Mycorrhizal specialization toward each distinct Oliveonia fungus in two closely related photosynthetic Dactylostalix orchids

Suetsugu, Kenji

Okada, Hidehito

(Citation)
Functional Ecology,39(1):199-208

(Issue Date)
2025-01

(Resource Type)
journal article

(Version)
Version of Record

Received: 20 June 2024 | Accepted: 15 October 2024

DOI: 10.1111/1365-2435.14690

Functional Ecology | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Mycorrhizal specialization toward each distinct *Oliveonia* fungus in two closely related photosynthetic *Dactylostalix* orchids

Kenji Suetsugu[1,2] | **Hidehito Okada**[1]

[1]Department of Biology, Graduate School of Science, Kobe University, Kobe, Japan

[2]Institute for Advanced Research, Kobe University, Kobe, Japan

Correspondence
Kenji Suetsugu
Email: kenji.suetsugu@gmail.com

Funding information
Precursory Research for Embryonic Science and Technology, Grant/Award Number: JPMJPR21D6; JSPS KAKENHI, Grant/Award Number: JP17H05016; Research Institute for Humanity and Nature

Handling Editor: Adam Frew

Abstract

1. Although rhizoctonias from Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae are typical orchid mycobionts, orchid mycorrhizal fungi exhibit vast taxonomic and ecological diversity. This diversity stems from the high specificity of orchid mycorrhizal associations and the remarkable diversity of over 28,000 orchid species. The subtribe Calypsoinae is particularly notable for its diverse mycorrhizal partnerships, including rhizoctonias, ectomycorrhizal and saprotrophic non-rhizoctonia fungi. However, the mycobionts within certain Calypsoinae lineages, such as the genus *Dactylostalix*, remain understudied. This study explores the physiological ecology of two photosynthetic Calypsoinae species, *Dactylostalix ringens* and *Dactylostalix uniflora*, to gain insight into potentially novel associations and their ecological implications.
2. We analysed the mycorrhizal communities of both *Dactylostalix* species using high-throughput ITS metabarcoding of root samples collected from multiple locations. Additionally, we measured the natural abundances of ^{13}C and ^{15}N isotopes in the leaves of the two *Dactylostalix* species and their co-occurring autotrophic reference plants, as well as in fungal pelotons isolated from *D. ringens*, to assess the potential for partial mycoheterotrophy.
3. Our findings revealed that *D. ringens* and *D. uniflora* form specialized mycorrhizal associations predominantly with distinct lineages of *Oliveonia* (Oliveoniaceae, Auriculariales), even in sympatric populations. Stable isotope analysis showed that both *Dactylostalix* species exhibited conflicting isotopic signals: elevated $\delta^{15}N$ values, supporting partial mycoheterotrophy, but lower $\delta^{13}C$ values compared to autotrophic plants, suggesting autotrophy. Peloton samples from *D. ringens* displayed only modest ^{13}C enrichment relative to autotrophic references.
4. These conflicting isotopic signals make it difficult to precisely determine whether both *Dactylostalix* species are autotrophic or partially mycoheterotrophic. Intriguingly, the ^{13}C and ^{15}N signatures of *Dactylostalix* species and their pelotons resemble those of many rhizoctonia-associated orchids. This isotopic evidence

implies a niche overlap with endophytic tendencies between rhizoctonias and *Oliveonia*, suggesting that potential endophytic traits may have facilitated the recruitment of *Oliveonia* as novel mycorrhizal partners. Furthermore, the mycorrhizal segregation between *D. ringens* and *D. uniflora* likely promotes their sympatric coexistence and may contribute to reproductive isolation through ecological specialization.

KEYWORDS
^{13}C and ^{15}N abundances, Auriculariales, Calypsoinae, mycoheterotrophy, mycorrhizal associations, mycorrhizal segregation, niche differentiation, stable isotopes

1 | INTRODUCTION

The family Orchidaceae is diverse, with over 28,000 recognized species, making it the most species-rich plant family (Christenhusz & Byng, 2016). A defining feature of this family is initial mycoheterotrophy, where all orchids rely on fungal-derived carbon for the germination of their minute seeds (Leake, 1994; Merckx, 2013). This reliance on fungi is considered a key evolutionary event for orchids, coinciding with a shift in mycobionts from Glomeromycota to Basidiomycota (Kristiansen et al., 2001, 2004; Yukawa et al., 2009).

Although many orchid species likely transition to full autotrophy in adulthood (initial mycoheterotrophy sensu Merckx, 2013), some green orchids continue to obtain carbon from both photosynthesis and fungi (partial mycoheterotrophy or mixotrophy) (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Hynson et al., 2013; Selosse & Roy, 2009). Additionally, over 250 non-photosynthetic orchid species remain fully mycoheterotrophic throughout adulthood (Jacquemyn & Merckx, 2019; Merckx, 2013). Although partial and full mycoheterotrophy occur in other plant families, no other group exhibits such frequent shifts in trophic modes as the Orchidaceae (Jacquemyn & Merckx, 2019; Merckx, 2013).

Historically, rhizoctonias, a polyphyletic group of putatively saprotrophic Basidiomycetes including Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae, have been considered the primary fungal symbionts of orchids (Bidartondo et al., 2004; Dearnaley et al., 2012; Warcup & Talbot, 1967). However, accumulating research has revealed a broader spectrum of fungal partners, including ectomycorrhizal (ECM) and saprotrophic non-rhizoctonia fungi (Bidartondo et al., 2004; Hynson et al., 2013; Jacquemyn & Merckx, 2019; Ogura-Tsujita et al., 2009). Notably, fully mycoheterotrophic orchids or partially mycoheterotrophic orchids with a high degree of heterotrophy primarily associate with ECM or wood- and litter-decaying fungi, while rhizoctonias are typically linked to initially mycoheterotrophic species or partially mycoheterotrophic orchids with a low degree of heterotrophy (Bidartondo et al., 2004; Gebauer et al., 2016; Ogura-Tsujita et al., 2012; Suetsugu & Matsubayashi, 2021a; Yagame et al., 2016). Overall, orchid mycorrhizal fungi exhibit species diversity comparable to that of ECM fungi, largely due to the typically high specificity of orchid mycorrhizal associations and the vast diversity of orchids themselves (Wang et al., 2022).

The subtribe Calypsoinae (Epidendroideae, Orchidaceae), a primarily temperate group comprising 13 genera and around 80 species, includes approximately 20 mycoheterotrophic species (Chase et al., 2015; Freudenstein et al., 2017). Phylogenetic analysis of plastid matK and nuclear rDNA ITS regions has shown that full mycoheterotrophy has evolved at least four times within this subtribe (Freudenstein et al., 2017). Members of Calypsoinae have become a model group for studying mycorrhizal partnerships and specificity, due to their diverse mycorrhizal relationships, which include rhizoctonias, ECM fungi, and saprotrophic non-rhizoctonia fungi (Barrett et al., 2010; Barrett & Freudenstein, 2011; Freudenstein & Barrett, 2014; McCormick et al., 2004; Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021b; Yagame et al., 2021).

