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RESEARCH

Partial mycoheterotrophy in the leafless orchid *Eulophia zollingeri* specialized on wood-decaying fungi

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Abstract

Although the absence of normal leaves is often considered a sign of full heterotrophy, some plants remain at least partially autotrophic despite their leafless habit. Leafless orchids with green stems and capsules probably represent a late evolutionary stage toward full mycoheterotrophy and serve as valuable models for understanding the pathways leading to this nutritional strategy. In this study, based on molecular barcoding and isotopic analysis, we explored the physiological ecology of the leafless orchid *Eulophia zollingeri*, which displays green coloration, particularly during its fruiting phase. Although previous studies had shown that *E. zollingeri*, in its adult stage, is associated with Psathyrellaceae fungi and exhibits high ^{13}C isotope signatures similar to fully mycoheterotrophic orchids, it remained uncertain whether this symbiotic relationship is consistent throughout the orchid's entire life cycle and whether the orchid relies exclusively on mycoheterotrophy for its nutrition during the fruiting season. Our study has demonstrated that *E. zollingeri* maintains a specialized symbiotic relationship with Psathyrellaceae fungi throughout all life stages. However, isotopic analysis and chlorophyll data have shown that the orchid also engages in photosynthesis to meet its carbon needs, particularly during the fruiting stage. This research constitutes the first discovery of partial mycoheterotrophy in leafless orchids associated with saprotrophic non-rhizoctonia fungi.

Keywords Autotrophy · Carbon acquisition · Chlorophyll · Leafless orchids · Mixotrophy · Protocorm · Saprotrophic fungi · Stable isotopes

Introduction

Orchidaceae, the most species-rich family in angiosperms, is renowned for its tiny seeds with minimal nutrients (Arditti and Ghani 2000). These plants rely entirely on mycorrhizal fungi for their carbon needs during their early underground growth, a phenomenon known as mycoheterotrophy (Leake 1994). Phylogenetic studies have revealed that the loss of photosynthesis in adulthood, coupled with full mycoheterotrophy, has independently occurred at least 40 times within Orchidaceae (Merckx and Freudenstein 2010; Jacquemyn and Merckx 2019). Recent advancements in our understanding of these intriguing plants have been facilitated by the application of molecular and isotopic approaches.

Stable isotope analysis is a crucial method for assessing orchid nutrition, relying on two key observations: (i) fungal tissues are enriched in heavy carbon and nitrogen isotopes compared to autotrophic plants (Gebauer and Dietrich 1993; Gleixner et al. 1993) and (ii) fully mycoheterotrophic orchids have isotopic signatures similar to or slightly higher than their fungal partners (Gebauer and Meyer 2003; Trudell et al. 2003). Gebauer and Meyer (2003) discovered that some green species of *Cephalanthera* and *Epipactis* exhibit higher ^{13}C and ^{15}N levels compared to nearby autotrophic plants, yet these levels are lower than those in fully mycoheterotrophic plants. This indicates partial mycoheterotrophy, a mixotrophic nutritional mode that combines autotrophy and mycoheterotrophy in the adult stage (Gebauer and Meyer 2003). Partial mycoheterotrophy has later been recognized in a wide array of orchids in most orchid subfamilies, including the basal clade (Bidartondo et al. 2004; Julou et al. 2005;

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Motomura et al. 2010; Gebauer et al. 2016; Suetsugu and Matsubayashi 2021a; Suetsugu et al. 2021b, c).

Furthermore, molecular identifications of mycobionts have established a clear link between the nutritional modes of orchids and their fungal associations. Most green orchids associate with "rhizoctonia" basidiomycetes, including Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae (Dearnaley et al. 2013; Rasmussen and Rasmussen 2014). However, fully mycoheterotrophic orchids, except for albino variants, specialize in associations with ectomycorrhizal or saprotrophic non-rhizoctonia fungi (Jacquemyn and Merckx 2019; Ogura-Tsujita et al. 2021). Additionally, in line with their intermediate isotopic signatures, partially mycoheterotrophic orchids with a relatively high degree of mycoheterotrophy also tend to affiliate with these fungi (Bidartondo et al. 2004; Selosse and Roy 2009; Hynson et al. 2013; Suetsugu et al. 2022). This suggests that the transition to exploiting ectomycorrhizal fungi or saprotrophic non-rhizoctonia fungi likely occurred before the loss of photosynthesis (Selosse and Roy 2009; Motomura et al. 2010; Jacquemyn and Merckx 2019; Suetsugu et al. 2022).

During the evolution of full mycoheterotrophy, another prominent trend has been the substantial reduction of leaves (Merckx et al. 2013; Tsukaya 2018). Notably, lineages of partial mycoheterotrophs exhibit a range of leaf development. Some species, such as *Cephalanthera damasonium* and *Epipactis helleborine*, retain well-developed foliage leaves (Julou et al. 2005; Stöckel et al. 2011; Suetsugu et al. 2017). In contrast, others, such as *Cephalanthera subaphylla*, *Epipactis microphylla*, and *Stigmatodactylus sikokianus*, produce highly reduced ones (Selosse et al. 2004; Sakamoto et al. 2016; Suetsugu et al. 2021a). The presence of these diverse phenotypes suggests that the process of leaf reduction during evolution was gradual rather than the result of a single-step mutation (Roy et al. 2013).

Moreover, some orchids, such as *Limodorum abortivum*, *Cymbidium macrorhizon*, and *Corallorhiza trifida*, lack foliage leaves but maintain green stems and capsules. While these leafless orchids have often been presumed to be fully mycoheterotrophic, several studies have indicated that they retain some degree of autotrophic capacity (Girlanda et al. 2005; Zimmer et al. 2008; Cameron et al. 2009). For instance, chlorophyll accumulation has been found in shoots and capsules of *C. macrorhizon*, especially during fruiting (Suetsugu et al. 2018; Kobayashi et al. 2021). Using a linear two-source mixing model with sprouting plants as 100% carbon gain from fungi and autotrophic plants as the 0% baseline, Suetsugu et al. (2018) estimated that fruiting *C. macrorhizon* plants obtain about 25% of their total carbon from autotrophy.

