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RESEARCH ARTICLE

The nexus of decay and birth: Ecological and evolutionary significance of wood-decaying fungi in green Calypsoinae orchid germination

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Abstract

1. A key feature of Orchidaceae is the production of dust-like seeds that depend on fungal carbon during early development. Although protocorms and mature green orchids typically associate with rhizoctonia fungi, many non-photosynthetic orchids and some photosynthetic green orchids maintain symbioses with saprotrophic (SAP), non-rhizoctonia fungi throughout their lives.
2. We examined the mycorrhizal communities of four green Calypsoinae orchids (*Calypso bulbosa*, *Cremastra variabilis*, *Oreorchis patens* and *Tipularia japonica*) at germination. Using seed baiting combined with molecular barcoding, we tested for potential associations with SAP, non-rhizoctonia fungi at the protocorm stage, given that conspecific adults with coralloid rhizomes, structures morphologically resembling protocorms, often associate with these fungi. We also measured natural abundances of ¹³C and ¹⁵N in *T. japonica* protocorms, coralloid rhizome-bearing adults, individuals lacking coralloid rhizomes and autotrophic reference plants to quantify the contribution of fungal carbon to plant nutrition.
3. Germination was observed exclusively near adult plants with coralloid rhizomes and decomposing wood, whereas seeds placed near adults lacking coralloid rhizomes failed to germinate. Molecular barcoding revealed that protocorms of all four species were predominantly associated with wood-decaying fungi. Their mycorrhizal communities closely matched those of conspecific adults with coralloid rhizomes, and the dominant operational taxonomic unit (OTU) was even shared across protocorms and coralloid rhizomes of *C. variabilis* and *O. patens*. Stable isotope analyses further indicated that *T. japonica* protocorms derived all their carbon from wood-decaying fungi, while coralloid rhizome-bearing adults obtained more than half of their carbon from these fungi.
4. These results underscore the role of SAP, non-rhizoctonia fungi in germination and suggest that wood-derived carbon facilitates seedling establishment in shaded forests. This broadens our understanding of plant adaptation to low-light environments and deepens our knowledge of the functional diversity of mycorrhizal

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interactions. Moreover, because many fully mycoheterotrophic Calypsoinae species possess coralloid rhizomes exclusively colonized by SAP non-rhizoctonia fungi, such germination-stage associations in green Calypsoinae may have promoted the repeated evolution of full mycoheterotrophy in this group.

KEYWORDS

mycoheterotrophy, mycorrhizal communities, orchid seedlings, protocorm, saprotrophic fungi, seed-sowing experiments

1 | INTRODUCTION

The family Orchidaceae is one of the most species-rich angiosperm families, exhibiting remarkable diversity across a wide range of habitats worldwide. A defining characteristic of orchids is their production of numerous dust-like seeds (Arditti & Ghani, 2000). In natural environments, orchid seeds rely on mycorrhizal fungi for essential nutrients such as carbon, nitrogen and phosphorus during early development, resulting in fully mycoheterotrophic underground seedlings (protocorms) (Jacquemyn & Merckx, 2019; Merckx, 2013; Rasmussen & Rasmussen, 2014).

At maturity, many orchids shift toward partial autotrophy, and some eventually achieve full autotrophy, even supplying carbon to their fungal partners (Cameron et al., 2008). However, some orchids continue to depend partially or entirely on mycorrhizal fungi for carbon in adulthood (Gebauer & Meyer, 2003; Hynson et al., 2013). Fully mycoheterotrophic plants, which are non-photosynthetic and completely reliant on fungi, are especially prevalent in Orchidaceae, including over 200 species and more than 30 independent evolutionary origins (Merckx & Freudenstein, 2010). This early dependence on fungal carbon may have facilitated multiple transitions to fully mycoheterotrophy in orchids (Hynson et al., 2013; Jacquemyn & Merckx, 2019; Merckx, 2013).

Due to their subterranean nature, empirical data on fungal associations at the seedling stage remain limited. However, seed baiting techniques (e.g. sowing seeds in mesh bags) combined with molecular barcoding have greatly advanced our understanding of mycoheterotrophic germination (Rasmussen & Whigham, 1993). The fungal partners of orchid seedlings generally correspond to those found in co-occurring adult plants (Jacquemyn et al., 2012; McKendrick et al., 2002; Rasmussen & Rasmussen, 2014). Orchid seedlings that transition to autotrophy or a low degree of mycoheterotrophy in adulthood typically associate with polyphyletic rhizoctonia fungi (Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae), which also colonize adult roots (Dearnaley et al., 2012; Rasmussen & Rasmussen, 2014). In contrast, both seedlings and adults of fully mycoheterotrophic orchids, as well as partially mycoheterotrophic orchids with high fungal carbon dependence, are predominantly associated with ectomycorrhizal (ECM) fungi or, less commonly documented, with saprotrophic non-rhizoctonia (SAP) fungi (Bidartondo & Read, 2008; Jacquemyn & Merckx, 2019).

At finer taxonomic scales, many orchids exhibit developmental shifts in fungal partners, although the fungal communities often overlap at the genus or family level across life stages (Bidartondo & Read, 2008; Meng et al., 2019; Ventre Lespiaucq et al., 2021). For example, a specific *Tulasnella* species consistently isolated from mature *Dendrobium moniliforme* promotes protocorm formation but not further development, whereas a different *Tulasnella* species supports seedling establishment (Meng et al., 2019). Similarly, in *Cephalanthera longifolia*, early developmental stages involve a subset of the fungal taxa found in germinating seeds and adult roots (Bidartondo & Read, 2008).

More pronounced symbiont shifts are often observed in orchids that associate at least partially with SAP fungi. For example, the fully mycoheterotrophic *Gastrodia elata* initially depends on litter-decomposing *Mycena* species for germination but later associates with wood-decomposing *Armillaria* species (Park & Lee, 2013). Similarly, protocorms of *Tipularia discolor* associate with specific Auriculariales fungi, while the photosynthetic adults form relationships with rhizoctonia fungi (McCormick et al., 2004). Likewise, *Oeceoclades maculata* germinates with a single *Psathyrella* species but later broadens its associations to include rhizoctonia fungi such as Ceratobasidiaceae and Tulasnellaceae (Bayman et al., 2016). Additionally, Psathyrellaceae fungi dominate in protocorms and seedlings of *Cremastra appendiculata*, while rhizoctonia fungi predominate in the roots of mature plants (Zahn et al., 2022). Nonetheless, SAP associations at the germination stage in green orchids are still considered exceptional (Bayman et al., 2016).

Interestingly, some green orchids such as *Cremastra variabilis*, a sister species of *C. appendiculata*, occasionally possess coralloid rhizomes in adulthood, structures commonly observed in fully mycoheterotrophic species (Maekawa, 1971). These rhizomes resemble conspecific protocorms morphologically and are often interpreted as persistent protocorms retaining juvenile features (Freudenstein, 1994; Gillman, 1876; MacDougal, 1899; Maekawa, 1971). Recent studies have linked coralloid rhizomes with both the degree of mycoheterotrophy and associations with SAP fungi (Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021; Yagame et al., 2021). We therefore hypothesize that protocorms in species whose adults occasionally produce coralloid rhizomes and associate with SAP fungi are colonized by the same fungal taxa found in conspecific coralloid rhizomes. Given that orchids fully depend on fungal-derived carbon during the protocorm stage (Merckx, 2013)

and that wood-decaying fungi are likely better carbon sources than rhizoctonia fungi (Suetsugu & Okada, 2025b), these interactions are also ecologically plausible.

Here, we focused on four green orchid species in the subtribe Calypsoinae: *Calypso bulbosa*, *C. variabilis*, *Oreorchis patens* and *Tipularia japonica*. A recent phylogenomic analysis revealed that the monotypic genus *Calypso* forms a clade with *Changnienia* and *Tipularia* (approximately five species), while *Cremastra* (approximately seven species) is grouped with *Aplectrum*, *Oreorchis* (approximately 17 species), *Kitigorhis* (approximately two species) and *Corallorhiza* (approximately 13 species) (Barrett et al., 2025). These Calypsoinae taxa have emerged as a model system for studying mycorrhizal associations and mycoheterotrophy due to their diverse fungal partners and nutritional strategies (Barrett et al., 2010; Barrett & Freudenstein, 2011; Freudenstein & Barrett, 2014; Suetsugu & Matsubayashi, 2021; Suetsugu & Okada, 2025a, 2025b). Among the species examined, three are known to occasionally develop coralloid rhizomes at maturity that are associated with SAP fungi: *C. bulbosa* with Auriculariales fungi, and *C. variabilis* and *O. patens* with Psathyrellaceae fungi (Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021; Suetsugu & Okada, 2025b; Yagame et al., 2021). In these species, the occurrence of coralloid rhizomes together with SAP associations is linked to increased levels of mycoheterotrophy (Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021; Suetsugu & Okada, 2025b; Yagame et al., 2021). *Tipularia japonica* may exhibit similar traits, as seed germination in the close relative *T. discolor* is stimulated by decaying wood and the protocorms of *T. discolor* associate with wood-decomposing Auriculariales fungi (McCormick et al., 2004, 2012; Rasmussen & Whigham, 1998; Whigham et al., 2002).