Freudenstein et al. (2017) identified two major clades within Calypsoinae: the *Calypso* clade and the *Corallorhiza* clade, with three subgroups within the *Calypso* clade: (i) *Dactylostalix* + *Ephippianthus*, (ii) *Calypso* + *Yoania* and (iii) *Changnienia* + *Tipularia*. *Dactylostalix* and *Ephippianthus* are likely sister taxa to the other subclades, although the exact placement of *Dactylostalix* + *Ephippianthus* remains somewhat unresolved (Freudenstein et al., 2017). An interesting aspect of the *Calypso* clade mycorrhizal associations is that *Calypso bulbosa* primarily associates with *Protomerulius* (Auriculariales, Basidiomycota) (Suetsugu & Matsubayashi, 2021b), and *Tipularia discolor* can associate with both Tulasnellaceae and two Auriculariales clades (McCormick et al., 2004, 2021). Consequently, although Auriculariales fungi, generally wood-decaying fungi, are seldom considered mycobionts of green orchids, they may represent the ancestral mycobionts within the *Calypso* clade. However, the mycorrhizal associations of *Dactylostalix* and *Ephippianthus*, both of which are endemic to Japan, the Kuril Islands, and Sakhalin Island and likely diverged early in the *Calypso* clade (Freudenstein et al., 2017), remain unexplored.

Although *Dactylostalix* has long been considered a monotypic genus (Freudenstein, 1994; Freudenstein et al., 2005), a second species, *D. uniflora*, was recently recognized based on morphological and phylogenetic data (Suetsugu, Hirota, et al., 2024). *Dactylostalix ringens* thrives in both subarctic and cool-temperate forests, while *D. uniflora* is restricted to subarctic regions, particularly in high-elevation areas of Honshu and Hokkaido. Even in areas where they coexist, *D. ringens* generally grows at lower

elevations than *D. uniflora*. Given the frequent habitat partitioning between these species (Suetsugu, Hirota, et al., 2024) and the high mycorrhizal specificity observed in some orchids, including green species (Freudenstein & Barrett, 2014; Shefferson et al., 2005), it is possible that these two *Dactylostalix* species host distinct mycorrhizal communities.

Accordingly, we investigated the fungal partners of the two *Dactylostalix* species to determine whether these species (i) interact with rhizoctonias, which are widely recognized as typical orchid mycorrhizal fungi (Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae), or Auriculariales fungi, and (ii) exhibit mycorrhizal segregation between the two species. Since ^{13}C and ^{15}N isotopic abundances have traditionally been used to infer the nutritional modes of green orchids, particularly those associated with ECM or saprotrophic non-rhizoctonia fungi (e.g. Bidartondo et al., 2004; Gebauer & Meyer, 2003; Julou et al., 2005), we analysed the isotopic compositions of *Dactylostalix* leaves and pelotons to assess the potential for partial mycoheterotrophy in these species.

2 | MATERIALS AND METHODS

2.1 | Study species and sampling locality

Dactylostalix ringens and *D. uniflora* are terrestrial orchids found in the shaded understory of cool-temperate and subarctic forests in Japan, the Kuril Islands, and Sakhalin Island (Figure 1) (Freudenstein, 1994; Freudenstein et al., 2005). These species share key traits, including a solitary flower, a pollinarium with four superposed pollinia, a viscidium with a rudimentary stipe, the absence of a corm or pseudobulb, and a flat, non-plicate leaf (Freudenstein, 1994; Freudenstein et al., 2005). However, *D. uniflora* differs from *D. ringens* in having a shorter scape, a smaller flower, fewer spots on the tepals, drooping sepals and lateral petals, and a labellum with smaller lateral lobes (Figure 2) (Suetsugu, Hirota, et al., 2024).

To minimize the influence of site-specific effects, we conducted our study at multiple locations, including a site where both species grow nearby (Mt. Fuji). Rhizomes of *Dactylostalix ringens* were collected from 32 plants across five populations between May 2012 and June 2023, while rhizomes of *D. uniflora* were collected from 17 plants across two populations between September 2021 and July 2023 (Table S1).

2.2 | Molecular identification of mycobionts

Rhizomes were excised and examined under a light microscope to confirm the presence of mycorrhizal colonization. Mycorrhizal fragments containing fungal pelotons, ranging from 3 to 5 mm in length, were extracted from each sample for molecular analysis. After surface sterilization to eliminate contaminants, DNA was extracted using the cetyltrimethylammonium bromide method (Doyle & Doyle, 1990).

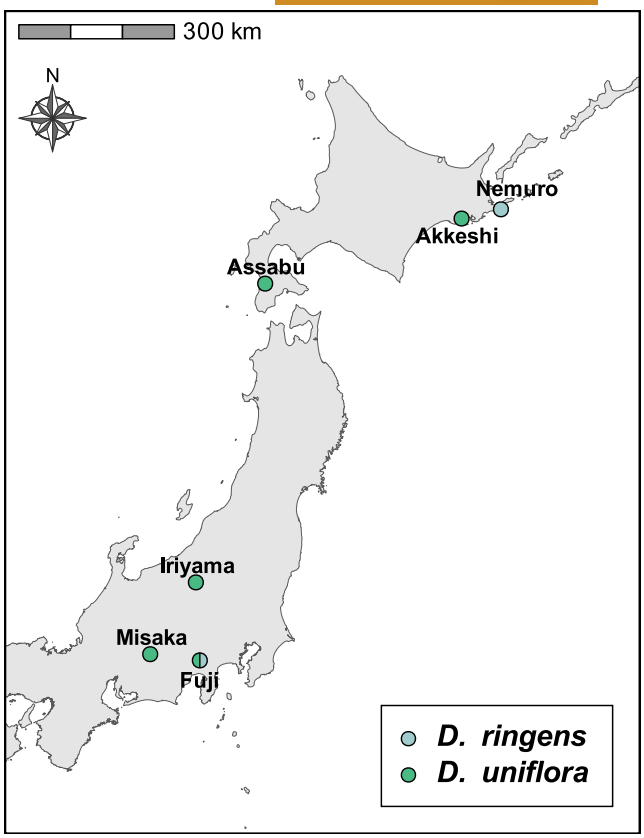

FIGURE 1 Map showing the sampling localities of *Dactylostalix ringens* and *D. uniflora*.

The ITS region sequences of the mycorrhizal fungi were amplified using the ITS86F/ITS4 primer set, which is suitable for analysing fungal communities in orchid roots (Waud et al., 2014). Primers were fused with 3–6-mer Ns and Illumina forward/reverse sequencing primers, following the method outlined by Suetsugu et al. (2021). PCR amplification was performed using the Q5 High-Fidelity DNA Polymerase kit under the following conditions: an initial denaturation at 98°C for 40 s, followed by 35 cycles of 98°C for 5 s, 58°C for 10 s and 72°C for 20 s, with a final extension at 72°C for 10 min. A supplemental PCR was conducted to add Illumina P5/P7 adapter sequences and sample-specific indices (Suetsugu & Matsubayashi, 2021a; Syed et al., 2009). Supplemental PCR conditions included an initial denaturation at 98°C for 40 s, followed by 12 cycles of 98°C for 5 s, 65°C for 10 s, and 72°C for 20 s, with a final extension at 72°C for 10 min. The pooled library was sequenced using the Illumina MiSeq system with the MiSeq Reagent Micro Kit v2 (300 cycles). The sequence data have been deposited in the NCBI Sequence Read Archive (SRA accession number PRJNA1160459).