While their contribution to net carbon acquisition may be limited, leafless orchids with green stems and capsules likely represent a late stage in the evolution of full mycoheterotrophy, offering valuable models for understanding the pathways leading to complete heterotrophy (Cameron et al. 2009; Suetsugu et al. 2018; Kobayashi et al. 2021). For instance, although fully mycoheterotrophic plants often exhibit higher mycorrhizal specificity than partially mycoheterotrophic ones (Hynson et al. 2013; Jacquemyn and Merckx 2019), these leafless species with reduced photosynthetic ability also tend to associate predominantly with narrow clades of ectomycorrhizal fungi (Girlanda et al. 2005; Zimmer et al. 2008; Ogura-Tsujita et al. 2012). This specialization mirrors that in fully mycoheterotrophic plants, indicating a closer physiological state to full mycoheterotrophy. However, the evolution of mycoheterotrophy involving saprotrophic non-rhizoctonia fungi is less understood. Although some orchids with developed foliage leaves are shown to exhibit partial mycoheterotrophy with saprotrophic non-rhizoctonia fungi (Suetsugu and Matsubayashi 2021b; Yagame et al. 2021; Zahn et al. 2022; Suetsugu et al. 2022), leafless orchids with green stems and capsules associated with these fungi, potentially representing further intermediate stages, are yet to be discovered.

To gain deeper insights into the diverse nutritional strategies employed by orchids, our study focused on *E. zollingeri*, a leafless orchid species characterized by faintly green stems. This species is part of the pantropical genus *Eulophia*, which includes approximately 230 species (Merckx et al. 2013). While most *Eulophia* species are terrestrial, a few can be found as epiphytes or lithophytes. Notably, at least 17 species within this genus lack leaves and are considered fully mycoheterotrophic (Merckx et al. 2013). Examples of these leafless species include *E. epiphanoides* in Southwest Tanzania, *E. galeoloides* in tropical Africa, *E. gastrodioides* in Mozambique and Zambia, *E. macrantha* in Malawi and Zimbabwe, *E. richardsiae* in Northern Zambia, and *E. zollingeri*, which is widespread across tropical and subtropical regions in Asia and Australia (Merckx et al. 2013).

Despite its wide distribution, *E. zollingeri* establishes a specialized association with a specific lineage of wood-decaying Psathyrellaceae fungi at least at the adult stage (Ogura-Tsujita and Yukawa 2008). This species is often considered to be fully mycoheterotrophic due to its lack of foliage leaves and violet stem coloration during the flowering stage (Ogura-Tsujita and Yukawa 2008; Ogura-Tsujita et al. 2021). This classification is further supported by its high $\delta^{13}C$ values (Suetsugu et al. 2020). Yet, its deeper green hue in the fruiting stage suggests chlorophyll accumulation, indicating possible partial autotrophy. Our study aimed to clarify the trophic status of *E. zollingeri* at different developmental stages, including protocorm, flowering, and fruiting stages, by analyzing $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures. Additionally, we measured chlorophyll content and chlorophyll fluorescence to

accurately assess the nutritional mode of this species. Finally, we investigated the fungal partners of *E. zollingeri* during these stages using high-throughput DNA sequencing.

Materials and methods

Study site and sampling procedures

Fieldwork was conducted in an evergreen broad-leaved forest located in Miyazaki City, Miyazaki Prefecture, Japan. Two visits were made to the site: the first on July 9, 2017, during the flowering season, and the second on September 16, 2017, during the fruiting season. The forest was predominantly composed of *Castanopsis sieboldii* trees, and the study population included approximately 30 individuals of *E. zollingeri* (Fig. 1).

At the study site, we collected stem tips, each approximately 3 cm long, from both flowering ($n = 8$) and fruiting ($n = 8$) individuals of *E. zollingeri*. These samples were then analyzed for chlorophyll content and isotopic composition. In addition, we gathered leaves from co-occurring autotrophic reference species for isotopic analysis. To minimize the influence of environmental variables, such as atmospheric CO_2 isotope composition, microscale light conditions, and soil type (Gebauer and Schulze 1991), we collected leaves from reference plants growing in close spatial proximity (< 1 m) at the same height as the focal *E. zollingeri* individuals. This criterion led us to select *C. sieboldii* and *Neolitsea sericea*, as they were the only plants growing in close spatial proximity with the same distance from the ground.

Additionally, we collected mycorrhizal samples from flowering individuals ($n = 5$) and fruiting individuals ($n = 5$) for molecular identification of mycorrhizal fungi. To minimize the sampling impact, we extracted the minimum required root samples, harvesting two – four root fragments by excavating soil about 10 cm away from the shoots and