Consequently, we characterized the mycorrhizal communities of these four species at the protocorm stage to improve our understanding of SAP-associated mycoheterotrophic germination. Specifically, we addressed the following questions: (i) Are the mycorrhizal communities at the protocorm stage dominated by SAP fungi, and if so, do they resemble those found in the coralloid rhizomes of conspecific adults? (ii) Is mycorrhizal community composition during the initial mycoheterotrophic stage similar across species, particularly between *C. variabilis* and *O. patens*, which are potentially associated with Psathyrellaceae fungi, and between *C. bulbosa* and *T. japonica*, which may be associated with Auriculariales fungi? (iii) Does germination occur more frequently near adult plants bearing coralloid rhizomes than near those lacking such structures? (iv) As in the other three species, do *T. japonica* individuals with coralloid rhizomes exhibit a high degree of mycoheterotrophy?

2 | MATERIALS AND METHODS

2.1 | Seed germination experiments and sample collection

Seeds of *C. variabilis* and *O. patens* were collected in early October 2010 in Ebetsu City, Hokkaido Prefecture, whereas those of *C. bulbosa* were obtained in mid-August 2012 in Kamiiso Town,

Hokkaido Prefecture. Seeds were enclosed in nylon mesh bags (108 µm mesh), sealed in 50×50 mm slide mounts and labelled with coloured strings (Rasmussen & Whigham, 1993). Each packet contained approximately 100 seeds. At each site, three seed packets were buried within a week of collection to minimize loss of viability, placed within 10 cm of adult plants either bearing coralloid rhizomes ($n=10$) or lacking them ($n=10$), resulting in 60 packets per species. The presence of deadwood near each planting site was recorded. Seed packets of *C. variabilis* and *O. patens* were retrieved in late May 2012, and those of *C. bulbosa* in mid-September 2013. To minimize disturbance, we carefully assessed the presence or absence of coralloid rhizomes by accessing the roots or coralloid rhizomes laterally, without excavating entire plants. After inspection, the holes were refilled with the original soil.

Because preliminary germination trials in *T. japonica* were unsuccessful, we collected soil samples in late December 2020 within 10 cm of adults with ($n=10$) or without ($n=10$) coralloid rhizomes in Fuji City, Shizuoka Prefecture. As in the seed burial experiments, the presence of nearby deadwood was recorded. Protocorms were isolated using a centrifugation method (Johnson-Groh et al., 2002; Mason & Farrar, 1989). Specifically, soil samples were disaggregated, sieved to remove coarse debris and centrifuged in water followed by 50% sucrose. Supernatants were examined under a Leica M420 stereomicroscope (Leica Microsystems, Wetzlar, Germany) to detect protocorms.

Germination was defined as seed coat rupture accompanied by the emergence of an enlarged embryo. In *C. variabilis* and *O. patens*, numerous well-developed protocorms were recovered and categorized as small (<5 mm, unbranched) or large (>5 mm, branched) to infer developmental shifts in fungal associations. In contrast, all or most protocorms of *C. bulbosa* and *T. japonica* were <5 mm, preventing size-based classification.

Additionally, mycorrhizal samples (root tips or coralloid rhizomes) were collected from adult plants of all four species, both with and without coralloid rhizomes, within their respective populations. For individuals with coralloid structures, only coralloid rhizomes were sampled, as previous studies have shown that fungal colonization primarily occurs in these tissues (Suetsugu & Okada, 2025b; Yagame et al., 2013). All protocorms and mycorrhizal tissues were preserved in 1.5 mL tubes containing 99.5% ethanol and stored at -20°C until DNA extraction.

2.2 | Molecular identification of mycobionts

Samples were rinsed to remove debris and surface-sterilized with 1% sodium hypochlorite for 30 s, followed by three rinses in sterile distilled water. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). Sample sizes were as follows: for *C. bulbosa*, 12 coralloid rhizome-bearing adults, 14 coralloid rhizome-lacking adults and 12 protocorms; for *C. variabilis*, 8 coralloid rhizome-bearing adults, 10 coralloid rhizome-lacking adults, 5 small protocorms and 17 large protocorms; for *O.*

patens, 16 coralloid rhizome-bearing adults, 9 coralloid rhizome-lacking adults, 7 small protocorms and 12 large protocorms; and for *T. japonica*, 12 coralloid rhizome-bearing adults, 7 coralloid rhizome-lacking adults and 6 protocorms.

Following Suetsugu and Okada (2025b), the fungal ITS region was amplified using two primer sets: ITS86F/ITS4, commonly used to profile fungal communities in orchid roots (Waud et al., 2014), and ITS86F/ITS4Tul2, specific to Tulasnellaceae fungi (Oja et al., 2015). The first PCR incorporated primers with 3–6-mer random nucleotides and Illumina sequencing primers; the second PCR added Illumina P5/P7 adapters and indices. Libraries were sequenced on an Illumina MiSeq platform using the MiSeq Reagent Micro Kit v2 (300 cycles). ITS86F/ITS4-based data for several adult *C. bulbosa* individuals and all *O. patens* adults were previously archived in the Sequence Read Archive (PRJDB8982 and PRJNA1213319; Suetsugu & Matsubayashi, 2021; Suetsugu & Okada, 2025b). Remaining sequence data, including all protocorms, all adult samples amplified with ITS86F/ITS4Tul2, and adults of *C. variabilis*, *T. japonica*, and the remaining *C. bulbosa* amplified with ITS86F/ITS4, were deposited under accession number PRJNA1257467.

Bioinformatic processing was conducted with Claident v0.9.2024.06.10 (Tanabe & Toju, 2013), following Suetsugu and Okada (2025b). In brief, after completing primer trimming, quality filtering and denoising, chimeric sequences and index-hopping artefacts were removed using the clemovechimev and clemovecontam commands in Claident. Reads were clustered into OTUs at a 97% similarity threshold, with the most abundant sequence in each OTU designated as the representative. Taxonomic classification was performed to the genus level where feasible. OTUs corresponding to orchid mycorrhizal fungi (Dearnaley et al., 2012; Wang et al., 2021) were retained for further analysis. Rare OTUs, defined as singletons or those with ≤ 50 reads across all datasets, were excluded. To normalize sequencing depth, abundance-based rarefaction was applied by standardizing all samples to a minimum of 1406 reads.

2.3 | Fungal community analyses

β -diversity in mycorrhizal communities was evaluated using weighted UniFrac distance matrices (WUDMs), which account for phylogenetic relationships among OTUs via the 'GUniFrac' package (Chen & Chen, 2021; Lozupone et al., 2011; Lozupone & Knight, 2005). OTU phylogenies were inferred by aligning sequences with MAFFT v7.475 (Katoh & Standley, 2013) and constructing a maximum likelihood tree using IQ-TREE v2.2.2 (Nguyen et al., 2015) under the HKY+I model selected by the Bayesian Information Criterion, with 1000 ultrafast bootstrap replicates (Hoang et al., 2018). Given the substantial interspecific divergence in mycorrhizal communities, analyses were conducted separately for each species or closely related pairs (*C. variabilis* and *O. patens*; *C. bulbosa* and *T. japonica*) to capture intraspecific variation among protocorms, coralloid rhizome-bearing adults and those lacking coralloid rhizomes.

A non-metric multidimensional scaling (NMDS) analysis based on WUDMs was performed using the metaMDS function in the 'vegan' package. Hierarchical clustering with average linkage was applied using the hclust function in 'stats' to assess the influence of developmental stages and phenotypes (protocorms, adults with coralloid rhizomes and those without) on fungal community composition. Group differences were tested using PERMANOVA with 9999 permutations (adonis2 in 'vegan'), and pairwise comparisons with Bonferroni-adjusted *p*-values were conducted using 'pairwiseAdonis' (Martinez Arbizu, 2020).

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

To investigate the nutritional mode of *T. japonica*, natural abundances of ^{13}C and ^{15}N in leaves of mature plants with and without coralloid rhizomes, protocorms, nearby deadwood, and autotrophic reference species within four 2 × 2 m quadrats were measured. Analyses were performed using a continuous-flow isotope-ratio mass spectrometer (Delta V Advantage; Thermo Fisher Scientific, Waltham, MA, USA) coupled with an elemental analyser (Flash EA 2000) at the Research Institute for Humanity and Nature (Kyoto, Japan). Relative isotope abundances were calculated as:

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

where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratio of each sample, and R_{standard} is that of Vienna Pee Dee Belemnite or atmospheric N_2 , respectively. Calibration was performed using laboratory standards CERKU 01, CERKU 02 and CERKU 03 (Tayasu et al., 2011). Analytical standard deviations were $<0.09\text{‰}$ for $\delta^{13}\text{C}$ ($n=24$) and $<0.22\text{‰}$ for $\delta^{15}\text{N}$ ($n=24$). Total C and N concentrations were determined from sample weights and the CO_2 and N_2 gas volumes measured against laboratory standards (Tayasu et al., 2011). Enrichment factors (ϵ) were calculated as $\epsilon = \delta_s - \delta_{\text{REF}}$, where δ_s is the $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ value of a *T. japonica* individual, and δ_{REF} is the mean value for reference plants in the same sampling plot (Preiss & Gebauer, 2008). The proportion of carbon derived from fungi (% Cdf) in mature *T. japonica* plants bearing coralloid rhizomes was estimated using a linear two-source mixing model: % Cdf = $(\epsilon^{13}\text{C}_{\text{PMH}} / \epsilon^{13}\text{C}_{\text{FMH}}) \times 100$, where $\epsilon^{13}\text{C}_{\text{PMH}}$ represents the ^{13}C enrichment factor of mature *T. japonica* plants and $\epsilon^{13}\text{C}_{\text{FMH}}$ is the mean ^{13}C enrichment factor of *T. japonica* protocorms. For mature *T. japonica* plants lacking coralloid rhizomes, we did not calculate the proportion of fungal carbon because their rhizoctonia associations, which show only weak ^{13}C enrichment, are unsuitable for the two-source mixing model (Schweiger et al., 2018).