After sequencing, bioinformatic analysis was performed using Claident v0.9.2020.12.06 (Tanabe & Toju, 2013), as described by Suetsugu and Okada (2021). Briefly, primer regions and low-quality reads were removed, and erroneous sequences were denoised using DADA2 (Callahan et al., 2016) implemented in Claident. Sequences potentially resulting from PCR chimera formation or index-hopping were excluded using the clremovechimev and clremovecontam

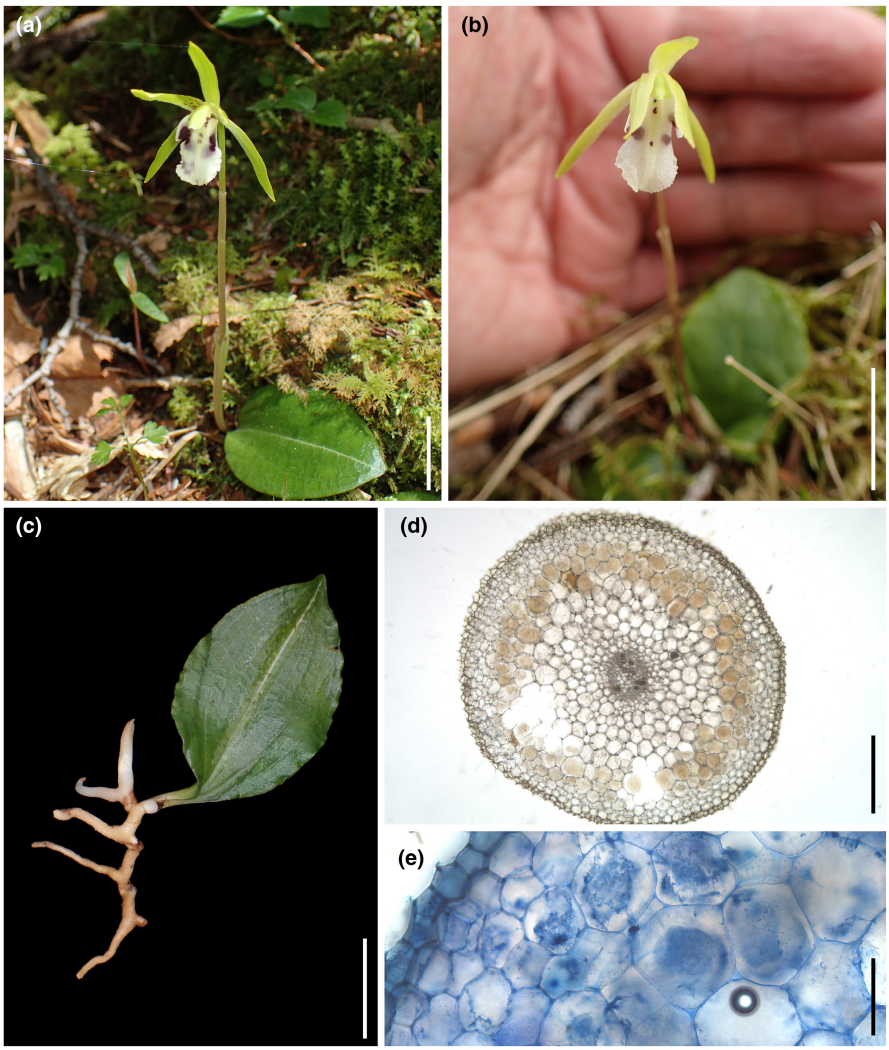

FIGURE 2 Two *Dactylostalix* species and their mycorrhizal interactions. (a) Flowering *D. ringens* plant. (b) Flowering *D. uniflora* plant. (c) *D. ringens* habit, including an underground system primarily composed of a rhizome. (d) Cross-section of the *D. ringens* rhizome. (e) Close-up of *D. ringens* rhizome cells with undegenerated and degenerated fungal coils stained with trypan blue. Scale bars: 20 mm (a, b), 30 mm (c), 500 μm (d) and 100 μm (e).

commands (Esling et al., 2015; Nilsson et al., 2019). Remaining sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold using VSEARCH v2.8.0 (Rognes et al., 2016), implemented in the clclassseqv command. The most abundant sequence in each OTU cluster was selected as the representative sequence.

Since all *Dactylostalix* specimens were primarily colonized by OTUs within the family Oliveoniaceae, we constructed a phylogenetic tree that included the detected Oliveoniaceae OTUs and closely related fungi to further explore their relationships. OTUs identified as *Dactylostalix* mycobionts were subjected to a BLAST search against the International Nucleotide Sequence Database Collaboration (INSDC) for comparison (Altschul et al., 1997). Several phylogenetically close sequences, along with representative sequences from the family Oliveoniaceae, were downloaded.

Sequences were aligned using MAFFT v7.475 (Katoh & Standley, 2013) with the linsi option, and a maximum-likelihood phylogenetic tree was constructed using IQ-TREE 1.6.12 (Nguyen et al., 2015). The best-fit substitution model (TIM2+F+G4) was selected by ModelFinder (Kalyaanamoorthy et al., 2017) based on AIC. Branch support was calculated using the Shimodaira–Hasegawa-like procedure (SH-aLRT) and ultrafast bootstrapping (UFboot) with 1000 replicates. Nodes with SH-aLRT values ≥80% and ultrafast bootstrap values ≥95% were considered to have strong support.

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

Partially mycoheterotrophic orchids, particularly those exploiting ECM fungi or saprotrophic non-rhizoctonia fungi, typically exhibit higher $\delta^{13}C$ and $\delta^{15}N$ values than co-occurring autotrophic plants, reflecting the enriched isotopic composition of their fungal hosts (Gomes et al., 2023; Zahn et al., 2023). To infer the nutritional mode of *Dactylostalix* species, we conducted $\delta^{13}C$ and $\delta^{15}N$ analyses on *D. ringens*, *D. uniflora*, and their co-occurring autotrophic plants. Four 2 m × 2 m quadrats containing *D. ringens* and *D. uniflora* were established in Awakura, Fujinomiya City, Shizuoka Prefecture, Japan, on June 15, 2021. This location was selected as a rare site where both species coexist sympatrically.

Leaf samples were collected from eight individuals of *D. ringens* and three individuals of *D. uniflora*. Additionally, leaves from *Maianthemum dilatatum*, *Leucosceptrum japonicum* f. *barvinerve*,

Pternopetalum tanakae and *Parasenecio adenostyloides* ($n=4$ for each species) were collected across the quadrats. These species were selected as autotrophic reference plants due to their occurrence at the same canopy height as the *Dactylostalix* plants. Since the estimation of the nutritional mode in green orchids can be refined through isotopic analysis of mycobionts (Gomes et al., 2023; Julou et al., 2005; Zahn et al., 2023), we also extracted pelotons from the rhizomes of three *D. ringens* individuals, following the methods of Gomes et al. (2023) and Zahn et al. (2023). The leaves and fungal pelotons were desiccated at 60°C and finely ground using an agate mortar.

The natural abundances of ^{13}C and ^{15}N isotopes in the samples were measured using a continuous-flow isotope-ratio mass spectrometer coupled to an elemental analyser (Thermo Fisher Scientific, Waltham, MA, USA), following the protocol outlined by Suetsugu and Matsubayashi (2021b).

Isotope abundances were calculated using the formula:

$$\delta^{13}C \text{ or } \delta^{15}N = \left(R_{sample} / R_{standard} - 1\right) \times 1000 \left[‰\right],$$

where R_{sample} represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio in each sample, and $R_{standard}$ represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios of Vienna PeeDee Belemnite or atmospheric N_2, respectively. Calibration of the C and N isotope ratios was performed using laboratory standards: CERKU-03 (glycine, $\delta^{13}C=-34.92‰$, $\delta^{15}N=2.18‰$) and CERKU-05 (threonine, $\delta^{13}C=-9.45‰$, $\delta^{15}N=-2.88‰$) (Tayasu et al., 2011). Analytical standard deviations (SDs) from repeated measurements of these standards were less than 0.06‰ for $\delta^{13}C$ ($n=54$) and 0.11‰ for $\delta^{15}N$ ($n=53$).