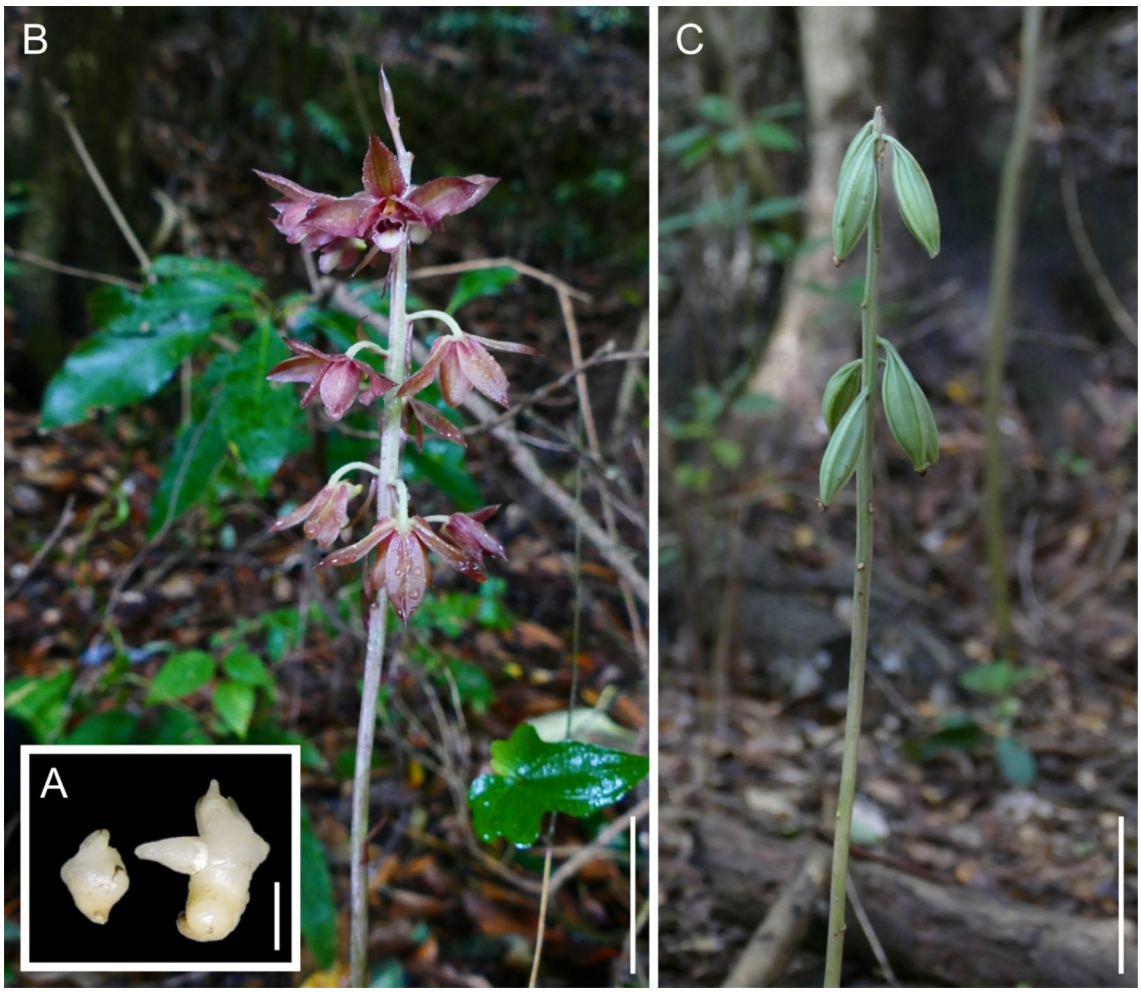

Fig. 1 Life history of *Eulophia zollingeri*. (**A**) Protocorms. (**B**) Flowering plant. (**C**) Fruiting plant. Scale bars: 5 mm (**A**) and 30 mm (**B**, **C**)

approaching the plant underground from one side. After sampling, the excavated holes were refilled with the same soil composition. During the process, we fortuitously collected *E. zollingeri* protocorms near the four *E. zollingeri* individuals at the fruiting season. These protocorms, originating around the same *E. zollingeri* individuals, were then combined and used for both mycobiont molecular identification and isotopic analysis.

Chlorophyll measurement

To measure chlorophyll fluorescence, both flowering ($n = 8$) and fruiting ($n = 8$) individuals of *E. zollingeri* were dark adapted for 15 min. Following this, the steady-state quantum yield of photosystem II was assessed using a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). This value was defined as the ratio of the actual fluorescence yield (Fv) to the maximum fluorescence (Fm).

Chlorophyll concentrations were measured following the methodology of Zimmer et al. (2008). Briefly, we collected the tips of stems from flowering or fruiting *E. zollingeri* specimens and finely cut them using small scissors and a surgical knife. Approximately half of each sample was allocated for chlorophyll measurement, while the remaining portion was used for stable isotope analysis. The finely chopped samples designated for chlorophyll measurement were mixed with N,N′-dimethylformamide and stored in the dark at – 23 °C for 8 days after recording their fresh weight. After centrifugation, the absorbance levels of the supernatants at 646.8, 663.8, and 750 nm were measured using a spectrophotometer (U-2010; Hitachi High Technologies, Tokyo, Japan). Chlorophyll concentrations were calculated according to the equation outlined by Porra et al. (1989):

$$\text{Chl}a\ (\mu\text{g/mg FW}) = 12.00(A_{663.8} - A_{750}) - 3.11(A_{646.8} - A_{750})$$

$$\text{Chl}b\ (\mu\text{g/mg FW}) = 20.78(A_{646.8} - A_{750}) - 4.88(A_{663.8} - A_{750})$$

where A represents absorbance at the specified wavelengths. Total chlorophyll concentration (Chl$a + b$) is expressed on a fresh mass basis (μg/mg FW). Differences in chlorophyll fluorescence and chlorophyll concentration between the flowering and fruiting stages of *E. zollingeri* were analyzed using Student's *t*-test.

Molecular identification of mycobionts

Excised roots or protocorms were examined under a light microscope to confirm mycorrhizal colonization. For molecular analysis, mycorrhizal fragments filled with fungal pelotons (measuring 3–5 mm in length) were collected from each *E. zollingeri* sample. Following surface sterilization, DNA was extracted from these mycorrhizal samples using the cetyltrimethylammonium bromide method (Doyle and Doyle 1990).

The nuclear ribosomal internal transcribed spacer 2 (ITS2) region was amplified using a basidiomycete-specific primer set, following the methodology outlined by Suetsugu and Matsubayashi (2021b). The first round of polymerase chain reaction (PCR) utilized the primer set ITS1OF/ITS4OF (Taylor and McCormick 2008) and was conducted under the following cycle conditions: an initial denaturation at 98 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s, with a final extension at 72 °C for 10 min. The Q5 High-Fidelity DNA Polymerase Kit (NEB, Ipswich, USA) was used for this process. Nested PCR was also performed using the primer set ITS86F/ITS4 (Waud et al. 2014), which was fused with 3–6-mer Ns and with Illumina forward/reverse sequencing primers. DNA metabarcoding with the selected primer pair (ITS86F/ITS4) is known to be highly suitable for studying mycorrhizal communities of orchids (e.g., Op De Beeck et al. 2014; Waud et al. 2014). The same polymerase kit was used under the following cycle conditions: an initial denaturation at 98 °C for 3 min, followed by 10 cycles of 98 °C for 10 s, 65 °C for 20 s, and 72 °C for 20 s, with a final extension at 72 °C for 10 min.

Supplemental PCR was additionally performed to incorporate Illumina P5/P7 adapter sequences and sample-specific indices (Syed et al. 2009; Suetsugu and Matsubayashi 2021a). The resulting ITS sequencing libraries were processed using an Illumina MiSeq sequencer, equipped with a 2 × 150 cycle sequencing kit (10% PhiX spike-in; Illumina, San Diego, CA, USA). The raw sequence data were deposited in the DRA (accession number DRA017590).