A linear mixed model was used to compare $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\epsilon^{13}\text{C}$, and $\epsilon^{15}\text{N}$ values, with plant identity (mature plants with or without coralloid rhizomes, protocorms and neighbouring references) as a fixed effect and plot as a random factor. Post hoc pairwise comparisons were performed using the Tukey–Kramer test. All statistical analyses were conducted in R (R Core Team, 2025) using the lme4 (Bates et al., 2015) and multcomp (Hothorn et al., 2008) packages.

3 | RESULTS

3.1 | Patterns of seed germination

Seed-sowing experiments demonstrated that germination success depended on the presence of nearby conspecific adult plants with coralloid rhizomes and deadwood. Of the 60 seed packets buried (3 packets $\times$ 10 coralloid rhizome-bearing vs. 10 coralloid rhizome-lacking adults), germination occurred only near coralloid rhizome-bearing adults and exclusively at sites where deadwood was present. Deadwood was more frequently observed near coralloid rhizome-bearing adults than those lacking coralloid rhizomes (6 vs. 3 individuals in *C. bulbosa*, 9 vs. 3 in *C. variabilis* and 9 vs. 2 in *O. patens*). However, even under this seemingly favourable combination of coralloid rhizome-bearing adult plus deadwood, more than half of the seed packets produced no protocorms. Specifically, only four packets near two coralloid rhizome-bearing individuals of *C. bulbosa*, five packets near three coralloid rhizome-bearing individuals of *C. variabilis*, and seven packets near three coralloid rhizome-bearing individuals of *O. patens* contained protocorms, indicating that these two factors alone were insufficient for successful recruitment. A similar pattern was observed in *T. japonica*, where soil sampling recovered only 10 protocorms, all located near two coralloid rhizome-bearing adults.

In all cases where germination occurred, the initial morphology of the emerging protocorms was a compact, bulbous structure.

Larger protocorms subsequently developed a coral-like architecture resembling the coralloid rhizomes observed in some conspecific adults (Figure 1; Figure S1).

3.2 | Molecular identification of mycobionts

Metabarcoding revealed that protocorms and coralloid rhizome-bearing adults of *C. bulbosa* formed highly specialized associations with a single OTU assigned to the wood-decaying genus *Protomerulius* (Auriculariales), accounting for 16,872 reads (100% of reads) in protocorms and 16,869 reads (99.98%) in coralloid rhizome-bearing adults (Table S1). This OTU was also frequently detected in coralloid rhizome-lacking adults (10,686 reads; 54.29%), although Tulasnellaceae OTUs were additionally present in these individuals (7592 reads; 38.57%; Figure 2).

In *C. variabilis* and *O. patens*, both protocorms and coralloid rhizome-bearing adults were predominantly associated with Psathyrellaceae fungi, which accounted for 30,458 reads (98.47%) and 11,238 reads (99.91%) in *C. variabilis* protocorms and adults, and 26,627 reads (99.67%) and 22,456 reads (99.82%) in *O. patens* protocorms and adults, respectively. *Coprinopsis* OTU1 comprised 57.68%, 50.00%, 80.88% and 99.32% of total reads in *C. variabilis* protocorms, *C. variabilis* adults, *O. patens* protocorms and *O. patens* adults, respectively (Figure 3). In contrast, coralloid rhizome-lacking adults more frequently associated with Ceratobasidiaceae and

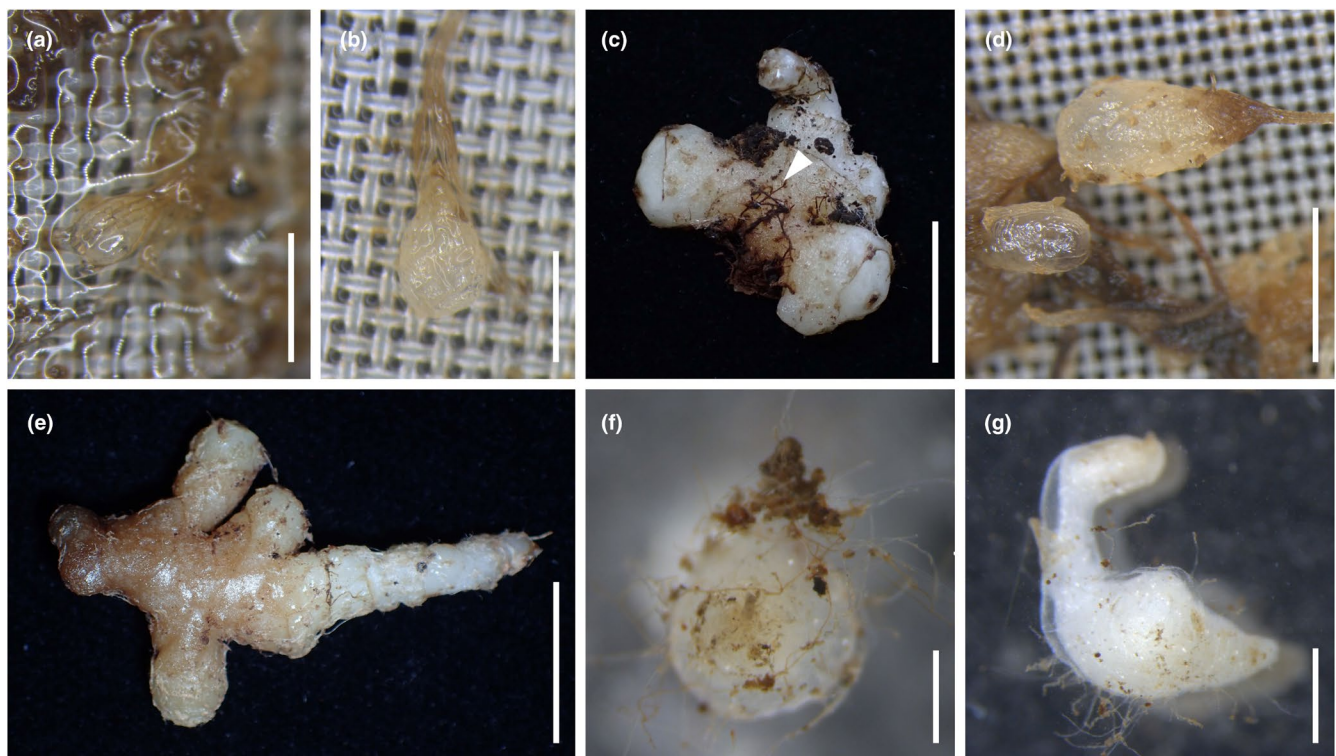


FIGURE 1 Protocorms of green Calypsoinae orchids investigated. (a, b) *Calypso bulbosa* protocorms. (c) Branched protocorm of *Cremastra variabilis*, with an arrow indicating mycorrhizal rhizomorphs. (d) *Oreorchis patens* protocorms. (e) Branched protocorm of *O. patens*. (f) *Tipularia japonica* protocorm. (g) *Tipularia japonica* protocorm with a developing leaf primordium. Scale bars: 1 mm (a, d, g), 0.5 mm (b, f) and 5 mm (c, e).