We compared the $\delta^{13}C$ and $\delta^{15}N$ values among groups using a linear mixed model, with 'identity' as a fixed effect and 'plot' as a random effect. A post hoc Tukey–Kramer test was used to assess significant differences in $\delta^{13}C$ and $\delta^{15}N$ between *Dactylostalix* leaves, *Dactylostalix* fungal pelotons, and autotrophic reference plants.

3 | RESULTS

3.1 | Molecular identification of mycobionts

In our analysis of the fungal community, the majority of ITS sequences from the mycobionts of both *D. ringens* and *D. uniflora* were assigned to *Oliveonia* species (four OTUs, 1,308,554 reads; 98.4% of all reads) (Dataset S1, Suetsugu & Okada, 2024). Notably, all *D. ringens* individuals predominantly harboured the same *Oliveonia* OTU, identified as *Oliveonia* OTU1, which accounted for 806,523 reads, representing 99.4% of all *D. ringens* sequences. This predominant association between *D. ringens* and *Oliveonia* OTU1 across multiple populations, spanning distances over 1000 km, highlights the specialized nature of this symbiotic relationship. The other OTUs, given their minimal read counts, are likely opportunistic fungi with no substantial role in *D. ringens*.

Similarly, all individuals of *D. uniflora* were primarily colonized by a distinct *Oliveonia* OTU, *Oliveonia* OTU2, which accounted for 501,986 reads, or 96.9% of all *D. uniflora* sequences. This consistent association was observed in both Awakura and Nemuro, despite their geographical separation by over 1000 km. Notably, *Oliveonia* OTU1, which dominates in *D. ringens*, was absent from all *D. uniflora* individuals, and conversely, *Oliveonia* OTU2 was absent from *D. ringens*, even at the sympatric Awakura site (Figure 3). Phylogenetic analysis indicated that the mycobionts of *D. ringens* and *D. uniflora* form a well-supported clade with sporadic mycobionts of green orchids from the genera *Dendrobium*, *Epipactis* and *Pleione* (Figure 4).

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

The foliar $\delta^{13}C$ values of *D. ringens* (−33.5 ± 1.8‰) did not significantly differ from those of the autotrophic reference plants (−32.8 ± 0.7‰, $p=0.51$). However, the $\delta^{13}C$ values of *D. uniflora* (−35.0 ± 1.1‰) were significantly lower than those of the autotrophic reference plants ($p<0.05$; Figure 5; Table S2). In contrast, the foliar $\delta^{15}N$ values of both *D. ringens* (−1.3 ± 0.4‰) and *D. uniflora* (−3.8 ± 0.6‰) were significantly higher than those of the autotrophic reference plants (−5.7 ± 0.9‰, $p<0.01$ for both). Additionally, a significant difference in $\delta^{15}N$ values was observed between *D. ringens* and *D. uniflora* ($p<0.05$), highlighting the distinct ^{15}N enrichment patterns in these species.

Meanwhile, the $\delta^{13}C$ values of *D. ringens* fungal pelotons (−30.0 ± 1.6‰) were significantly higher than those of both the autotrophic reference plants and *D. ringens* leaves ($p<0.01$). Conversely, although the $\delta^{15}N$ values of *D. ringens* fungal pelotons (−1.8 ± 4.1‰) were significantly higher than those of the autotrophic reference plants ($p<0.001$), no statistically significant difference in $\delta^{15}N$ values was observed between *D. ringens* pelotons and *D. ringens* leaves ($p=0.97$).

4 | DISCUSSION

Our fungal community profiling through metabarcoding revealed a dominant association between both *Dactylostalix* species and the genus *Oliveonia* (Oliveoniaceae, Auriculariales, Basidiomycota), distinct from rhizoctonia and ECM fungi. This newly identified partnership with *Oliveonia* fungi broadens our understanding of the diversity of orchid mycobionts. Auriculariales fungi, typically known as wood decomposers, are widespread across tropical to subarctic regions (Malysheva & Spirin, 2017). The position of the mycobiont among potential saprophytes suggests physiological flexibility between saprotrophism and symbiosis, a probable common trait of orchid mycobionts (Kottke et al., 2010; Selosse et al., 2022).

Intriguingly, saprotrophic fungi, excluding rhizoctonia fungi from the families Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae, have sporadically been found in the roots of photosynthetic orchids (e.g. Marasmiaceae spp. in *Cremastra variabilis*) as facultative biotrophs or endophytes (Jacquemyn & Merckx, 2019; Selosse & Cameron, 2010; Selosse & Martos, 2014; Yagame et al., 2021). It has also been suggested that the evolutionary pathway from

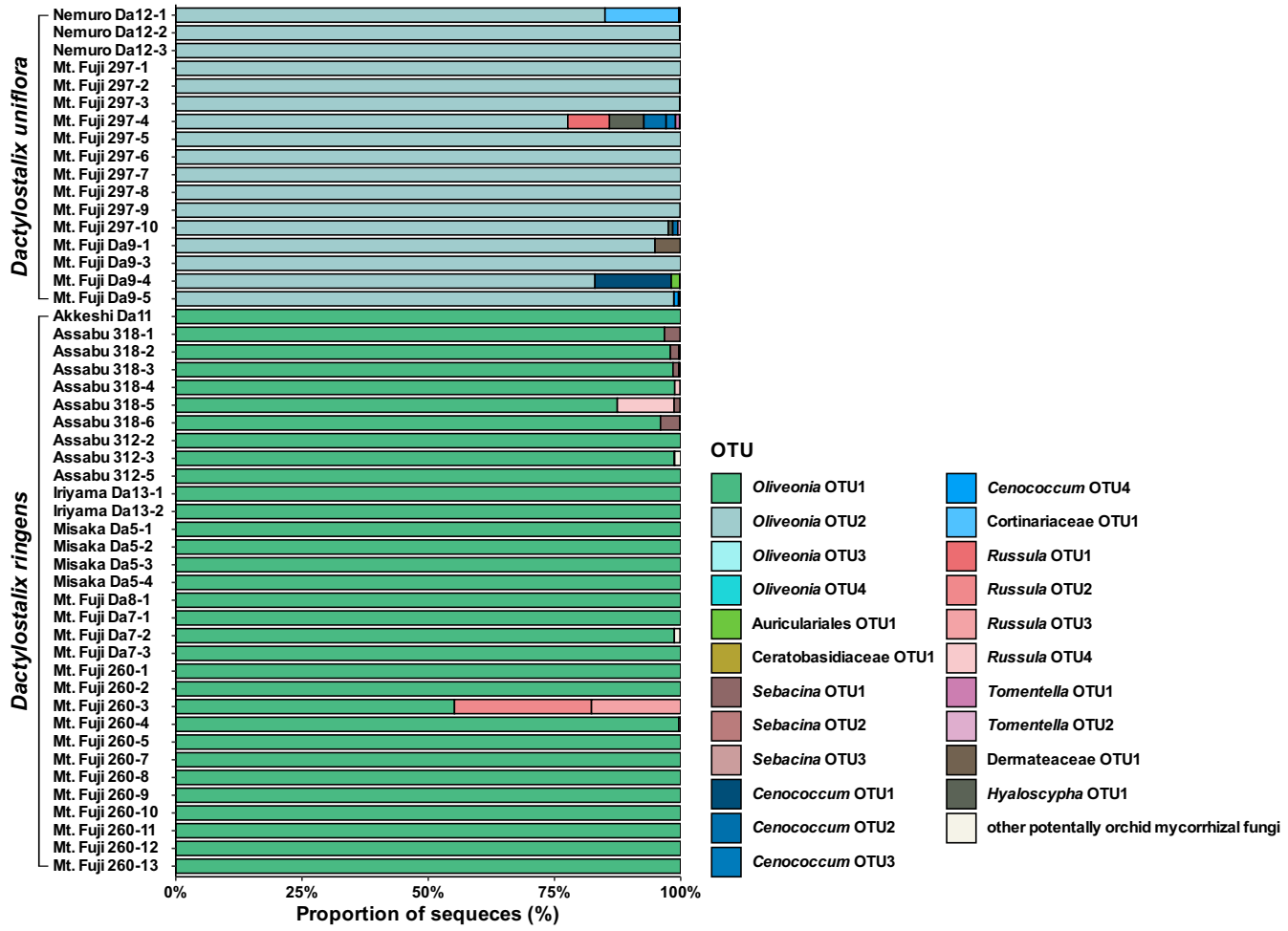

FIGURE 3 Relative abundance of mycorrhizal communities associated with *Dactylostalix ringens* and *D. uniflora*, at the operational taxonomic unit (OTU) level.