Bioinformatic analysis was carried out using Claident v.0.2.2019.05.10 (Tanabe and Toju 2013), as described by Suetsugu et al. (2021b) and Suetsugu and Matsubayashi (2021b). Briefly, operational taxonomic units (OTUs) were defined based on a sequence similarity cutoff of 97%, and potentially chimeric OTUs were eliminated using UCHIME version 7.2 (Nilsson et al. 2019). Taxonomic assignments of the OTUs were conducted using the query-centric auto-*k*-nearest-neighbor (QCauto) and lowest common ancestor algorithms (Tanabe and Toju 2013). The most abundant sequence within each OTU cluster was designated as the representative sequence for that OTU.

Because all *E. zollingeri* specimens were predominantly colonized by OTUs classified within the family Psathyrellaceae, a phylogenetic tree comprising the detected Psathyrellaceae OTUs and closely related fungi was constructed. Specifically, the OTUs identified as *E. zollingeri* mycobionts were queried against the International Nucleotide Sequence Database Collaboration (INSDC) using BLAST (Altschul et al. 1997). Several phylogenetically close sequences, along

with other representative sequences from the family Psathyrellaceae, were downloaded. Finally, these sequences were aligned using ClustalW, and a maximum likelihood tree was constructed using MEGA X (Kumar et al. 2018) using the maximum likelihood (ML) method with a GTR + I + G model and 1000 bootstrap replicates.

δ^{13}C and δ^{15}N analysis

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

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

where R_{sample} represents the ^{13}C/^{12}C or ^{15}N/^{14}N ratio of each sample, and R_{standard} represents the ^{13}C/^{12}C or ^{15}N/^{14}N ratios of Vienna PeeDee Belemnite or atmospheric N_2, respectively. The C and N isotope ratios were calibrated using the following laboratory standards: CERKU-01 (DL-alanine, $\delta^{13}\mathrm{C} = -25.36‰$, $\delta^{15}\mathrm{N} = -2.89‰$), CERKU-02 (L-alanine, $\delta^{13}\mathrm{C} = -19.04‰$, $\delta^{15}\mathrm{N} = 22.71‰$), and CERKU-05 (L-threonine, $\delta^{13}\mathrm{C} = -9.45‰$, $\delta^{15}\mathrm{N} = -2.88‰$) for C and CERKU-01 and CERKU-02 for N (Tayasu et al. 2011). The analytical standard deviations (SD) of these standards were 0.03‰ (δ^{13}C, $n = 5$) and 0.06‰ (δ^{15}N, $n = 5$) for DL-alanine, 0.04‰ (δ^{13}C, $n = 5$) and 0.07‰ (δ^{15}N, $n = 5$) for L-alanine, and 0.03‰ (δ^{13}C, $n = 5$) for L-threonine.

To facilitate seasonal comparisons, we employed a conversion of δ values into site-independent enrichment factors (ε) for both seasons. Initially, we computed the average δ^{13}C and δ^{15}N values for reference plants within each season. Subsequently, we subtracted these average values from the respective δ^{13}C and δ^{15}N values of the *E. zollingeri* samples and reference plants, resulting in site-independent enrichment factors ($\varepsilon = \delta^{\mathrm{x}}\mathrm{S} - \delta^{\mathrm{x}}\mathrm{R}$) for each sample. Here, δ^{x}S represents the δ^{13}C or δ^{15}N value of a specific *E. zollingeri* specimen, and δ^{x}R signifies the mean δ^{13}C or δ^{15}N of all reference plants within the same season.

To determine the proportion of carbon derived from fungi (% Cdf) in fruiting *E. zollingeri* specimens, we applied a linear two-source mixing model: % Cdf = (ε^{13}Cfruit/ε^{13}C FMH) × 100. In this equation, ε^{13}Cfruit corresponds to the ^{13}C enrichment factor of a fruiting *E. zollingeri* plant, while ε^{13}CFMH represents the mean ^{13}C enrichment factor of *E. zollingeri* at the protocorm stage or the flowering stage.

After verifying the normal distribution of the δ^{13}C, δ^{15}N, ε^{13}C, and ε^{15}N datasets with Bartlett's test, the variations in their values among protocorms, flowering, and fruiting individuals were assessed through a one-way ANOVA. Subsequently, post hoc multiple comparisons were conducted among them using the Tukey–Kramer test.

Results

Chlorophyll fluorescence and concentration

The total chlorophyll concentrations (Chl*a* + *b*) of fruiting *E. zollingeri* individuals were quantified at 80.3 ± 10.0 μg gFW^{-1} (mean ± SD), while their flowering counterparts exhibited a concentration of 29.1 ± 5.2 ± g gFW^{-1}. Statistical analysis revealed a significant difference, with the chlorophyll concentration of fruiting *E. zollingeri* individuals significantly higher than that of the flowering *E. zollingeri* individuals ($P < 0.001$). However, there was no significant difference in the Chl*a*:Chl*b* ratios between the flowering (2.0 ± 0.1) and fruiting (2.1 ± 0.1) specimens ($P = 0.21$).

Simultaneously, the evaluation of the maximum quantum yield of photosystem II (Fv/Fm) revealed that the flowering *E. zollingeri* specimens exhibited a value of 0.65 ± 0.04, similar to the value recorded for the fruiting *E. zollingeri* specimens at 0.67 ± 0.04 ($P = 0.37$). However, the mean Fv/Fm values recorded in *E. zollingeri* during both the flowering and fruiting stages were lower than the typical range of 0.7 − 0.83 observed in normal autotrophic plants (Maxwell and Johnson 2000; Ritchie 2006). Despite this, as Ritchie (2006) suggests, these Fv/Fm values are not so depressed as to imply the complete absence of photosynthesis. This finding confirms that *E. zollingeri* maintains a PSII apparatus that, while somewhat degenerated, remains functional.

Molecular identification of mycobionts

Fungal ITS sequences were successfully obtained from a total of 22 mycorrhizal samples collected from 14 *E. zollingeri* specimens. As expected, *E. zollingeri* was predominantly associated with wood-decaying fungi from the Psathyrellaceae family throughout the entire life cycle (Fig. 2; Table S1).