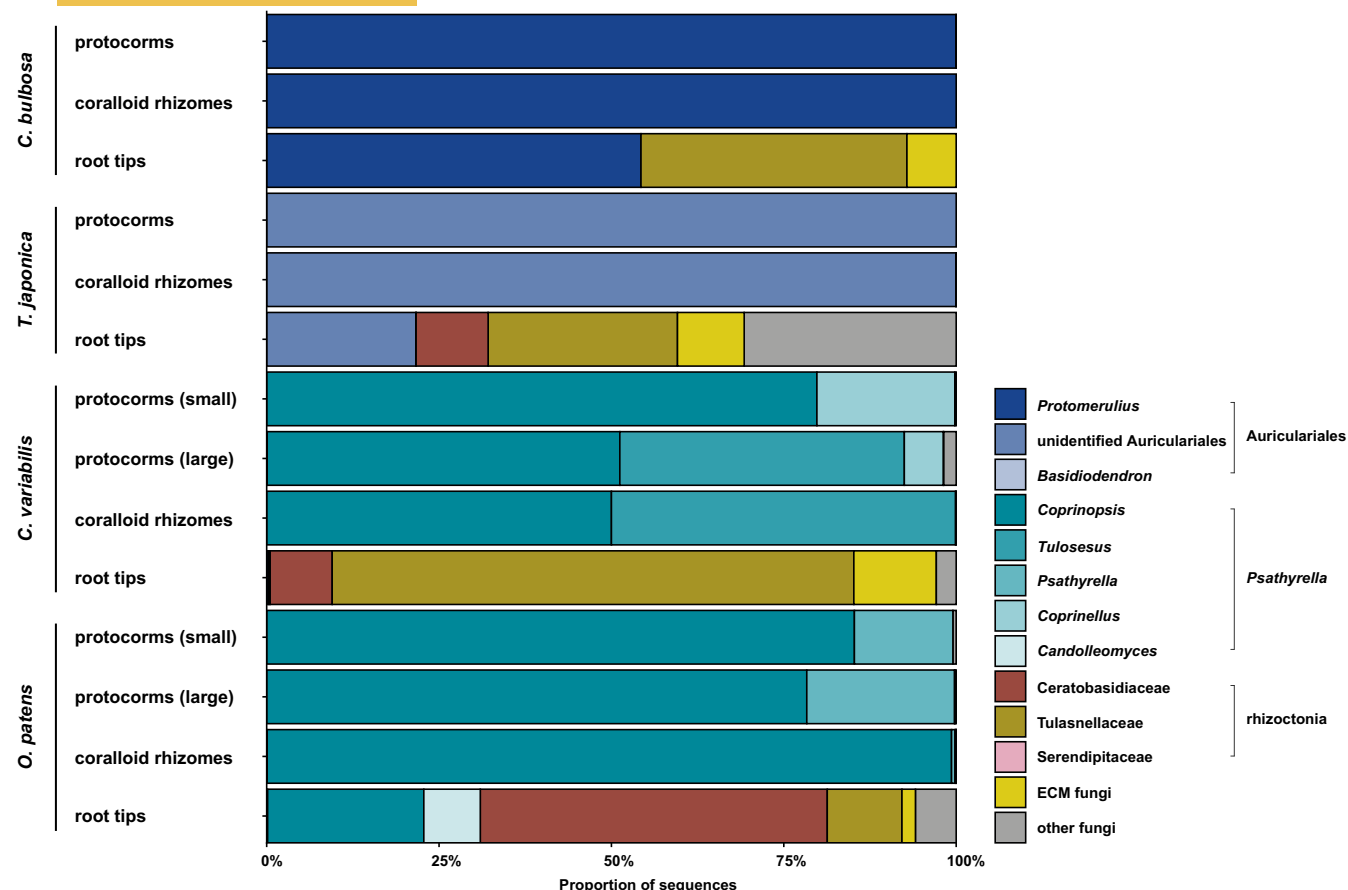


FIGURE 2 Relative abundance of mycorrhizal communities associated with the four Calypsoinae species investigated.

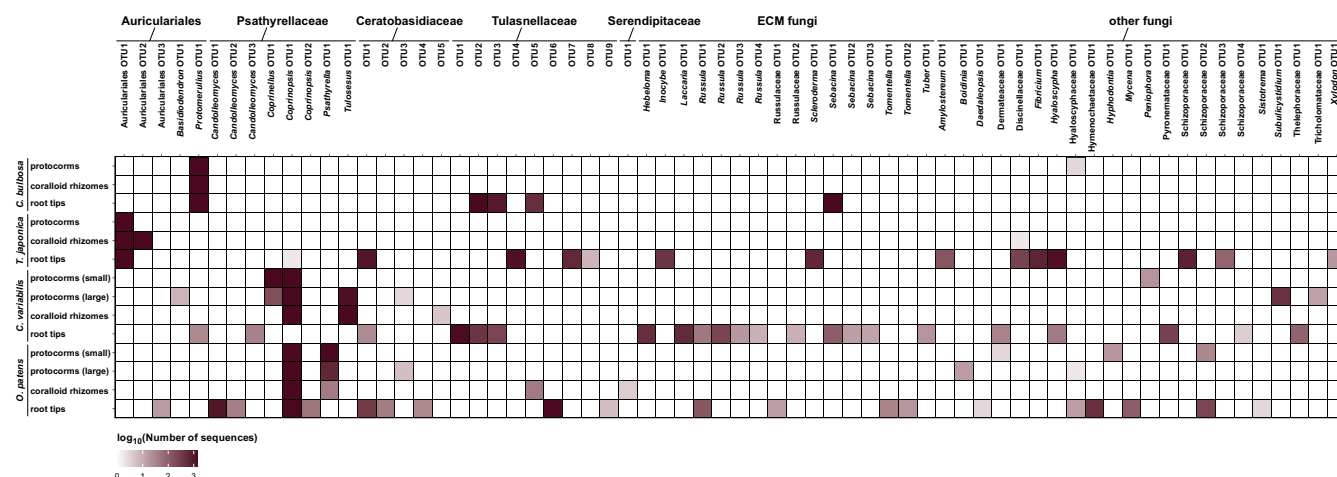


FIGURE 3 Interaction matrix showing associations between the four Calypsoinae species and their mycorrhizal fungi. Colour intensity represents the relative abundance of sequencing reads.

Tulasnellaceae OTUs (11,904 reads, 84.66% and 7739 reads, 61.16% in *C. variabilis* and *O. patens*, respectively) than with Psathyrellaceae OTUs (33 reads, 0.23% and 3936 reads, 30.87%).

Protocorms and coraloid rhizome-bearing adults of *T. japonica* were also predominantly associated with two Auriculariales OTUs, accounting for 8436 reads (100%) and 16,867 reads (99.99%),

respectively. These OTUs represented unidentified Auriculariales lineages. Based on BLAST search results using criteria of $\geq 90\%$ sequence similarity and $\geq 80\%$ query coverage, only one match was found for OTU1 (uncultured fungus from New Zealand, KX220116.1; 90.22% similarity), and no match was found for OTU2. Although OTU1 was also present in some coraloid rhizome-lacking

adults (2131 reads, 21.65%), these individuals more frequently associated with Ceratobasidiaceae and Tulasnellaceae OTUs (3731 reads, 37.91%).

3.3 | Fungal community analyses

The NMDS plot based on WUDMs revealed that the mycorrhizal communities of protocorms and conspecific adults with coralloid rhizomes were closely clustered, with many samples exhibiting complete overlap, indicating identical or nearly identical fungal assemblages in some cases (Figure 4).

Hierarchical clustering supported this pattern, showing that all protocorms, all coralloid rhizome-bearing adults, and some coralloid rhizome-lacking adults clustered together, while most remaining coralloid rhizome-lacking adults formed a distinct outgroup (Figure S2). PERMANOVA detected no significant differences in fungal community composition between protocorms and coralloid rhizome-bearing adults in all species except *T. japonica*, where Auriculariales OTU turnover was observed; protocorms were exclusively associated with OTU1, whereas coralloid rhizomes more frequently

harboured OTU2. Significant differences were detected between protocorms and coralloid rhizome-lacking adults, and between coralloid rhizome-bearing and coralloid rhizome-lacking adults in all species ($p < 0.05$ for all comparisons; Table S2). Protocorm size had no significant effect on fungal composition (PERMANOVA $p = 1$ in both comparisons; Table S2; Figure 4; Figure S2).

When *C. variabilis* and *O. patens* were analysed jointly, NMDS again showed close clustering between protocorms and coralloid rhizome-bearing adults, with many interspecific samples exhibiting overlap (Figure 5). Hierarchical clustering confirmed that all protocorms, all coralloid rhizome-bearing adults and some coralloid rhizome-lacking adults formed a single cluster, while the remaining coralloid rhizome-lacking adults grouped separately (Figure 6). PERMANOVA also found no significant differences between protocorms and coralloid rhizome-bearing adults across species (Table S3). Although interspecific similarity between *C. bulbosa* and *T. japonica* was less pronounced, NMDS, hierarchical clustering and PERMANOVA consistently showed that samples sharing the same phenotype (i.e. protocorms or adults with coralloid rhizomes) were generally more similar in mycorrhizal composition than conspecific samples differing in phenotype (Figures 5 and 6; Table S3).