saprotrophism to endophytism, and eventually to mycorrhizal association, is common (Martino et al., 2018; Selosse et al., 2018, 2022; Selosse & Cameron, 2010; Thoen et al., 2020). Notably, some members of the Auriculariales, including *Oliveonia* species, have been identified as incidental mycobionts in green orchids (Ogura-Tsujita et al., 2012; Qin et al., 2019; Yao et al., 2022), indicating a predisposition for such associations. Considering that closely related species such as *Calypso bulbosa* and *Tipularia discolor* also associate with other members of the Auriculariales, the interaction with Auriculariales fungi may even represent an ancestral trait within the *Calypso* clade.

Our metabarcoding data also revealed that *D. ringens* consistently associates with *Oliveonia* OTU1, even at a site where *D. uniflora*, which is linked to *Oliveonia* OTU2, occurs nearby. Thus, we can confidently conclude that these two species harbour different *Oliveonia* OTUs with high specificity. Combined with recent findings (Suetsugu, Hirota, et al., 2024), it is now clear that *D. ringens* and *D. uniflora* are not only morphologically distinct but also represent ecologically distinct lineages.

Our findings suggest that differences in mycorrhizal communities play a role in niche partitioning, facilitating the coexistence of *D. ringens* and *D. uniflora*. This hypothesis is supported by classical ecological theory, which predicts that two species competing for the same resources cannot coexist stably (Gause, 1934). Previous research has shown that sympatric orchid species often possess distinct mycorrhizal communities and exhibit spatial segregation, even when sharing certain OTUs (Jacquemyn et al., 2014; McCormick & Jacquemyn, 2014; Taylor & Bruns, 1999). These results align with ours, indicating that niche differentiation through mycorrhizal segregation contributes to sympatric coexistence in orchids. One possible mechanism facilitating coexistence in species with different mycorrhizal associations involves access to different resources mediated by distinct fungal taxa (Waterman et al., 2011). This is indirectly supported by the ^{15}N abundances of *D. ringens* and *D. uniflora*, which suggest that both species utilize different nitrogen sources.

Moreover, specialization on different fungal hosts may promote reproductive isolation between *D. ringens* and *D. uniflora*. Host-shift speciation is a common mode of ecological speciation (Calcagno et al., 2007; Fry, 2003). Disruptive selection on host specificity (e.g. performance trade-offs) can drive further specialization, leading to the emergence of distinct species (Jacquemyn et al., 2018; Rundle & Nosil, 2005). Therefore, differences in

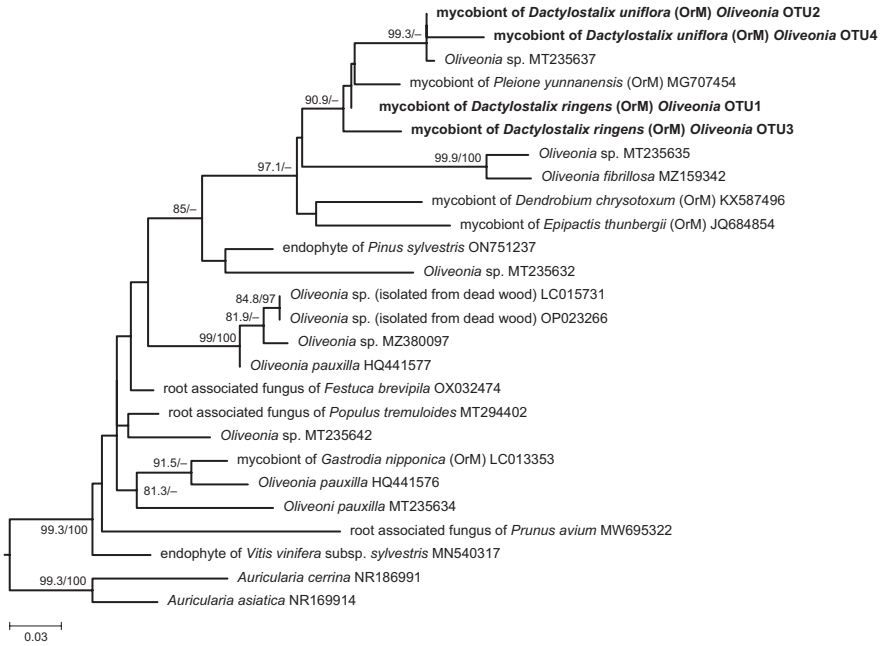

FIGURE 4 Phylogenetic tree of partial internal transcribed spacer rDNA sequences from mycorrhizal fungi of the *Dactylostalix ringens* and *D. uniflora* plants investigated in this study (in bold) and from the International Nucleotide Sequence Database Collaboration (INSDC) database. Nodes with SH-aLRT values <80% and ultrafast bootstrap values <95% are not shown. OrM, orchid mycorrhizal fungi.

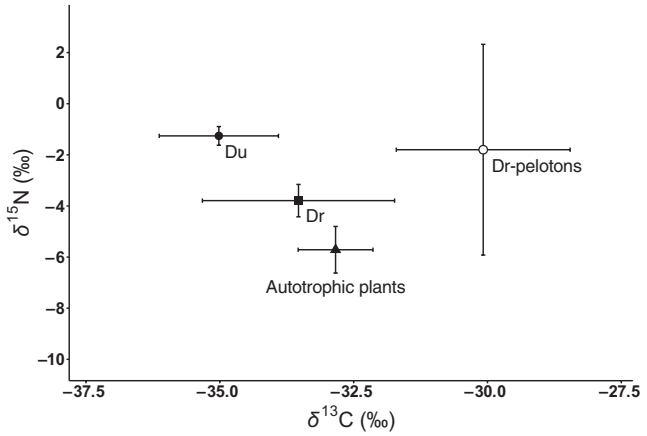

FIGURE 5 Mean (±SD) values of $\delta^{13}C$ and $\delta^{15}N$ in the leaves of two *Dactylostalix* species and their neighbouring autotrophic plants. Dr, *Dactylostalix ringens*; Du, *Dactylostalix uniflora*.

mycorrhizal communities likely play a role in reproductive isolation and speciation in orchids (Barrett & Freudenstein, 2011; Freudenstein & Barrett, 2014; Jacquemyn et al., 2018). Mycorrhizal segregation in *Dactylostalix* species may also contribute to reproductive isolation between them. Further research, including artificial interspecific cross-pollination and in-situ seed baiting, is needed to determine whether mycorrhizal associations function as a post-mating barrier.