Following quality filtering, our analysis revealed the presence of five OTUs, accounting for a total of 22,629 sequencing reads in mycorrhizal tissues of *E. zollingeri* during the protocorm stage. Two of these OTUs, encompassing 17,454 reads (constituting 77.1% of all reads), were assigned to the family Psathyrellaceae. A similar pattern emerged in mycorrhizal tissues of *E. zollingeri* during the flowering stage, where 15 OTUs (64,390 sequencing reads) were identified, with seven OTUs (comprising 61,346 reads, 95.3% of all reads) attributed to Psathyrellaceae. Similarly, 22 OTUs (68,970 sequencing reads) were detected in mycorrhizal

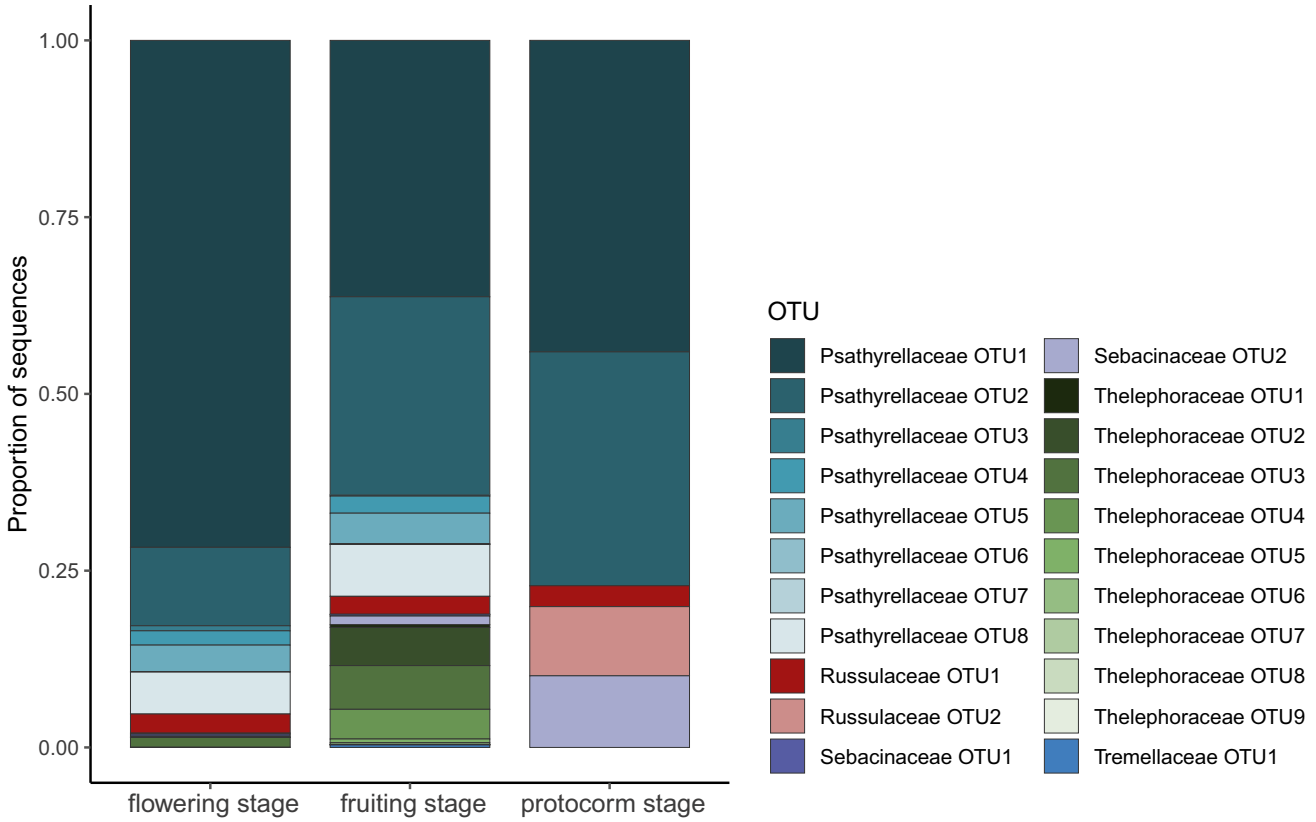

Fig. 2 Relative abundance of mycorrhizal fungi DNA sequence reads amplified from *Eulophia zollingeri* across three stages (protocorm, flowering, and fruiting stages) at the operational taxonomic unit (OTU) level

tissues of *E. zollingeri* during the fruiting stage, with eight OTUs (accounting for 54,229 reads, 78.6% of all reads) belonging to Psathyrellaceae.

Given the predominance of Psathyrellaceae in the mycobionts of *E. zollingeri*, we conducted a phylogenetic analysis using representative sequences of the OTUs obtained from our study and closely related Psathyrellaceae species. The ML phylogenetic analysis demonstrated that the dominant OTUs in *E. zollingeri* formed a clade with the mycobionts previously identified in *E. zollingeri*, specifically within the *Candolleomyces candolleanus* (=*Psathyrella candolleana*) species complex (Fig. 3). This clade also included the mycobionts of an unrelated fully mycoheterotrophic orchid *Epipogium roseum*.

Stable isotope analysis

The δ^{13}C values of *E. zollingeri* specimens during the fruiting stage (-25.8 ± 0.4‰) were significantly lower than those of *E. zollingeri* specimens during both the protocorm stage (-25.2 ± 0.4‰; $P < 0.05$; Fig. 4) and during the flowering stage (-25.0 ± 0.3‰; $P < 0.01$). On the other hand, there were no significant differences in the δ^{13}C values of neighboring autotrophic reference plants sampled during the flowering stage (-32.6 ± 1.0‰) and the fruiting stage (-32.1 ± 1.2‰; $P = 0.37$). Consequently, the ε^{13}C values of *E. zollingeri* specimens during the fruiting stage (6.4 ± 0.4‰) were significantly lower than those observed in *E. zollingeri* specimens during the protocorm stage (6.9 ± 0.4‰; $P < 0.05$) or the flowering stage (7.6 ± 0.3‰; $P < 0.001$; Table S2).