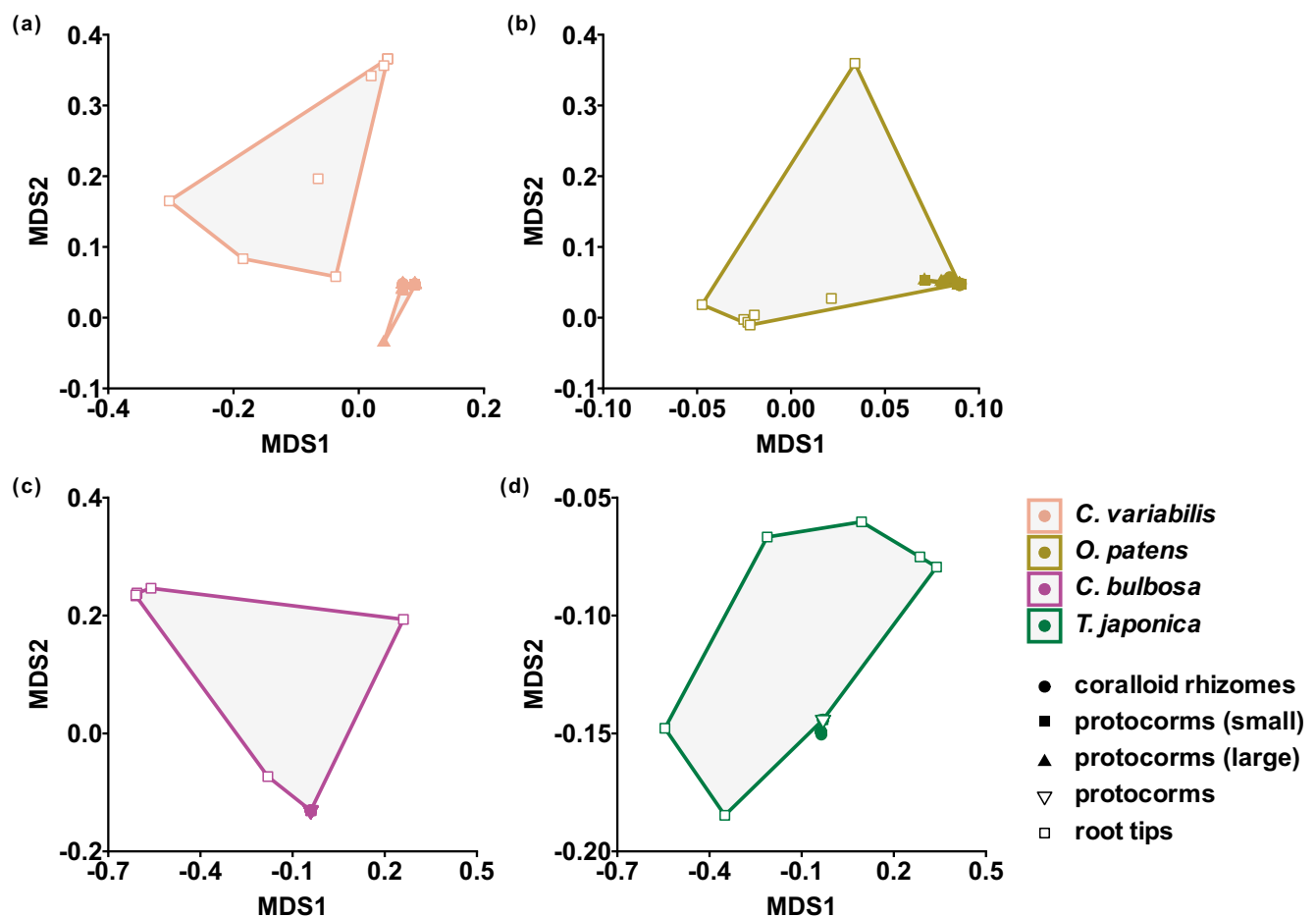


FIGURE 4 Non-metric multidimensional scaling plots of mycorrhizal communities in (a) *Cremastra variabilis*, (b) *Calypso bulbosa*, (c) *Oreorchis patens* and (d) *Tipularia japonica*, based on weighted UniFrac distance matrices. In the plots, many data points corresponding to protocorms and coralloid rhizome-bearing adults overlap due to nearly identical coordinates (Stress = 0.064).

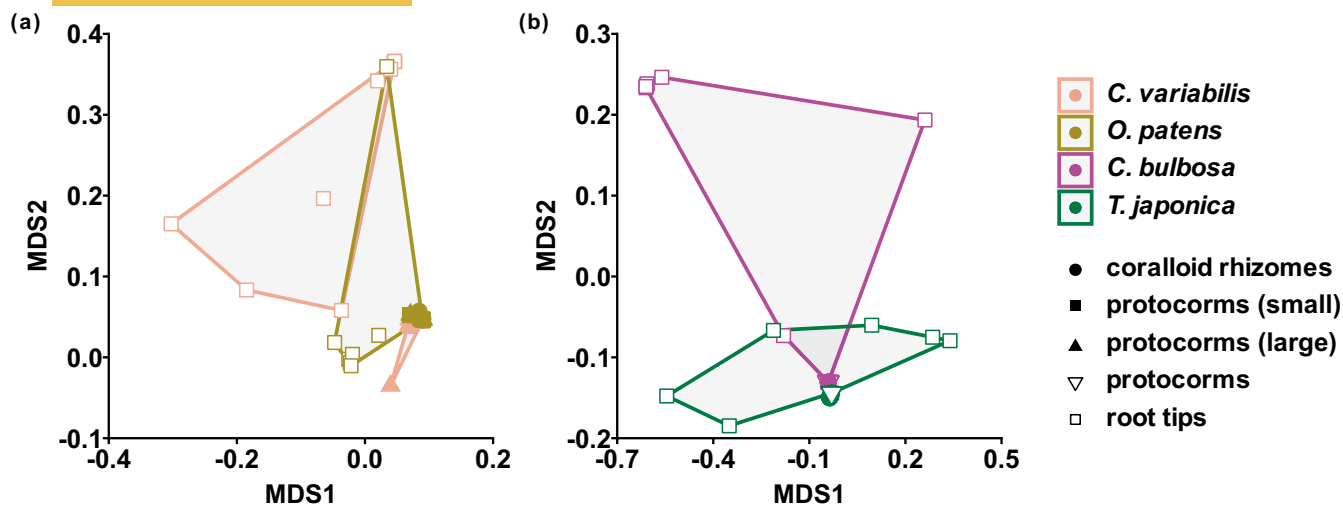


FIGURE 5 Non-metric multidimensional scaling plots of mycorrhizal communities in (a) *Crematstra variabilis* and *Oreorchis patens*, and (b) *Calypso bulbosa* and *Tipularia japonica*, based on weighted UniFrac distance matrices.

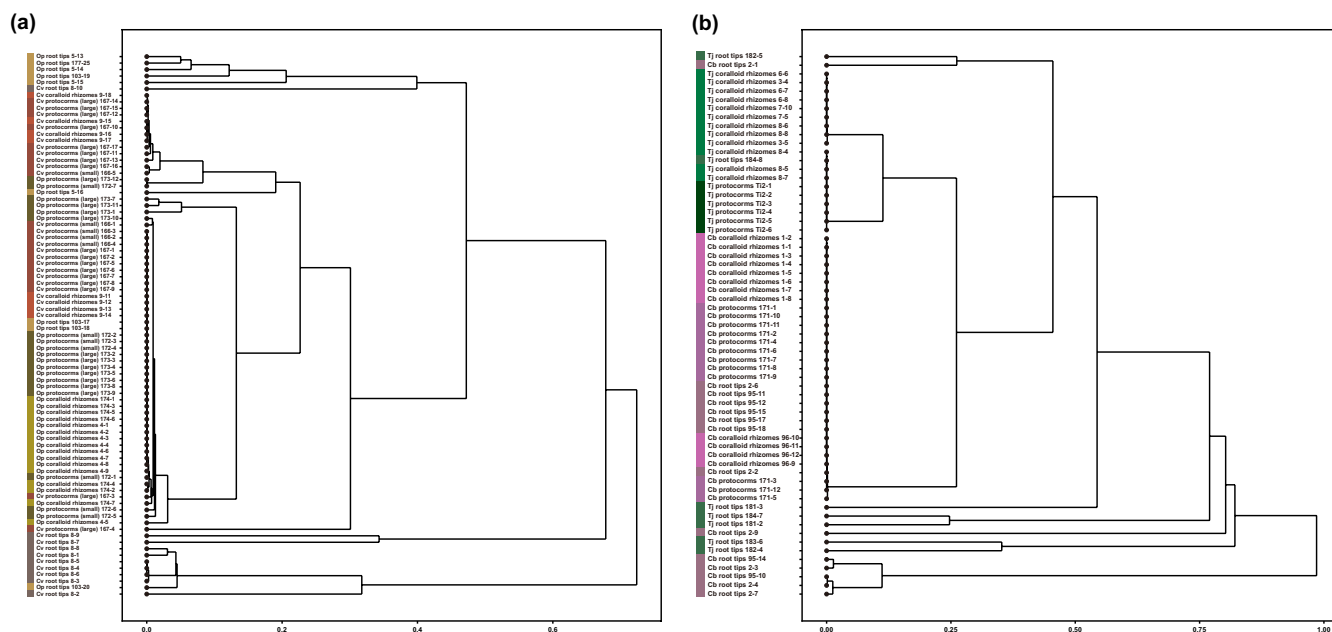


FIGURE 6 Hierarchical clustering of mycorrhizal communities associated with (a) *Crematstra variabilis* and *Oreorchis patens*, and (b) *Calypso bulbosa* and *Tipularia japonica*, based on weighted UniFrac distance matrices. The x-axis displays the cophenetic distance. Sampling ID details are provided in Table S1.

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

The $\delta^{13}\text{C}$ values of mature *T. japonica* individuals with coraloid rhizomes ($-29.2 \pm 0.6\text{‰}$; mean $\pm$ SD, $n=4$) were significantly higher than those of autotrophic reference plants ($-35.7 \pm 1.6\text{‰}$, $n=12$) and *T. japonica* individuals lacking coraloid rhizomes ($-35.0 \pm 0.6\text{‰}$, $n=5$; $p<0.001$; Figure 7; Table S4). In contrast, $\delta^{13}\text{C}$ values did not differ between individuals without coraloid rhizomes and autotrophic reference plants ($p=0.86$). The $\delta^{13}\text{C}$ value of deadwood ($-26.4 \pm 1.5\text{‰}$, $n=3$), which likely serves as an indirect carbon source for fungal

symbionts of protocorms and coraloid rhizome-bearing individuals, was intermediate between those of protocorms ($-23.2 \pm 0.7\text{‰}$, $n=3$) and coraloid rhizome-bearing adults.

In contrast, $\delta^{15}\text{N}$ values did not differ significantly among coraloid rhizome-bearing ($-3.0 \pm 1.1\text{‰}$) and coraloid rhizome-lacking adults ($-3.8 \pm 0.6\text{‰}$), protocorms ($-4.5 \pm 0.2\text{‰}$), deadwood ($-3.4 \pm 0.5\text{‰}$) and autotrophic reference plants ($p>0.05$ for all comparisons).