Another fascinating aspect of the *Oliveonia* association with the two *Dactylostalix* species is that *Oliveonia* produces a rhizoctonia-like anamorph (Kotiranta & Saarenoksa, 2005; Roberts, 1998). Morphologically, *Oliveonia* closely resembles Ceratobasidiaceae due to its resupinate holobasidia and the presence of self-replicating (repetitive) basidiospores (Kotiranta & Saarenoksa, 2005; Roberts, 1998). As a result, it was initially classified under Ceratobasidiaceae, but molecular data subsequently placed *Oliveonia* in the distinct family Oliveoniaceae, now part of Auriculariales (Roberts, 1998). The convergence of rhizoctonia-like fungi, including Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and *Oliveonia*, as orchid symbionts is remarkable, suggesting that their shared traits likely facilitate their recruitment as orchid mycorrhizal partners. Further investigation is required to identify these convergent characteristics that enhance their suitability as orchid symbionts.

Additionally, both *Dactylostalix* species show lower ^{13}C and higher ^{15}N abundances compared to surrounding autotrophs, unlike most green orchids associated with saprotrophic non-rhizoctonia fungi, which typically exhibit much higher ^{13}C and moderately higher ^{15}N enrichment (Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021b; Yagame et al., 2021; Zahn et al., 2022). Moreover, although *Oliveonia* fungi are likely saprotrophic and expected to be enriched in ^{13}C, our peloton samples from *D. ringens* showed only modest ^{13}C enrichment (2.6 ± 1.8‰) compared to autotrophic reference plants. This is much lower than the enrichment reported for pelotons associated with wood- or litter-decomposing fungi (around 10‰) (Suetsugu, Matsubayashi, et al., 2024). This could reflect the endophytic nature of *Oliveonia* (e.g. Durán et al., 2018; Mesny et al., 2021), as endophytic associations often result in lower ^{13}C enrichment (Halbwachs et al., 2013; Selosse & Martos, 2014).

Interestingly, rhizoctonia-associated orchids also show conflicting signals: elevated $\delta^{15}N$ values, supporting partial mycoheterotrophy, but similar or lower $\delta^{13}C$ values compared to autotrophic plants, suggesting autotrophy (Hynson et al., 2013). The observed ^{13}C depletion in these orchids may be due to the uptake of ^{13}C-depleted carbon from rhizoctonias (Gomes et al., 2023; Zahn et al., 2023). Indeed, pelotons of rhizoctonia-associated orchids often exhibit significantly lower ^{13}C enrichment compared to those associated with typical saprotrophic fungi (Gomes et al., 2023; Zahn

et al., 2023; but also see Suetsugu, Matsubayashi, et al., 2024). The minimal ^{13}C enrichment in pelotons (<3‰) limits the utility of ^{13}C enrichment as a marker for mycoheterotrophic carbon acquisition in many rhizoctonia-associated orchids (Gomes et al., 2023; Zahn et al., 2023; but also see Suetsugu, Matsubayashi, et al., 2024), as well as in *Dactylostalix* species associated with *Oliveonia* fungi. Further research, including hydrogen isotope analysis (Gebauer et al., 2016; Yagi et al., 2024), will be necessary to determine the precise nutritional mode of *Dactylostalix* species. This method has shown that partial mycoheterotrophy is more common in rhizoctonia-associated orchids than previously thought (Gebauer et al., 2016; Yagi et al., 2024).

Overall, our study revealed that both orchid species do not associate with saprotrophic rhizoctonia fungi from Serendipitaceae, Ceratobasidiaceae or Tulasnellaceae. Instead, they associate with *Oliveonia* fungi (Oliveoniaceae, Auriculariales). Although *Oliveonia* fungi were not previously recognized as primary orchid symbionts, it is noteworthy that they were only recently separated from rhizoctonias, typical orchid mycobionts (Kotiranta & Saarenoksa, 2005; Roberts, 1998). Furthermore, we showed that the ^{13}C and ^{15}N signatures of *Dactylostalix* species and their pelotons closely resemble those of many rhizoctonia-associated orchids (Gomes et al., 2023; Zahn et al., 2023). This isotopic evidence suggests a niche overlap with endophytic tendencies between rhizoctonias and *Oliveonia*, and these potential endophytic traits have likely facilitated the recruitment of both rhizoctonias and *Oliveonia* fungi as mycorrhizal partners (Selosse et al., 2022). Additionally, *D. ringens* and *D. uniflora* form specialized mycorrhizal associations with distinct *Oliveonia* lineages across multiple locations. Thus, mycorrhizal segregation between the two *Dactylostalix* species may facilitate their sympatric coexistence and potentially contribute to reproductive isolation.

AUTHOR CONTRIBUTIONS

Kenji Suetsugu planned and designed the research, conducted field and laboratory experiments, performed analyses, and drafted the initial manuscript. Hidehito Okada conducted analyses, revised the manuscript, and approved the final version.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Katsumi Iwahori, Masayuki Sato, Yasutomi Sugawahara, Masayuki Matsui and Kohtaro Shutoh for providing specimens or photographs for this study. We also thank Ryuta Yagi, Chikage Yoshimizu, Kazuma Takizawa, Takako Shizuka and Makoto Taniguchi for their technical support. We are grateful to Ministry of the Environment, the Forestry Agency and *Agency for Cultural Affairs* for their generous support and permissions. This study was financially supported by PRESTO (JPMJPR21D6, KS) from the Japan Science and Technology Agency, the JSPS KAKENHI (17H05016, KS), and a Joint Research Grant for the Environmental Isotope Study from the Research Institute for Humanity and Nature.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA accession number PRJNA1160459). The processed data are available from Figshare: https://doi.org/10.6084/m9.figshare.27261825 (Suetsugu & Okada, 2024).

ORCID

Kenji Suetsugu https://orcid.org/0000-0002-7943-4164

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.

Barrett, C. F., & Freudenstein, J. V. (2011). An integrative approach to delimiting species in a rare but widespread mycoheterotrophic orchid. *Molecular Ecology*, *20*, 2771–2786.

Barrett, C. F., Freudenstein, J. V., Taylor, D. L., & Koljalg, U. (2010). Rangewide analysis of fungal associations in the fully mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. *American Journal of Botany*, *97*, 628–643.

Bidartondo, M. I., Burghardt, B., Gebauer, G., Bruns, T. D., & Read, D. J. (2004). Changing partners in the dark: Isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *271*, 1799–1806.

Calcagno, V., Thomas, Y., & Bourguet, D. (2007). Sympatric host races of the European corn borer: Adaptation to host plants and hybrid performance: Adaptation to host plants in ECB. *Journal of Evolutionary Biology*, *20*, 1720–1729.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*, 581–583.

Chase, M. W., Cameron, K. M., Freudenstein, J. V., Pridgeon, A. M., Salazar, G., Van den Berg, C., & Schuiteman, A. (2015). An updated classification of Orchidaceae. *Botanical Journal of the Linnean Society*, *177*, 151–174.

Christenhusz, M. J. M., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa*, *261*, 201–217.

Dearnaley, J. D. W., Martos, F., & Selosse, M. A. (2012). Orchid mycorrhizas: Molecular ecology, physiology, evolution and conservation aspects. In B. Hock (Ed.), *The Mycota. Fungal associations* (pp. 207–230). Springer.

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, *12*, 13–15.

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., & Hacquard, S. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell*, *175*, 973–983. e14.

Esling, P., Lejzerowicz, F., & Pawlowski, J. (2015). Accurate multiplexing and filtering for high-throughput amplicon-sequencing. *Nucleic Acids Research*, *43*, 2513–2524.