Using the mean ε^{13}C values of *E. zollingeri* specimens during the protocorm stage as a reference point for fully mycoheterotrophic conditions, we estimated that *E. zollingeri* specimens at the fruiting stage derived approximately 92.0 ± 5.2% of their organic matter from mycorrhizal fungi. Meanwhile, when employing the mean ε^{13}C values of *E. zollingeri* specimens during the flowering stage as a reference for fully mycoheterotrophic conditions, it was calculated that *E. zollingeri* obtains approximately 83.9 ± 4.8% of its carbon from the fungal association during the fruiting stage.

In contrast, there were no statistically significant disparities observed in the δ^{15}N values between the flowering (2.5 ± 1.3‰) and fruiting stages of *E. zollingeri* (2.2 ± 1.5‰; $P = 0.75$; Fig. 4). Additionally, both stages exhibited significantly elevated δ^{15}N values in comparison

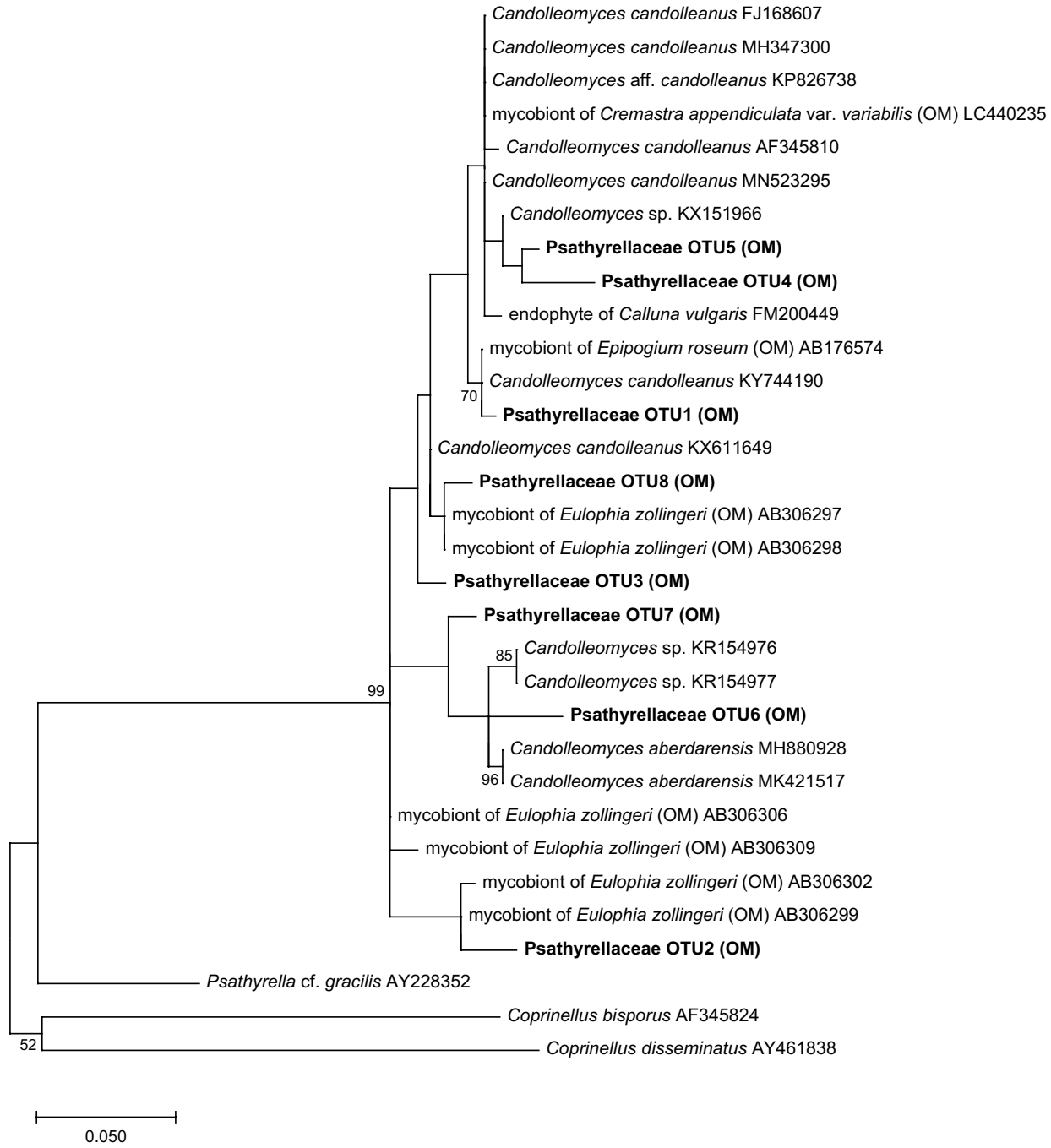

Fig. 3 Maximum likelihood phylogenetic tree of ITS2 rDNA sequences from Psathyrellaceae fungi detected in mycorrhizal samples of *Eulophia zollingeri* in the present study (bold typeface), along with sequences obtained from the INSDC database. Accession numbers are provided for all INSDC sequences. The maximum likelihood tree was rooted using *Coprinellus bisporus* and *C. disseminatus* (Psathyrellaceae). Node values indicate bootstrap values (1000 replicates) of 50% or greater. The scale bar indicates the number of substitutions per site

to the protocorms ($0.1 \pm 0.3‰$; $P < 0.05$). Similarly, the ε^{15}N values of *E. zollingeri* specimens during the flowering ($5.4 \pm 1.3‰$) or fruiting stage ($6.3 \pm 1.5‰$) were significantly higher than those observed in *E. zollingeri* specimens during the protocorm stage ($4.2 \pm 0.3‰$; $P < 0.05$).

