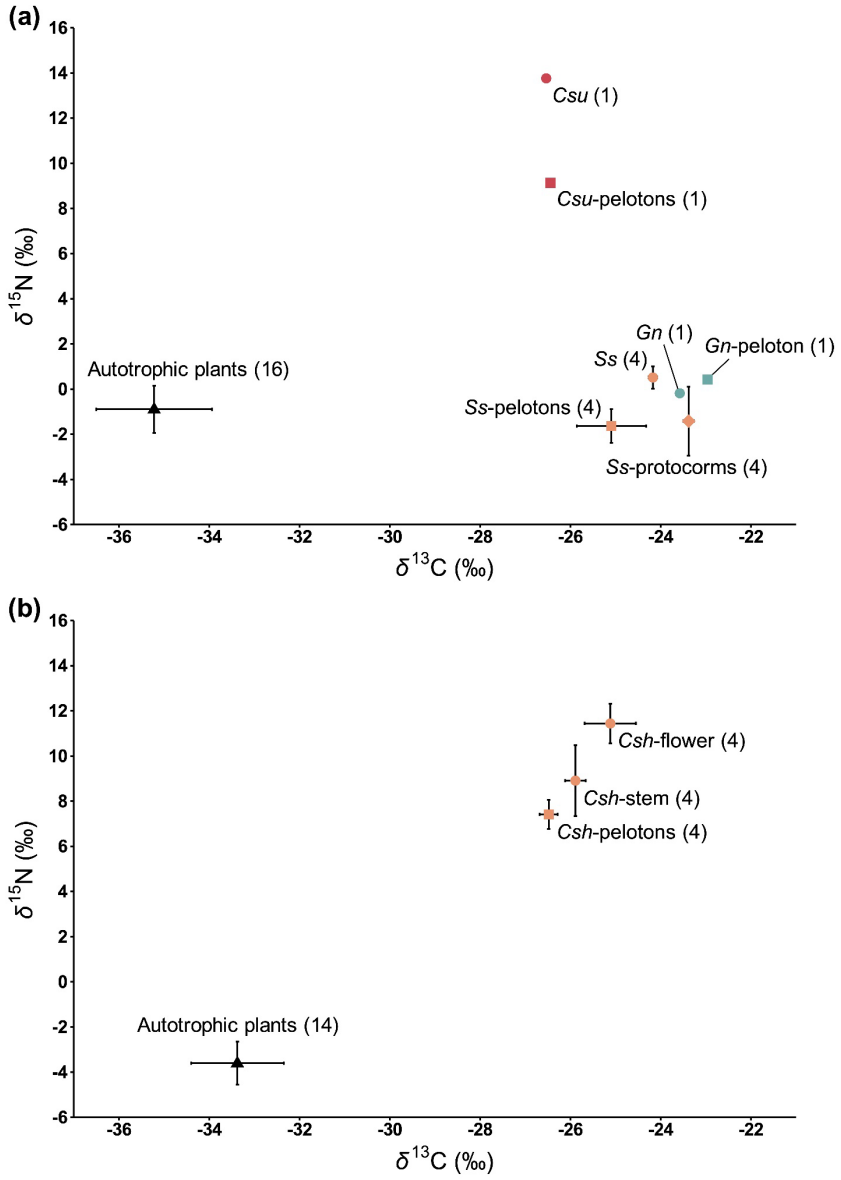


Fig. 4. Maximum-likelihood phylogenetic tree of partial ITS rDNA sequences from *Chamaegastrodia shikokiana*-associated fungus and its related taxa. Bold text indicates the sequence obtained in the present study. Other sequences, with their respective accession numbers, were obtained from the INSDC database. Node values indicate bootstrap support (1000 replicates) of ≥50%. The scale bar indicates the number of substitutions per site. OM, orchid mycorrhizal; ECM, ectomycorrhizal.