Freudenstein, J. V. (1994). Gynostemium structure and relationships of the Corallorhizinae (Orchidaceae: Epidendroideae). *Plant Systematics and Evolution*, *193*, 1–19.

Freudenstein, J. V., & Barrett, C. F. (2014). Fungal host utilization helps circumscribe leafless coralroot orchid species: An integrative analysis of *Corallorhiza odontorhiza* and *C. wisteriana*. *Taxon*, *63*, 759–772.

Freudenstein, J. V., Garcia-Cruz, J., Grayer, R. J., Pridgeon, A. M., & Veitch, N. C. (2005). Tribe Calypsoeae. In A. M. Pridgeon, P. J. Cribb, M. W. Chase, & F. N. Rasmussen (Eds.), *Genera Orchidacearum: Epidendroideae (part one)* (pp. 89–115). Oxford University Press.

Freudenstein, J. V., Yukawa, T., & Luo, Y. (2017). A reanalysis of relationships among Calypsoinae (Orchidaceae: Epidendroideae): Floral and vegetative evolution and the placement of *Yoania*. *Systematic Botany*, *42*, 17–25.

Fry, J. D. (2003). Multilocus models of sympatric speciation: Bush versus Rice versus Felsenstein. *Evolution*, *57*, 1735–1746.

Gause, G. F. (1934). *The struggle for existence*. The Williams & Wilkins Company.

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

Gebauer, G., Preiss, K., & Gebauer, A. C. (2016). Partial mycoheterotrophy is more widespread among orchids than previously assumed. *New Phytologist*, *211*, 11–15.

Gomes, S. I. F., Giesemann, P., Klink, S., Hunt, C., Suetsugu, K., & Gebauer, G. (2023). Stable isotope natural abundances of fungal hyphae extracted from the roots of arbuscular mycorrhizal mycoheterotrophs and rhizoctonia-associated orchids. *New Phytologist*, *239*, 1166–1172.

Halbwachs, H., Dentinger, B. T., Detheridge, A. P., Karasch, P., & Griffith, G. W. (2013). Hyphae of waxcap fungi colonise plant roots. *Fungal Ecology*, *6*, 487–492.

Hynson, N. A., Madsen, T. P., Selosse, M. A., Adam, I. K. U., Ogura-Tsujita, Y., Roy, M., & Gebauer, G. (2013). The physiological ecology of mycoheterotrophy. In V. S. F. T. Merckx (Ed.), *Mycoheterotrophy: The biology of plants living on fungi* (pp. 297–342). Springer.

Jacquemyn, H., Brys, R., Merckx, V. S. F. T., Waud, M., Lievens, B., & Wiegand, T. (2014). Coexisting orchid species have distinct mycorrhizal communities and display strong spatial segregation. *New Phytologist*, *202*, 616–627.

Jacquemyn, H., Kort, H. D., Broeck, A. V., & Brys, R. (2018). Immigrant and extrinsic hybrid seed inviability contribute to reproductive isolation between forest and dune ecotypes of *Epipactis helleborine* (Orchidaceae). *Oikos*, *127*, 73–84.

Jacquemyn, H., & Merckx, V. S. F. T. (2019). Mycorrhizal symbioses and the evolution of trophic modes in plants. *Journal of Ecology*, *107*, 1567–1581.

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

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, *14*, 587–589.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*, 772–780.

Kotiranta, H., & Saarenoksa, R. (2005). *Ceratobasidium* and *Oliveonia* (Basidiomycota, Aphyllophorales) in Finland. *Annales Botanici Fennici*, *42*, 237–245.

Kottke, I., Suárez, J. P., Herrera, P., Cruz, D., Bauer, R., Haug, I., & Garnica, S. (2010). Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. *Proceedings of the Royal Society B: Biological Sciences*, *277*, 1289–1298.

Kristiansen, K. A., Freudenstein, J. V., Rasmussen, F. N., & Rasmussen, H. N. (2004). Molecular identification of mycorrhizal fungi in *Neuwiedia veratrifolia* (Orchidaceae). *Molecular Phylogenetics and Evolution*, *33*, 251–258.

Kristiansen, K. A., Rasmussen, F. N., & Rasmussen, H. N. (2001). Seedlings of *Neuwiedia* (Orchidaceae subfamily Apostasioideae) have typical orchidaceous mycotrophic protocorms. *American Journal of Botany*, *88*, 956–959.

Leake, J. R. (1994). The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist*, *127*, 171–216.

Martino, E., Morin, E., Grelet, G.-A., Kuo, A., Kohler, A., Daghino, S., Barry, K. W., Cichocki, N., Clum, A., Dockter, R. B., Hainaut, M., Kuo, R. C., LaButti, K., Lindahl, B. D., Lindquist, E. A., Lipzen, A., Khouja, H. R., Magnuson, J., Murat, C., … Perotto, S. (2018). Comparative genomics and transcriptomics depict ericoid mycorrhizal fungi as versatile saprotrophs and plant mutualists. *New Phytologist*, *217*, 1213–1229.

Malysheva, V., & Spirin, V. (2017). Taxonomy and phylogeny of the Auriculariales (Agaricomycetes, Basidiomycota) with stereoid basidiocarps. *Fungal Biology*, *121*, 689–715.

McCormick, M., Burnett, R., & Whigham, D. (2021). Protocorm-supporting fungi are retained in roots of mature *Tipularia discolor* orchids as mycorrhizal fungal diversity increases. *Plants*, *10*, 1251.

McCormick, M. K., & Jacquemyn, H. (2014). What constrains the distribution of orchid populations? *New Phytologist*, *202*, 392–400.

McCormick, M. K., Whigham, D. F., & O'Neill, J. (2004). Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist*, *163*, 425–438.

Merckx, V. S. F. T. (2013). *Mycoheterotrophy: The biology of plants living on fungi*. Springer.

Mesny, F., Miyauchi, S., Thiergart, T., Pickel, B., Atanasova, L., Karlsson, M., Hüttel, B., Barry, K. W., Haridas, S., & Chen, C. (2021). Genetic determinants of endophytism in the *Arabidopsis* root mycobiome. *Nature Communications*, *12*, 7227.

Nguyen, L.-T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, *32*, 268–274.

Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F. O., & Tedersoo, L. (2019). The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, *47*, D259–D264.

Ogura-Tsujita, Y., Gebauer, G., Hashimoto, T., Umata, H., & Yukawa, T. (2009). Evidence for novel and specialized mycorrhizal parasitism: The orchid *Gastrodia confusa* gains carbon from saprotrophic *Mycena*. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 761–767.

Ogura-Tsujita, Y., Yokoyama, J., Miyoshi, K., & Yukawa, T. (2012). Shifts in mycorrhizal fungi during the evolution of autotrophy to mycoheterotrophy in *Cymbidium* (Orchidaceae). *American Journal of Botany*, *99*, 1158–1176.

Qin, J., Zhang, W., Ge, Z. W., & Zhang, S. B. (2019). Molecular identifications uncover diverse fungal symbionts of *Pleione* (Orchidaceae). *Fungal Ecology*, *37*, 19–29.

Roberts, P. (1998). *Oliveonia* and the origin of the holobasidiomycetes. *Folia Cryptogamica Estonica*, *33*, 127–132.

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *4*, e2584.

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters*, *8*, 336–352.

Selosse, M., & Cameron, D. D. (2010). Introduction to a Virtual Special Issue on mycoheterotrophy: New Phytologist sheds light on non-green plants. *New Phytologist*, *185*, 591–593.