Discussion

The evolutionary process of full mycoheterotrophy involving saprotrophic non-rhizoctonia fungi has been poorly understood, and it was only recently revealed that certain orchids

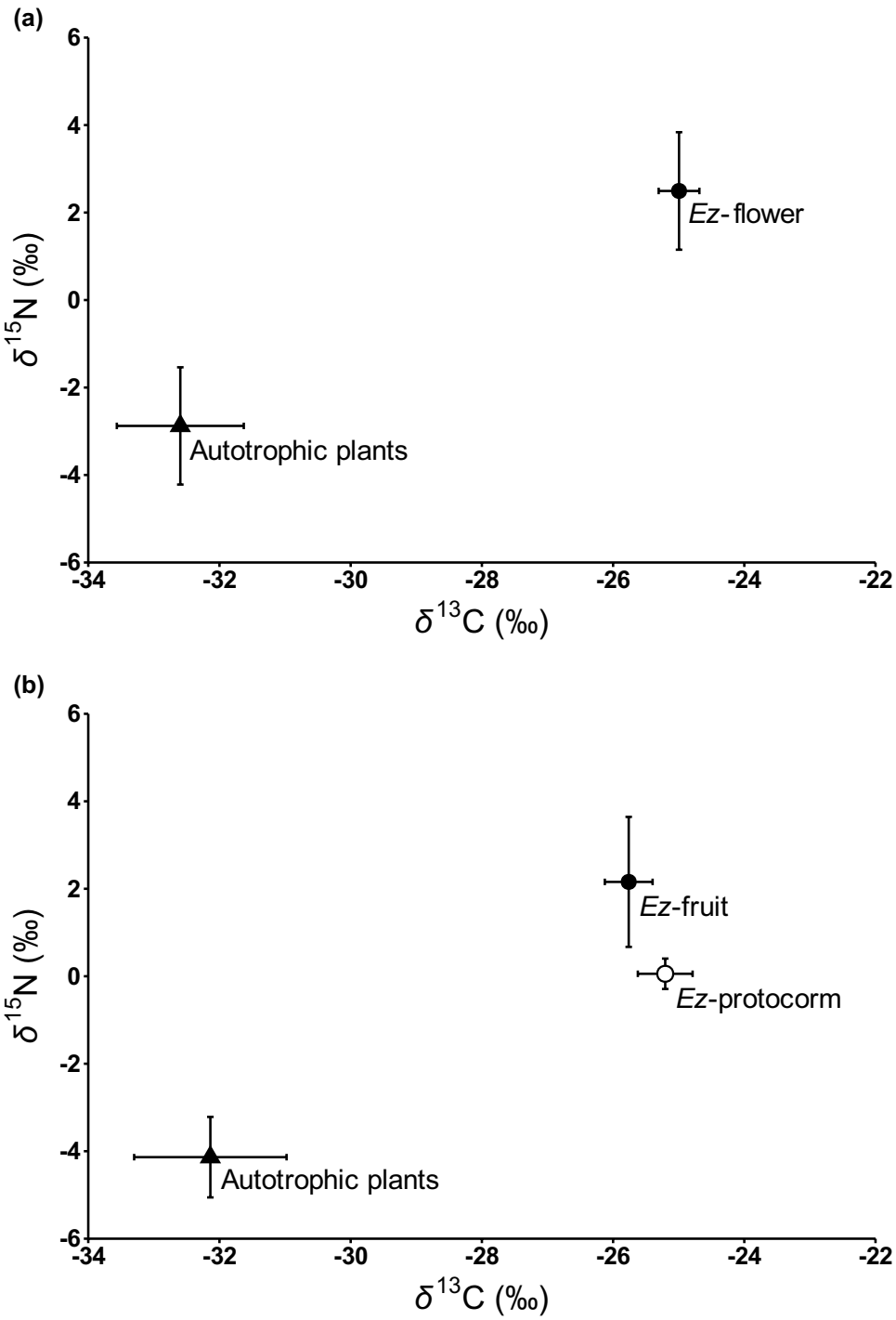

Fig. 4 Mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values of *Eulophia zollingeri* and its neighboring autotrophic plants during the **a** flowering season and **b** fruiting season. *Ez*-protocorm, *E. zollingeri* specimens at the protocorm stage; *Ez*-flower, *E. zollingeri* specimens at the flowering stage; *Ez*-fruit, *E. zollingeri* specimens at the fruiting stage

with well-developed foliage leaves exhibit partial mycoheterotrophy with these fungi. This study marks the first discovery of partial mycoheterotrophy among leafless orchids associated with saprotrophic non-rhizoctonia fungi, potentially representing further stages toward full mycoheterotrophy.

Molecular identification in our study revealed that *E. zollingeri* is predominantly colonized by Psathyrellaceae fungi throughout its entire life cycle. These findings align with the previous research indicating that *E. zollingeri* adult plants are exclusively associated with the *C. candolleanus* species complex across regions spanning Japan, Myanmar, and Taiwan (Ogura-Tsujita and Yukawa 2008). Our findings expand the understanding of the partnership across different life stages, going beyond previous studies targeting mycorrhizal communities during the adult stage (Ogura-Tsujita and Yukawa 2008; Suetsugu et al. 2020).

In addition, our molecular analysis suggests that *E. zollingeri* may form simultaneous associations with

ectomycorrhizal families such as Sebacinaceae, Thelephoraceae, and Russulaceae during certain stages of its life cycle. Interestingly, ectomycorrhizal Sebacinaceae and Russulaceae are predominantly associated with leafless orchids closely related to *E. zollingeri*, such as *Cymbidium macrorhizon* and *Cymbidium aberrans*, which are also members of the tribe Cymbidieae (Ogura-Tsujita et al. 2012). However, due to the limited sequence reads in our study and the lack of information on peloton formation by these fungi, it remains unknown whether ECM associations contribute to the carbon budget of *E. zollingeri*. Overall, we consider that the wood-decaying *Candolleomyces* likely plays a significant role in the carbon supply of *E. zollingeri*, given its dominant association. This is supported by previous studies showing the predominant association of the *C. candolleanus* species complex with adult plants of *E. zollingeri* (Ogura-Tsujita and Yukawa 2008) and the high ^{14}C enrichment pattern in *E. zollingeri*, which reflects ^{14}C-enriched bomb carbon from deadwood (Suetsugu et al. 2020).