819

820 **Fig. 5.** (a) Mean (± SD) $\delta^{13}C$ and $\delta^{15}N$ values of *Stigmatodactylus sikokianus*, its pelotons,
821 its neighboring mycoheterotrophic orchids with their pelotons, and its neighboring
822 autotrophic reference plants. (b) Mean (± SD) $\delta^{13}C$ and $\delta^{15}N$ values of *Chamaegastrodia*
823 *shikokiana*, its pelotons, and its neighboring autotrophic reference plants. *Csu*:
824 *Cephalanthera subaphylla*. *Gn*: *Gastrodia nipponica. Ss*: *Stigmatodactylus sikokianus.*
825 *Csh*: *Chamaegastrodia shikokiana.*

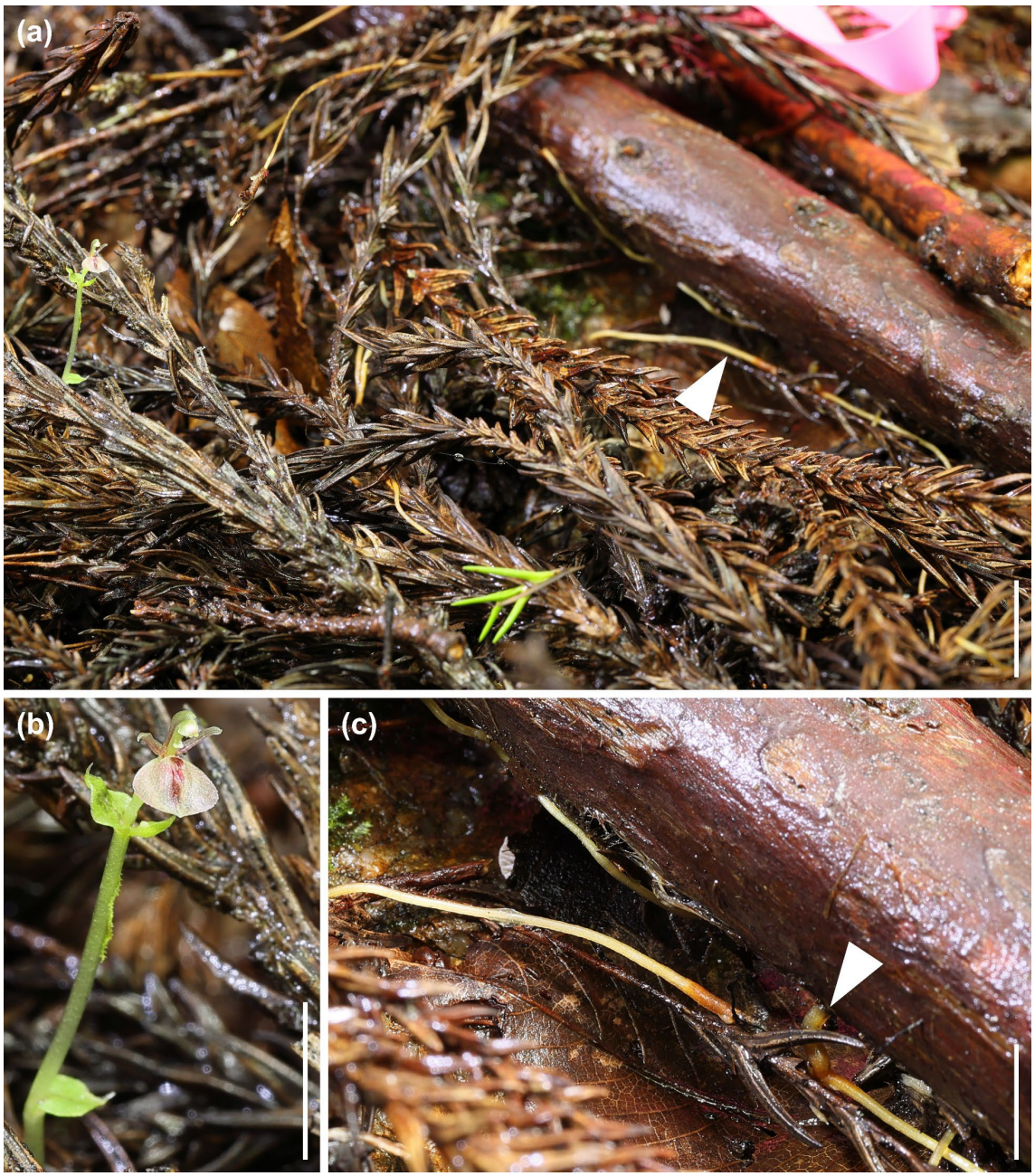


Fig. S1. *Stigmatodactylus sikokianus* and its co-occurring *Gastrodia nipponica*. (a) Flowering plant of *Stigmatodactylus sikokianus* and root of *Gastrodia nipponica*. The white arrow indicates *G. nipponica* root. (b) Close-up of *S. sikokianus* plant. (c) Close-up of *G. nipponica* root. The white arrow indicates mycorrhizal formation. Scale bars: 2 cm and 1 cm (b–c).