Mean $\varepsilon^{13}\text{C}$ and $\varepsilon^{15}\text{N}$ values for coraloid rhizome-bearing *T. japonica* were $6.6 \pm 0.5\text{‰}$ and $0.6 \pm 1.2\text{‰}$, respectively, whereas those for individuals lacking coraloid rhizomes were $0.6 \pm 0.7\text{‰}$

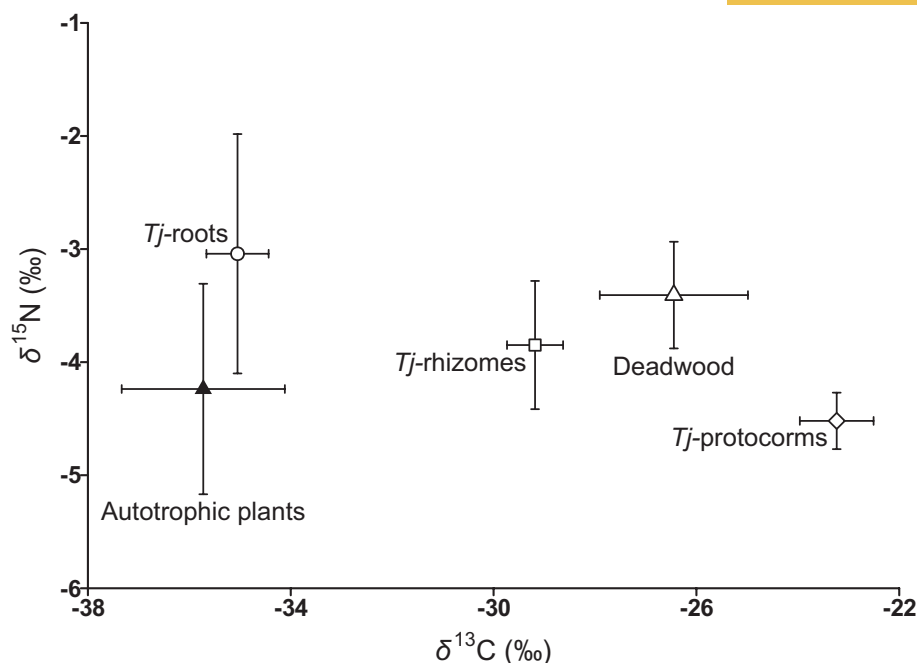


FIGURE 7 Mean ($\pm$ SD) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of *Tipularia japonica* and neighbouring autotrophic plants. Tj-protocorms, *T. japonica* protocorms; Tj-rhizomes, leaves of mature *T. japonica* with coralloid rhizomes; Tj-roots, leaves of mature *T. japonica* without coralloid rhizomes.

and $1.0 \pm 0.9\%$, respectively. A two-source mixing model estimated that coralloid rhizome-bearing individuals acquired approximately $52.2 \pm 4.3\%$ of their carbon from fungi.

4 | DISCUSSION

Our study showed that the four orchid species primarily associate with wood-decaying fungi during the protocorm stage. As decomposing wood holds substantial carbon in forest ecosystems (Rasmussen & Whigham, 1998), the ability to exploit this resource during early development likely aids seedling establishment in shaded understories. Previous studies also emphasize the role of decaying wood in germination and mycorrhizal colonization, even in rhizoctonia-associated orchids such as *Goodyera* and *Liparis* (McCormick et al., 2012). Since decaying wood has been identified as a favourable substrate for early development in several terrestrial orchid species, its role warrants consideration in conservation efforts. Additionally, while understory vegetation is already recognized as a key component of carbon storage (Dirnböck et al., 2020), our study further highlights the potential importance of organic carbon entering green orchids through wood-decaying fungi from decomposing wood. Our findings suggest that wood-derived carbon supports seedling establishment in shaded forests, offering new insight into plant adaptation to low light and mycorrhizal functional diversity.

Fully mycoheterotrophic orchids associated with wood-decaying fungi exhibit higher ^{13}C enrichment ($11.0 \pm 2.3\%$, $n=43$) than those exploiting ECM ($7.8 \pm 1.6\%$, $n=163$) or litter-decaying fungi

($8.2 \pm 0.5\%$, $n=15$) (Zahn et al., 2022), likely reflecting the high $\delta^{13}\text{C}$ values of woody substrates (Gebauer & Schulze, 1991). Consistent with previous findings for *C. bulbosa*, *C. variabilis*, and *O. patens* (Suetsugu et al., 2022; Suetsugu & Matsubayashi, 2021; Suetsugu & Okada, 2025b; Yagame et al., 2021), we observed pronounced ^{13}C enrichment in protocorms of *T. japonica* ($12.7 \pm 0.9\%$), exceeding that of the adjacent deadwood ($9.3 \pm 1.7\%$). Mature individuals of *T. japonica* with coralloid rhizomes also exhibited elevated ^{13}C enrichment ($6.6 \pm 0.5\%$), similar to levels reported in fully mycoheterotrophic orchids (Hynson et al., 2013), whereas those without coralloid rhizomes showed only minimal enrichment ($0.6 \pm 0.7\%$). Notably, protocorms of *T. discolor*, a congener distributed in North America, also displayed isotopic signatures resembling those of decaying wood (Whigham et al., 2002). This suggests that these congeners rely on fungi inhabiting similar wood-decaying niches. Indeed, both associate with Auriculariales fungi, which typically decompose wood, although their fungal partners belong to phylogenetically distinct clades (McCormick et al., 2004).

We found that fungal communities in protocorms closely resembled those in mature individuals bearing coralloid rhizomes across all four species. In *C. bulbosa*, both life stages were dominated by *Protomerulius* (Auriculariales); in *T. japonica*, by an unidentified Auriculariales lineage; and in *C. variabilis* and *O. patens*, by Psathyrellaceae fungi. Fine-scale turnover at the fungal species level was minimal, and NMDS and hierarchical clustering confirmed that fungal communities of protocorms and coralloid rhizomes clustered together. Given the morphological resemblance between protocorms and coralloid rhizomes, the latter have been interpreted as persistent protocorms retaining pedomorphic traits

(Freudenstein, 1994; Maekawa, 1971). The similarity in fungal composition supports this hypothesis.

Our prediction that mycorrhizal communities at the protocorm stage are similar between *C. variabilis* and *O. patens*, and between *C. bulbosa* and *T. japonica*, was partially supported. NMDS and hierarchical clustering analyses revealed that interspecific similarities in protocorm fungal communities were greater than the intraspecific differences between protocorms and mature plants lacking coralloid rhizomes. From an evolutionary perspective, the association between Auriculariales fungi and *C. bulbosa*, *T. discolor* and *T. japonica* (McCormick et al., 2004; Suetsugu & Matsubayashi, 2021) suggests that these wood-decaying fungi may represent ancestral mycobionts during germination within the clade comprising *Calypso*, *Tipularia* and *Changnienia*, even though no OTUs are shared. The mycorrhizal associations of the monotypic genus *Changnienia* and other *Tipularia* species merit further investigation.

Moreover, *C. variabilis* and *O. patens* shared a Psathyrellaceae fungus even at the OTU level during the protocorm stage. Given that *Cremastra* is sister to *Oreorchis*, *Kitigorchis* and *Corallorhiza*, the latter two being exclusively ECM-associated (Barrett et al., 2025; Suetsugu et al., 2021), one possible scenario is a transition from rhizoctonia to Psathyrellaceae fungi, followed by a shift to ECM fungi. Alternatively, independent transitions to Psathyrellaceae fungi may have occurred. If recruitment of the same fungal partner occurred independently, it may have been facilitated by the utilization of fungi that are less capable of detecting exploitation by mycoheterotrophic orchids (Perez-Lamarque et al., 2020; Zhao et al., 2021). Expanded taxonomic sampling and ancestral state reconstruction will be essential for a more precise understanding of mycorrhizal partner shifts in Calypsoinae.

Orchids with strong mycoheterotrophic tendencies frequently associate with ECM or SAP fungi, likely due to their greater carbon availability (Wang et al., 2021). As woody debris represents a major carbon source in forests (Rasmussen & Whigham, 1998), associations with wood-decaying fungi may enhance carbon acquisition, especially in warm, humid habitats (Martos et al., 2009; Ogura-Tsujita et al., 2009; Suetsugu et al., 2020). Despite its modest species diversity (~80 spp.), Calypsoinae has experienced at least four independent origins of full mycoheterotrophy (Freudenstein et al., 2017). Frequent association with SAP fungi during early development, followed by lifelong specialization on these fungi, probably facilitates such evolutionary shifts. For instance, the fully mycoheterotrophic *Cremastra aphylla* exclusively associates with wood-decaying Psathyrellaceae fungi, whereas its leafy relatives *C. variabilis* and *C. appendiculata* germinate with phylogenetically similar Psathyrellaceae fungi and later associate with both rhizoctonia and Psathyrellaceae fungi (Suetsugu et al., 2022; Yagame et al., 2013, 2021; Zahn et al., 2022).

Although the mechanisms underlying the shift from SAP to rhizoctonia fungi remain unclear, our data provide insight through germination patterns. Germination occurred only at a few sites, specifically near adult individuals with coralloid rhizomes and dead wood, and was absent near those lacking such structures. Even at

favourable sites, germination was infrequent. These patterns suggest that shifts in fungal partners may be driven by the limited availability of fungi required for germination (Rasmussen, 2002; Suetsugu & Okada, 2025b). Notably, wood-decaying fungi that support germination are often late-stage decomposers involved in humus formation (Oliver et al., 2010; Rasmussen & Whigham, 1998). As decomposition progresses and plants mature, compatible fungi likely become increasingly scarce. Thus, even where adult plants with coralloid rhizomes persist, they may retain previously acquired SAP fungi, while compatible fungi in surrounding substrates may have already declined, potentially explaining the frequent absence of germination. Similarly, some adults lacking coralloid rhizomes were found to associate with SAP fungi, possibly representing a transitional phase toward rhizoctonia fungi as SAP fungi diminish.

Such spatial and temporal instability likely promotes a shift to rhizoctonia fungi, while SAP fungi support optimal early development. Notably, orchids that retain wood-decaying fungal partners into maturity tend to have shorter lifespans than those associating with ECM fungi (Ogura-Tsujita et al., 2021). This may explain why green orchids that initially associate with SAP fungi often transition to rhizoctonia fungi (Bayman et al., 2016; Suetsugu & Okada, 2025b; Yagame et al., 2013; Zahn et al., 2022), whereas species forming ECM associations at germination usually maintain them (Bidartondo & Read, 2008; Jacquemyn et al., 2018; Těšitelová et al., 2012). Quantifying the abundance and distribution of SAP and rhizoctonia fungi near protocorms and mature plants, with or without coralloid rhizomes, would help clarify the mechanisms driving these transitions.