The comparison of ^{13}C isotopic data showed that fruiting *E. zollingeri* individuals are characterized by a lower enrichment in ^{13}C, suggesting the contribution of photosynthesis to its C nutrition. Recent studies involving stable isotope analysis have also suggested the capacity for photosynthesis in leafless orchids with chlorophyll-containing stems (Zimmer et al. 2008; Suetsugu et al. 2018). On the other hand, we note that mycoheterotrophic plants and their fungal partners exhibit tremendous variations in their C and N isotope profiles, influenced by their distinct physiological characteristics, such as soil nutrient acquisition strategies (Mayor et al. 2009; Hynson et al. 2016; Schiebold et al. 2017; Zahn et al. 2023). These variations can mask the actual contributions of photosynthesis and mycoheterotrophy, potentially leading to inaccuracies in determining the nutritional mode of some leafless orchids. Therefore, to accurately assess the ecophysiology of such orchids, it is crucial to measure stable isotope abundance throughout their entire life cycle, ideally including analysis at the fully mycoheterotrophic stage (Schweiger et al. 2018; Suetsugu et al. 2018). In this context, our study, which distinctly focuses on both flowering plants with low chlorophyll expression and the protocorm stage of the same species collected from the same site, presents several advantages over previous research in this field.

In our assessment, which utilizes flowering *E. zollingeri* plants with minimal chlorophyll accumulation as a reference for fully mycoheterotrophic status, we estimate that *E. zollingeri* obtains approximately 16% of its carbon through autotrophy during the fruiting stage. However, this method might underestimate the contribution of fungal associations to the carbon gain in fruiting *E. zollingeri.* This potential underestimation arises because (1) photosynthates acquired during previous years can be stored in the underground structures of flowering *E. zollingeri* plants and (2) these plants may already perform low levels of photosynthesis (Suetsugu et al. 2018).

Therefore, it might be more appropriate to use the non-photosynthetic underground protocorm stage as the fully mycoheterotrophic endpoint. However, protocorms consist of both plant and mycorrhizal fungi, leading to intermediary isotope ratios between mycoheterotrophic plants and fungi. Given the systematic increase in ^{13}C and ^{15}N abundance at each trophic level within a food chain (DeNiro and Epstein 1976), the isotopic composition of mycoheterotrophic plants often exceeds the δ^{13}C and δ^{15}N values found in their associated fungi (Schiebold et al. 2017). Therefore, using protocorms as a fully mycoheterotrophic reference may also result in underestimation (Johansson et al. 2015). In fact, when using the ε^{13}C values of *E. zollingeri* protocorms as a fully mycoheterotrophic endpoint, it was estimated that fruiting stage specimens derived only 8% of their carbon via photosynthesis. Yet, all eight fruiting individuals of *E. zollingeri* exhibited slightly lower ε^{13}C values, indicating some degree of autotrophic contribution. Thus, despite challenges in precisely estimating the level of mycoheterotrophy, our study provides evidence for partial autotrophy in *E. zollingeri.*

It is also noteworthy that the ε^{15}N values of flowering or fruiting *E. zollingeri* specimens were significantly higher than those observed in *E. zollingeri* protocorms. This difference is likely influenced by the presence of fungal tissue within the protocorm. Notably, the average ^{15}N enrichment from mycobionts to fully mycoheterotrophic orchids (Zahn et al. 2023) often surpasses the typical increase of 2.2–3.4‰ in δ^{15}N values observed in consumers relative to their diet (Minagawa and Wada 1984; Zanden and Rasmussen 2001; McCutchan Jr et al. 2003). This notable enrichment pattern could be attributed to the selective incorporation of ^{15}N-enriched nitrogen by orchids (Gomes et al. 2023; Zahn et al. 2023). Our observations of *E. zollingeri* further corroborate this, providing indirect evidence for the selective transport of ^{15}N-enriched nitrogen from mycobionts to the orchid.

Chlorophyll analysis has shown that *E. zollingeri* does produce chlorophyll, but the amount is relatively low, constituting approximately 1–3% of the levels typically found in the leaves of green understory orchids (Julou et al. 2005; Suetsugu and Matsubayashi 2022). Nonetheless, there is an accumulation of chlorophyll in *E. zollingeri* during its fruiting stage, possibly indicating a shift toward partial autotrophy, a notion supported by the observed ^{13}C enrichment pattern. We also detected chlorophyll-dependent photochemical activity, specifically in photosystem II. Although low Fv/Fm values indicate suboptimal light utilization and reduced efficiency of photosystem II, these values do not suggest a complete absence of photosynthesis (Ritchie 2006). The similarity between the plastome of *E. zollingeri* and other

photosynthetic orchids with well-developed leaves further supports the hypothesis that this species retains some photosynthetic capabilities (Kim et al. 2020). Given the strong induction of chlorophyll accumulation and the $\delta^{13}C$ values observed during the fruiting stage, *E. zollingeri* arguably engages in partial autotrophy at this stage, with photosynthesis likely contributing to seed production.

Interestingly, some leafless orchids such as *Limodorum abortivum* (Neottieae) and *C. macrorhizon* (Cymbidieae) also accumulate chlorophyll during the fruiting season (Bellino et al. 2014; Suetsugu et al. 2018). These trends suggest that the use of photosynthesis for reproductive purposes is a recurring strategy across different phylogenetic lineages of orchids. Such reliance on photosynthates could even act as a constraint potentially hindering the transition from partial to full mycoheterotrophy.

In summary, our study demonstrates that the leafless orchid *E. zollingeri* maintains a specialized relationship with Psathyrellaceae fungi, which probably plays a critical role in providing it with a carbon supply throughout its entire life cycle. However, our investigation, using isotopic and chlorophyll fluorescence data, revealed that this orchid still performs photosynthesis during its fruiting stage. These findings support the hypothesis that transitions to mycoheterotrophy are achieved through a gradual increase in the level of mycoheterotrophy, rather than by abrupt shifts of trophic mode (Selosse and Roy 2009; Motomura et al. 2010; Jacquemyn and Merckx 2019; Suetsugu et al. 2022). Our research has added another piece to the understanding of the spectrum of nutritional strategies among partially mycoheterotrophic plants, ranging from almost complete autotrophy to nearly complete mycoheterotrophy.

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Author contribution KS planned and designed the research, conducted field and laboratory experiments, carried out analyses, and wrote the initial draft. TO conducted isotopic experiments, performed analyses, and contributed to manuscript revisions. IT supervised the isotopic experiments conducted by TO and revised the manuscript. All authors have approved the final version.

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Data availability The sequence data have been deposited in the Sequence Read Archive of the DNA Data Bank of Japan (accession no. DRA017590). Additional supporting information is available online in the Supporting Information section at the end of the article.

Declarations

Conflict of interest The authors declare no competing interests.

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