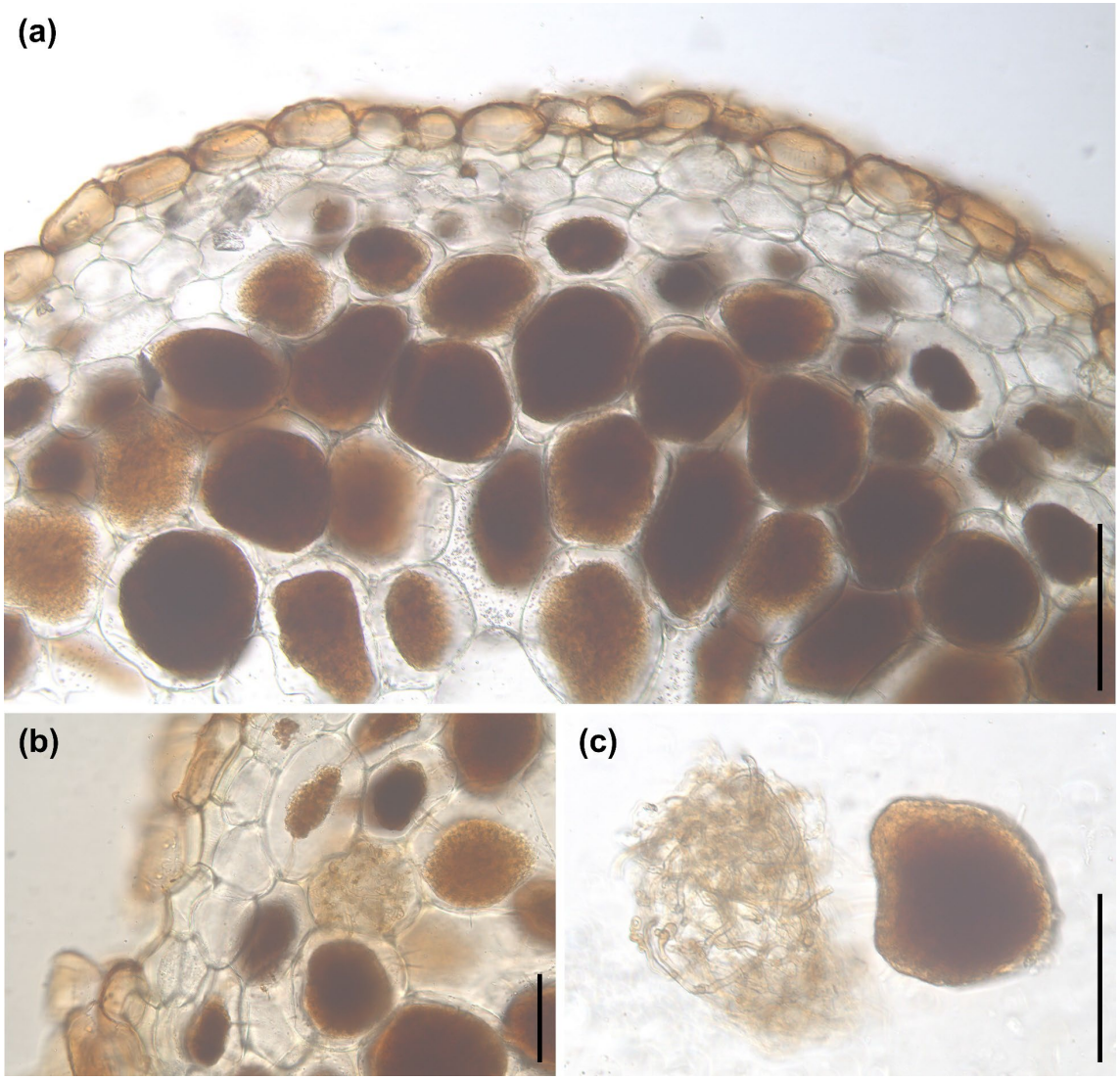


Fig. S2. Mycorrhizal interaction of *Cephalanthera subaphylla*. (a) Cross-section of the root. (b) Close-up of root cells with undegenerated and degenerated fungal coils. (c) Fungal pelotons released from root cells. Scale bars: 500 μm (a) and 200 μm (b–c).