However, comparable ontogenetic transitions are also documented in other plant lineages associated with ECM or arbuscular mycorrhizal fungi, which are typically more stable than wood-decaying fungi. For example, *Pyrola asarifolia* (Ericaceae) initially associates with Serendipitaceae fungi before transitioning to a wider range of ECM fungi, while *Sceptridium* ferns begin with Entrophosporaceae and later shift to Glomeraceae (Hashimoto et al., 2012; Suetsugu et al., 2025). These ontogenetic shifts likely reflect altered physiological demands between the fully mycoheterotrophic and autotrophic phases (Leake et al., 2008; Suetsugu et al., 2025). During the mycoheterotrophic stage, carbon acquisition is essential, while mineral uptake becomes increasingly important during autotrophic growth, as observed in typical mycorrhizal symbioses. Thus, shifts in fungal partners may partly reflect the distinct functional roles of each group, with wood-decaying fungi ensuring carbon uptake and rhizoctonia fungi contributing to mineral acquisition. Further investigations, including controlled culture experiments (e.g. Yagame et al., 2024), are needed to clarify the physiological roles of each fungal group.

In summary, our findings underscore the role of wood-decaying fungi in supporting early mycoheterotrophic development in green orchids. These associations demonstrate that carbon from decomposing wood, a key resource in forest ecosystems, can be transferred to green plants through fungal associations. This likely plays a crucial role in seedling establishment on shaded forest floors and

broadens our understanding of mycorrhizal functional diversity. Nevertheless, as this carbon source is likely transient, a subsequent shift to rhizoctonia fungi, which presumably exhibit reduced SAP capacity (Schweiger et al., 2018), often occurs. The persistence of coralloid rhizomes, which retain fungal communities resembling those in protocorms, enables mycoheterotrophic traits to extend into adulthood. This persistence likely predisposes certain orchids, such as those in Calypsoinae, to evolve fully mycoheterotrophic lifestyles.

AUTHOR CONTRIBUTIONS

Kenji Suetsugu conceived the ideas, designed the methodology and collected the data; Kenji Suetsugu and Hidehito Okada analysed the data; Kenji Suetsugu led the writing of the manuscript. All authors contributed critically to the drafts and approved the final version for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The sequence data have been deposited in the NCBI Sequence Read Archive (accession no. PRJNA1257467). The processed data are available from Figshare (<https://doi.org/10.6084/m9.figshare.30163951>).

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REFERENCES

- Arditti, J., & Ghani, A. K. A. (2000). Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist*, 145(3), 367–421. <https://doi.org/10.1046/j.1469-8137.2000.00587.x>
- Barrett, C. F., & Freudenstein, J. V. (2011). An integrative approach to delimiting species in a rare but widespread mycoheterotrophic orchid. *Molecular Ecology*, 20(13), 2771–2786.
- Barrett, C. F., Freudenstein, J. V., Skibicki, S. V., Sinn, B. T., Chung, S.-W., Hsu, T.-C., Liao, W., Lee, S. Y., Luo, Y.-B., Yukawa, T., & Suetsugu, K. (2025). Phylogenomics and intergenomic conflict in a challenging orchid clade (Calypsoinae): Monophyly of *Corallorhiza*, paraphyly of *Oreorchis*, and resurrection of *Kitigorhchis*. *Botanical Journal of*

- the Linnean Society, 209(1), 20–38. <https://doi.org/10.1093/botlinnean/boae092>
- Barrett, C. F., Freudenstein, J. V., Taylor, D. L., & Kõljalg, 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(4), 628–643. <https://doi.org/10.3732/ajb.0900230>
- Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1–48. <https://doi.org/10.18637/jss.v067.i01>
- Bayman, P., Mosquera-Espinosa, A. T., Saladini-Aponte, C. M., Hurtado-Guevara, N. C., & Viera-Ruiz, N. L. (2016). Age-dependent mycorrhizal specificity in an invasive orchid, *Oeceoclades maculata*. *American Journal of Botany*, 103(11), 1880–1889. <https://doi.org/10.3732/ajb.1600127>
- Bidartondo, M. I., & Read, D. J. (2008). Fungal specificity bottlenecks during orchid germination and development. *Molecular Ecology*, 17(16), 3707–3716.
- Cameron, D. D., Johnson, I., Read, D. J., & Leake, J. R. (2008). Giving and receiving: Measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. *New Phytologist*, 180(1), 176–184. <https://doi.org/10.1111/j.1469-8137.2008.02533.x>
- Chen, J., & Chen, M. J. (2021). Package ‘GUniFrac.’ The Comprehensive R Archive Network (CRAN).
- Dearnaley, J. D. W., Martos, F., & Selosse, M.-A. (2012). Orchid mycorrhizas: Molecular ecology, physiology, evolution and conservation aspects. In B. Hock (Ed.), *Fungal associations* (Vol. 9, pp. 207–230). Springer.
- Dirnböck, T., Kraus, D., Grote, R., Klatt, S., Kobler, J., Schindlbacher, A., Seidl, R., Thom, D., & Kiese, R. (2020). Substantial understory contribution to the C sink of a European temperate mountain forest landscape. *Landscape Ecology*, 35(2), 483–499. <https://doi.org/10.1007/s10980-019-00960-2>
- Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, 12(1), 13–15.
- Freudenstein, J. V. (1994). Character transformation and relationships in *Corallorhiza* (Orchidaceae: Epidendroideae). II. Morphological variation and phylogenetic analysis. *American Journal of Botany*, 81(11), 1458–1467.
- 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(4), 759–772.
- Freudenstein, J. V., Yukawa, T., & Luo, Y. (2017). A reanalysis of relationships among Calypsoinae (Orchidaceae: Epidendroideae): Floral and vegetative evolution and the placement of *Yuania*. *Systematic Botany*, 42(1), 17–25.
- Gebauer, G., & Meyer, M. (2003). ¹⁵N and ¹³C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist*, 160(1), 209–223. <https://doi.org/10.1046/j.1469-8137.2003.00872.x>
- Gebauer, G., & Schulze, E. D. (1991). Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia*, 87(2), 198–207.
- Gillman, H. (1876). *Aplectrum* with coral-like root. *Bulletin of the Torrey Botanical Club*, 6(18), 94–95.
- Hashimoto, Y., Fukukawa, S., Kunishi, A., Suga, H., Richard, F., Sauve, M., & Selosse, M. (2012). Mycoheterotrophic germination of *Pyrola asarifolia* dust seeds reveals convergences with germination in orchids. *New Phytologist*, 195(3), 620–630.
- Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, 35(2), 518–522.
- Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3), 346–363. <https://doi.org/10.1002/bimj.200810425>