Selosse, M.-A., & Martos, F. (2014). Do chlorophyllous orchids heterotrophically use mycorrhizal fungal carbon? *Trends in Plant Science*, *19*, 683–685.

Selosse, M.-A., Petrolli, R., Mujica, M. I., Laurent, L., Perez-Lamarque, B., Figura, T., Bourceret, A., Jacquemyn, H., Li, T., Gao, J., Minasiewicz, J., & Martos, F. (2022). The waiting room hypothesis revisited by orchids: Were orchid mycorrhizal fungi recruited among root endophytes? *Annals of Botany*, *129*, 259–270.

Selosse, M. A., & Roy, M. (2009). Green plants that feed on fungi: Facts and questions about mixotrophy. *Trends in Plant Science*, *14*, 64–70.

Selosse, M.-A., Schneider-Maunoury, L., & Martos, F. (2018). Time to rethink fungal ecology? Fungal ecological niches are often prejudged. *New Phytologist*, *217*, 968–972.

Shefferson, R. P., WEIß, M., Kull, T., & Taylor, D. L. (2005). High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology*, *14*, 613–626.

Suetsugu, K., Haraguchi, T. F., Tanabe, A. S., & Tayasu, I. (2021). Specialized mycorrhizal association between a partially mycoheterotrophic orchid *Oreorchis indica* and a *Tomentella* taxon. *Mycorrhiza*, *31*, 243–250.

Suetsugu, K., Haraguchi, T. F., & Tayasu, I. (2022). Novel mycorrhizal cheating in a green orchid: *Cremastra appendiculata* depends on carbon from deadwood through fungal associations. *New Phytologist*, *235*, 333–343.

Suetsugu, K., Hirota, S. K., Yagame, T., Yukawa, T., & Suyama, Y. (2024). Taxonomic revision of the formerly monotypic orchid genus *Dactylostalix*. *Phytotaxa*, *652*, 83–99.

Suetsugu, K., & Matsubayashi, J. (2021a). Evidence for mycorrhizal cheating in *Apostasia nipponica*, an early-diverging member of the Orchidaceae. *New Phytologist*, *229*, 2302–2310.

Suetsugu, K., & Matsubayashi, J. (2021b). Subterranean morphology modulates the degree of mycoheterotrophy in a green orchid *Calypso bulbosa* exploiting wood-decaying fungi. *Functional Ecology*, *35*, 2305–2315.

Suetsugu, K., Matsubayashi, J., & Okada, H. (2024). Stable isotope signatures illuminate diverse nutritional strategies in rhizoctonias and their orchid partners. *Plant, Cell & Environment*.

Suetsugu, K., & Okada, H. (2021). Symbiotic germination and development of fully mycoheterotrophic plants convergently targeting similar Glomeraceae taxa. *Environmental Microbiology*, *23*, 6328–6343.

Suetsugu, K., & Okada, H. (2024). Data for Mycorrhizal specialization toward each distinct *Oliveonia* fungus in two closely related photosynthetic *Dactylostalix* orchids. *Figshare*. https://doi.org/10.6084/m9.figshare.27261825

Syed, F., Grunenwald, H., & Caruccio, N. (2009). Optimized library preparation method for next-generation sequencing. *Nature Methods*, *6*, 1–2.

Tanabe, A. S., & Toju, H. (2013). Two new computational methods for universal DNA barcoding: A benchmark using barcode sequences of bacteria, archaea, animals, fungi, and land plants. *PLoS One*, *8*, e76910.

Tayasu, I., Hirasawa, R., Ogawa, N. O., Ohkouchi, N., & Yamada, K. (2011). New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. *Limnology*, *12*, 261–266.

Taylor, D. L., & Bruns, T. D. (1999). Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchids *Corallorhiza maculata* and *C. mertensiana*. *Molecular Ecology*, *8*, 1719–1732.

Thoen, E., Harder, C. B., Kauserud, H., Botnen, S. S., Vik, U., Taylor, A. F., Menkis, A., & Skrede, I. (2020). *In vitro* evidence of root colonization suggests ecological versatility in the genus *Mycena*. *New Phytologist*, *227*, 601–612.

Wang, D., Lerou, J., Nuytinck, J., Gomes, S. I., Jacquemyn, H., & Merckx, V. S. (2022). Root-associated fungi in Orchidaceae: Diversity, phylogeny, ecology, and outstanding questions. *bioRxiv*. https://doi.org/10.1101/2022.12.16.519622

Warcup, J. H., & Talbot, P. H. B. (1967). Perfect states of rhizoctonias associated with orchids. *New Phytologist*, *66*, 631–641.

Waterman, R. J., Bidartondo, M. I., Stofberg, J., Combs, J. K., Gebauer, G., Savolainen, V., Barraclough, T. G., & Pauw, A. (2011). The effects of above- and belowground mutualisms on orchid speciation and coexistence. *The American Naturalist*, *177*, E54–E68.

Waud, M., Busschaert, P., Ruyters, S., Jacquemyn, H., & Lievens, B. (2014). Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. *Molecular Ecology Resources*, *14*, 679–699.

Yagame, T., Lallemand, F., Selosse, M.-A., Funabiki, E., & Yukawa, T. (2021). Mycobiont diversity and first evidence of mixotrophy associated with Psathyrellaceae fungi in the chlorophyllous orchid *Cremastra variabilis*. *Journal of Plant Research*, *134*, 1213–1224.

Yagame, T., Ogura-Tsujita, Y., Kinoshita, A., Iwase, K., & Yukawa, T. (2016). Fungal partner shifts during the evolution of mycoheterotrophy in *Neottia*. *American Journal of Botany*, *103*, 1630–1641.

Yagi, R., Haraguchi, T., Tayasu, I., & Suetsugu, K. (2024). Do exchangeable hydrogens affect the evaluation of partial mycoheterotrophy in orchids?: Insights from δ^2H analysis in bulk, α-cellulose, and cellulose nitrate samples. *New Phytologist*, *243*, 2430–2441.

Yao, N., Wang, T., & Cao, X. (2022). *Epidendrum radicans* fungal community during ex situ germination and isolation of germination-enhancing fungi. *Microorganisms*, *10*, 1841.

Yukawa, T., Ogura-Tsujita, Y., Shefferson, R. P., & Yokoyama, J. (2009). Mycorrhizal diversity in *Apostasia* (Orchidaceae) indicates the origin and evolution of orchid mycorrhiza. *American Journal of Botany*, *96*, 1997–2009.

Zahn, F. E., Lee, Y.-I., & Gebauer, G. (2022). Fungal association and root morphology shift stepwise during ontogenesis of orchid *Cremastra appendiculata* towards autotrophic nutrition. *AoB Plants*, *14*, 1–10.

Zahn, F. E., Söll, E., Chapin, T. K., Wang, D., Gomes, S. I. F., Hynson, N. A., Pausch, J., & Gebauer, G. (2023). Novel insights into orchid mycorrhiza functioning from stable isotope signatures of fungal pelotons. *New Phytologist*, *239*, 1449–1463.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Mycorrhizal samples of *Dactylostalix ringens* and *D. uniflora* used in this study.

Table S2. Values of δ^{13}C and δ^{15}N in each sample of *Dactylostalix* species, their pelotons and neighbouring autotrophic reference plants.

Data S1. The sequencing reads of OTUs detected in each *Dactylostalix* individual.

How to cite this article: Suetsugu, K., & Okada, H. (2024). Mycorrhizal specialization toward each distinct *Oliveonia* fungus in two closely related photosynthetic *Dactylostalix* orchids. *Functional Ecology*, *00*, 1–10. https://doi.org/10.1111/1365-2435.14690