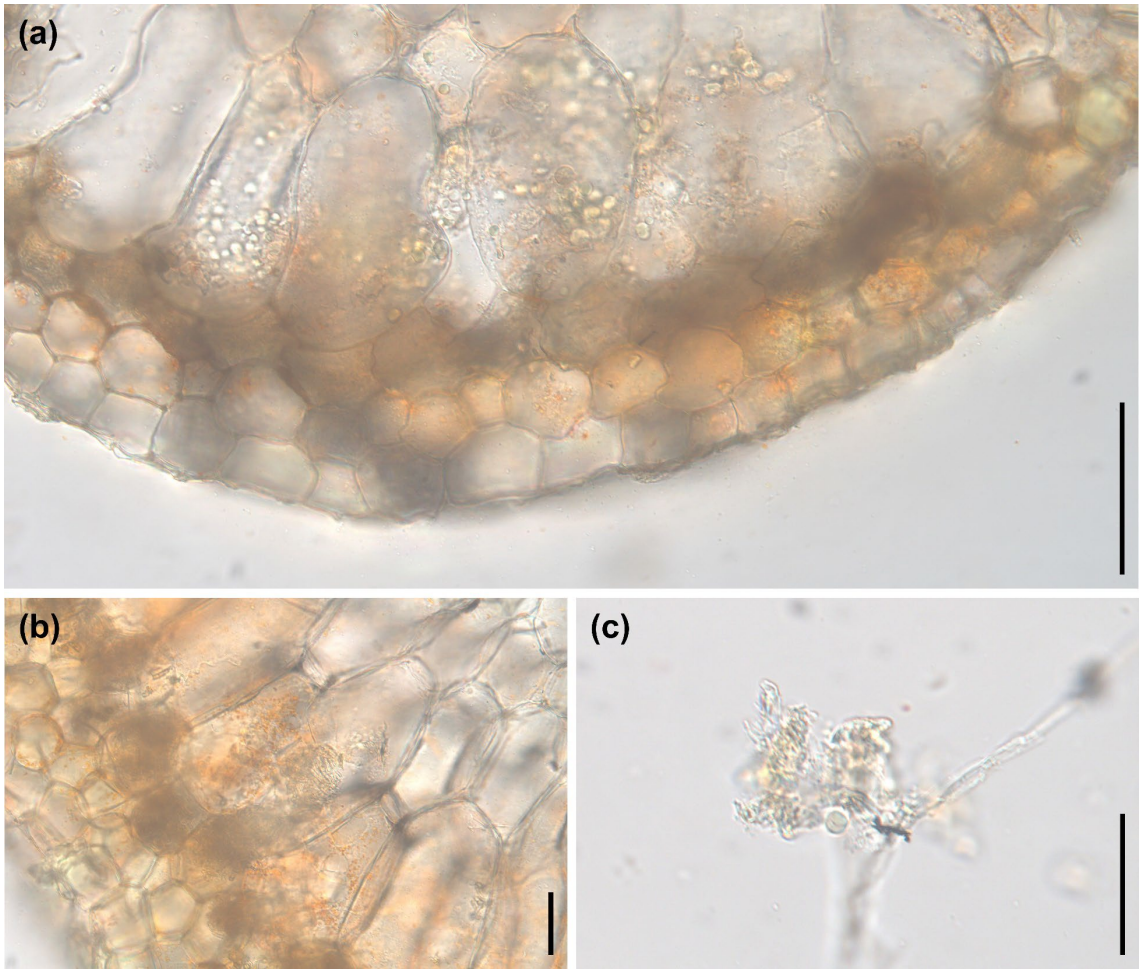


Fig. S3. Mycorrhizal interaction of *Gastrodia nipponica*. (a) Cross-section of the root. (b) Close-up of root cells with undegenerated and degenerated fungal coils. (c) Fungal peloton released from a root cell. Scale bars: 500 μm (a) and 100 μm (b–c).