- 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., Lievens, B., & Wiegand, T. (2012). Spatial variation in below-ground seed germination and divergent mycorrhizal associations correlate with spatial segregation of three co-occurring orchid species. *Journal of Ecology*, 100(6), 1328–1337. <https://doi.org/10.1111/j.1365-2745.2012.01998.x>
- 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(1), 73–84. <https://doi.org/10.1111/oik.04329>
- Jacquemyn, H., & Merckx, V. S. F. T. (2019). Mycorrhizal symbioses and the evolution of trophic modes in plants. *Journal of Ecology*, 107(4), 1567–1581. <https://doi.org/10.1111/1365-2745.13165>
- Johnson-Groh, C., Riedel, C., Schoessler, L., & Skogen, K. (2002). Belowground distribution and abundance of *Botrychium gametophytes* and juvenile sporophytes. *American Fern Journal*, 92(2), 80–92. [https://doi.org/10.1640/0002-8444\(2002\)092\[0080:BDAAOB\]2.0.CO;2](https://doi.org/10.1640/0002-8444(2002)092[0080:BDAAOB]2.0.CO;2)
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780. <https://doi.org/10.1093/molbev/mst010>
- Leake, J. R., Cameron, D. D., & Beerling, D. J. (2008). Fungal fidelity in the mycoheterotroph-to-autotroph life cycle of Lycopodiaceae: A case of parental nurture? *New Phytologist*, 177, 572–576.
- Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, 71(12), 8228–8235.
- Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. *The ISME Journal*, 5(2), 169–172.
- MacDougal, D. T. (1899). Symbiosis and saprophytism. *Bulletin of the Torrey Botanical Club*, 26(10), 511–530.
- Maekawa, F. (1971). *The wild orchids of Japan in color*. Seibundo-shinkosha.
- Martinez Arbizu, P. (2020). *PairwiseAdonis: Pairwise multilevel comparison using adonis*. R package version 0.4.0. <https://github.com/pmartinezarbizu/pairwiseAdonis>
- Martos, F., Dulormne, M., Pailler, T., Bonfante, P., Faccio, A., Fournel, J., Dubois, M. P., & Selosse, M. A. (2009). Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous orchids. *New Phytologist*, 184(3), 668–681.
- Mason, N. A., & Farrar, D. R. (1989). Recovery of *Botrychium gametophytes*, gemmae and immature sporophytes by centrifugation. *American Journal of Botany*, 79(4), 143–145.
- McCormick, M. K., Lee Taylor, D., Juhaszova, K., Burnett, R. K., Whigham, D. F., & O'Neill, J. P. (2012). Limitations on orchid recruitment: Not a simple picture. *Molecular Ecology*, 21(6), 1511–1523. <https://doi.org/10.1111/j.1365-294X.2012.05468.x>
- McCormick, M. K., Whigham, D. F., & O'Neill, J. (2004). Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist*, 163(2), 425–438. <https://doi.org/10.1111/j.1469-8137.2004.01114.x>
- McKendrick, S. L., Leake, J. R., Taylor, D. L., & Read, D. J. (2002). Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytologist*, 154(1), 233–247.
- Meng, Y.-Y., Fan, X.-L., Zhou, L.-R., Shao, S.-C., Liu, Q., Selosse, M.-A., & Gao, J.-Y. (2019). Symbiotic fungi undergo a taxonomic and functional bottleneck during orchid seeds germination: A case study on *Dendrobium moniliforme*. *Symbiosis*, 79(3), 205–212. <https://doi.org/10.1007/s13199-019-00647-x>
- Merckx, V. S. F. T. (2013). *Mycoheterotrophy: The biology of plants living on fungi* (1st ed.). Springer.
- Merckx, V. S. F. T., & Freudenstein, J. V. (2010). Evolution of mycoheterotrophy in plants: A phylogenetic perspective. *New Phytologist*, 185(3), 605–609. <https://doi.org/10.1111/j.1469-8137.2009.03155.x>
- 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(1), 268–274.
- 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(1657), 761–767. <https://doi.org/10.1098/rspb.2008.1225>
- Ogura-Tsujita, Y., Tetsuka, K., Tagane, S., Kubota, M., Anan, S., Yamashita, Y., Tone, K., & Yukawa, T. (2021). Differing life-history strategies of two mycoheterotrophic orchid species associated with leaf litter and wood-decaying fungi. *Diversity*, 13(4), 161.
- Oja, J., Kohout, P., Tedersoo, L., Kull, T., & Kõljalg, U. (2015). Temporal patterns of orchid mycorrhizal fungi in meadows and forests as revealed by 454 pyrosequencing. *New Phytologist*, 205(4), 1608–1618. <https://doi.org/10.1111/nph.13223>
- Oliver, J. P., Perkins, J., & Jellison, J. (2010). Effect of fungal pretreatment of wood on successional decay by several inky cap mushroom species. *International Biodeterioration & Biodegradation*, 64(7), 646–651.
- Park, E. J., & Lee, W. Y. (2013). In vitro symbiotic germination of mycoheterotrophic *Gastrodia elata* by *Mycena* species. *Plant Biotechnology Reports*, 7(2), 185–191.
- Perez-Lamarque, B., Selosse, M.-A., Öpik, M., Morlon, H., & Martos, F. (2020). Cheating in arbuscular mycorrhizal mutualism: A network and phylogenetic analysis of mycoheterotrophy. *New Phytologist*, 226(6), 1822–1835. <https://doi.org/10.1111/nph.16474>
- Preiss, K., & Gebauer, G. (2008). A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes in Environmental and Health Studies*, 44(4), 393–401. <https://doi.org/10.1080/10256010802507458>
- R Core Team. (2025). *R: A language and environment for statistical computing* [Computer software]. R foundation for statistical computing. <https://www.R-project.org/>
- Rasmussen, H. N. (2002). Recent developments in the study of orchid mycorrhiza. *Plant and Soil*, 244(1), 149–163.
- Rasmussen, H. N., & Rasmussen, F. N. (2014). Seedling mycorrhiza: A discussion of origin and evolution in Orchidaceae. *Botanical Journal of the Linnean Society*, 175(3), 313–327.
- Rasmussen, H. N., & Whigham, D. (1998). Importance of woody debris in seed germination of *Tipularia discolor* (Orchidaceae). *American Journal of Botany*, 85(6), 829–834.
- Rasmussen, H. N., & Whigham, D. F. (1993). Seed ecology of dust seeds in situ: A new study technique and its application in terrestrial orchids. *American Journal of Botany*, 80(12), 1374–1378.
- Schweiger, J. M. I., Bidartondo, M. I., & Gebauer, G. (2018). Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi. *Functional Ecology*, 32(4), 870–881. <https://doi.org/10.1111/1365-2435.13042>
- 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(2), 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(1), 333–343. <https://doi.org/10.1111/nph.17313>
- Suetsugu, K., & Matsubayashi, J. (2021). Subterranean morphology modulates the degree of mycoheterotrophy in a green orchid *Calypso bulbosa* exploiting wood-decaying fungi. *Functional Ecology*, 35(10), 2305–2315. <https://doi.org/10.1111/1365-2435.13864>

- Suetsugu, K., Matsubayashi, J., & Tayasu, I. (2020). Some mycoheterotrophic orchids depend on carbon from dead wood: Novel evidence from a radiocarbon approach. *New Phytologist*, 227(5), 1519–1529. <https://doi.org/10.1111/nph.16409>
- Suetsugu, K., & Okada, H. (2025a). Green, variegated, and albino *Cremastra variabilis* provide insight into mycoheterotrophic evolution associated with wood-decaying fungi. *Plant Biology*, 27(4), 602–613. <https://doi.org/10.1111/plb.70014>
- Suetsugu, K., & Okada, H. (2025b). Subterranean morphology underpins the degree of mycoheterotrophy, mycorrhizal associations, and plant vigor in the green orchid *Oreorchis patens*. *The Plant Journal*, 121(4), e70045. <https://doi.org/10.1111/tpj.70045>
- Suetsugu, K., Okada, H., Hirota, S. K., Yamasaki, M., Imaichi, R., & Ebihara, A. (2025). Drastic mycorrhizal community shifts in *Sceptridium* ferns during the generation transition from fully mycoheterotrophic gametophytes to photosynthetic sporophytes. *New Phytologist*, 245(4), 1705–1717. <https://doi.org/10.1111/nph.20330>
- 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(10), e76910. <https://doi.org/10.1371/journal.pone.0076910>
- 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(3), 261–266. <https://doi.org/10.1007/s10201-011-0345-5>
- Těšitelová, T., Těšitel, J., Jersáková, J., Říhová, G., & Selosse, M.-A. (2012). Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology. *American Journal of Botany*, 99(6), 1020–1032. <https://doi.org/10.3732/ajb.1100503>
- Ventre Lespiaucq, A., Jacquemyn, H., Rasmussen, H. N., & Méndez, M. (2021). Temporal turnover in mycorrhizal interactions: A proof of concept with orchids. *New Phytologist*, 230(5), 1690–1699.
- Wang, D., Jacquemyn, H., Gomes, S. I. F., Vos, R. A., & Merckx, V. S. F. T. (2021). Symbiont switching and trophic mode shifts in Orchidaceae. *New Phytologist*, 231(2), 791–800. <https://doi.org/10.1111/nph.17414>
- 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(4), 679–699. <https://doi.org/10.1111/1755-0998.12229>
- Whigham, D. F., O'Neill, J., McCormick, M. K., Smith, C., Rasmussen, H. R., Caldwell, B., & Daniell, T. (2002). Interactions between decomposing wood, mycorrhizas, and terrestrial orchid seeds and protocorms. In P. Kindlmann, J. H. Willems, & D. F. Whigham (Eds.), *Trends and fluctuations and underlying mechanisms in terrestrial orchid populations* (pp. 117–132). Backhuys Publishers.
- Yagame, T., Figura, T., Tanaka, E., Selosse, M.-A., & Yukawa, T. (2024). Mycobiont identity and light conditions affect belowground morphology and physiology of a mixotrophic orchid *Cremastra variabilis* (Orchidaceae). *Mycorrhiza*, 34(1–2), 19–31. <https://doi.org/10.1007/s00572-024-01138-8>
- Yagame, T., Funabiki, E., Nagasawa, E., Fukiharu, T., & Iwase, K. (2013). Identification and symbiotic ability of Psathyrellaceae fungi isolated from a photosynthetic orchid, *Cremastra appendiculata* (Orchidaceae). *American Journal of Botany*, 100(9), 1823–1830. <https://doi.org/10.3732/ajb.1300099>
- 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(6), 1213–1224.
- 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(3), 1–10. <https://doi.org/10.1093/aobpla/plac021>
- Zhao, Z., Li, X., Liu, M. F., Merckx, V. S. F. T., Saunders, R. M. K., & Zhang, D. (2021). Specificity of assemblage, not fungal partner species, explains mycorrhizal partnerships of mycoheterotrophic *Burmanna* plants. *The ISME Journal*, 15, 1614–1627.

SUPPORTING INFORMATION

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

Figure S1. Dimorphic subterranean morphology of *Tipularia japonica*. (a) Mature plant lacking coralloid rhizomes. (b) Mature plant bearing coralloid rhizomes. Scale bar: 3 cm.

Figure S2. Hierarchical clustering of mycorrhizal communities associated with (a) *Cremastra variabilis*, (b) *Calypso bulbosa*, (c) *Oreorchis patens* and (d) *Tipularia japonica*, based on weighted UniFrac distance matrices. The x-axis displays the cophenetic distance. Sampling ID details are provided in Table S1.

Table S1. Sequencing read counts for OTUs detected in each sample after abundance-based rarefaction.

Table S2. Results of pairwise PERMANOVA comparisons among intraspecific plant phenotypes, based on weighted UniFrac distance matrices.

Table S3. Results of pairwise PERMANOVA comparisons among interspecific plant phenotypes, based on weighted UniFrac distance matrices.

Table S4. Values of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\epsilon^{13}\text{C}$, $\epsilon^{15}\text{N}$ and C and N concentrations in *Tipularia japonica* and neighboring deadwood and autotrophic reference plants.

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