



Impact of mycoheterotrophy on the growth of *Gentiana zollingeri* (Gentianaceae), as suggested by size variation, morphology, and ^{13}C abundance of flowering shoots

Masahide Yamato¹ · Mai Yagita¹ · Ryota Kusakabe² · Keito Shimabukuro¹ · Kohei Yamana³ · Kenji Suetsugu³

Received: 3 February 2023 / Accepted: 3 September 2023

© The Author(s) under exclusive licence to The Botanical Society of Japan 2023, corrected publication 2023

Abstract

Gentiana zollingeri is an annual photosynthetic plant that employs a mycoheterotrophic growth strategy during its underground seedling stage (initial mycoheterotrophy). Notably, the morphological characteristics of its flowering shoots, such as shoot size, leaf size, and leaf color, are highly variable, and it was hypothesized that these variations may be linked to nutritional mode. The morphological characteristics of *G. zollingeri* individuals were thus investigated alongside environmental factors, ^{13}C abundance, and diversity of colonizing arbuscular mycorrhizal (AM) fungi. The majority of *G. zollingeri* flowering individuals were found to exhibit a high affinity for the specific AM fungi that exclusively colonize roots of the mycoheterotrophic seedlings, while other phylogenetically diverse AM fungi could also be detected. The leaves to shoot dry weight ratio (leaf ratio) was negatively correlated with the canopy openness in the habitat, suggesting that leaf development is impeded in sunny conditions. Furthermore, the shoot weight of *G. zollingeri* was positively correlated with leaf ^{13}C abundance. Given that ^{13}C enrichment can provide indirect evidence of mycoheterotrophy in AM plants, the results suggest that the utilization of carbon obtained through mycoheterotrophy, at least during the underground seedling stage, is crucial for *G. zollingeri*.

Keywords Arbuscular mycorrhiza · ^{13}C abundance · Gentianaceae · *Gentiana zollingeri* · Glomeraceae · Mycoheterotrophy · Stable isotopes · SSU rDNA

Introduction

In mycorrhizal symbioses, the same fungus often colonizes multiple host plants to form hyphal bridges among the plant roots. Such hyphal bridges develop into fungal networks, from which some plants can obtain carbon compounds and nitrogen. This nutritional mode is known as mycoheterotrophy, which has evolved among forest understory plants

in families such as Orchidaceae, Ericaceae, Burmanniaceae, Gentianaceae, Triuridaceae, and Petrosaviaceae (Leake 1994; Merckx et al. 2013a). The Dikarya (Basidiomycota and Ascomycota) are the mycorrhizal fungi of mycoheterotrophic plants in the Orchidaceae and Ericaceae, while Glomeromycotina (Spatafora et al. 2016) are the fungi found with other plant families, and these form the arbuscular mycorrhiza (AM).

Although partial mycoheterotrophy (mixotrophy), with both photosynthesis and mycoheterotrophy, has been confirmed in some Orchidaceae and Ericaceae plants using ^{13}C and ^{15}N abundances, it is often difficult to confirm partial mycoheterotrophy in AM-forming plants, as the ^{13}C and ^{15}N isotopic signatures of the AM fungi are similar to those of the host plants (Courty et al. 2011; Merckx et al. 2010; Nakano et al. 1999). Nonetheless, the enrichment of ^{13}C , ^{15}N , and ^2H isotopes is still found in many AM-forming fully mycoheterotrophic plants (Courty et al. 2011; Gomes et al. 2020). The enrichment of ^{13}C in particular can provide

✉ Masahide Yamato
myamoto@chiba-u.jp

¹ Faculty of Education, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

² Graduate School of Horticulture, Chiba University, 648, Matsudo, Chiba 271-8510, Japan

³ Department of Biology, Graduate School of Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe, Hyogo 657-8501, Japan

indirect evidence of partial mycoheterotrophy in AM-forming green plants (Cameron and Bolin 2010; Giesemann et al. 2020, 2021).

The angiosperm family Gentianaceae is comprised of 1750 species, in 102 genera and 7 tribes (Stevens 2017). Of these, fully mycoheterotrophic species have been found in three tribes: Voyriaceae, Saccifolieae, and Exaceae (Merckx et al. 2013a, b). Some photosynthetic species in the Gentianeae tribe, such as *Bartonia virginica* and *Obolaria virginica*, are thought to be partially mycoheterotrophic based on ^{15}N or ^{13}C enrichment and reduced sizes of leaves and root (Cameron and Bolin 2010). Partial mycoheterotrophy has also been suggested in *Pterygocalyx volubilis* in Gentianeae based on ^{13}C and ^{15}N enrichment in a habitat with C_4 plants, *Miscanthus sinensis* (Suetsugu et al. 2020). The ^{13}C abundance in C_4 plants is significantly higher than that in C_3 plants due to the different photosynthetic mechanisms involved, and thus the ^{13}C -enriched photosynthetic products of C_4 plants would contribute to ^{13}C enrichment in the *P. volubilis* shoots.

Gentiana zollingeri Fawc., is a small green spring-flowering herbaceous plant in the Gentianaceae. Recently, the mycoheterotrophic seedling growth (initial mycoheterotrophy) of *G. zollingeri* was demonstrated by Yamato et al. (2021). The small leaves in some adult individuals also suggested partial mycoheterotrophy, but this has not yet been confirmed. In the preliminary observations of *G. zollingeri* in several habitats, the morphological characteristics of the flowering shoots, such as shoot size, leaf size, and leaf color, were variable among individuals. Considering the initial and subsequent putative partial mycoheterotrophy of this plant, the morphological variations were hypothesized to be related to the dependency on mycoheterotrophy and photosynthesis, which could be influenced by environmental variables. Especially, it is hypothesized that ^{13}C enrichment is positively correlated with mycoheterotrophy as described above. Furthermore, since leaves are reduced to achlorophyllous scales in fully mycoheterotrophic plants (Merckx et al. 2013c), leaf size may be negatively correlated with dependency on mycoheterotrophy.

Accordingly, this study investigated the morphological characteristics of *G. zollingeri* individuals, alongside environmental factors, chlorophyll content, ^{13}C abundance, and diversity of colonizing AM fungi.

Materials and methods

Sampling and light condition measurements

Adult *G. zollingeri* individuals were collected in April 2021 from seven habitats (Table S1): “Nariyama,” a bamboo

forest in Yotsukaido, Chiba Prefecture; “Muzai,” a grassland in Inzai, Chiba Prefecture; “Amakubo A,” a deciduous woodland, and “Amakubo B,” a grassland, which were separated by ca. 70 m in Tsukuba Botanical Garden, Ibaraki Prefecture; “Obara,” a mixed forest of bamboo and deciduous broad-leaved trees in Tanba Sasayama, Hyogo Prefecture; “Kamihonjo” a tree plantation of *Cryptomeria japonica* D. Don in Mita, Hyogo Prefecture; and “Sumada” a broad-leaved forest in Mita, Hyogo Prefecture. At each site, three or four flowering *G. zollingeri* individuals, which were separated by > 1 m, were collected. At each sampling of the individuals, a soil core under the shoot, 5 cm in diameter and 10 cm in depth, was collected using a stainless-steel cylinder to obtain the root system.

To estimate the light conditions for *G. zollingeri* individuals, hemispherical photographs were taken from 1 m above the flowering shoot using a 4.5 mm F2.8 EX DC Circular Fisheye HSM lens (Sigma, Kawasaki, Japan). Each picture was then analyzed using a Gap Light Analyzer (Frazer et al. 1999) to determine canopy openness.

Characterization of shoot morphologies

All leaves isolated from the shoots were scanned, and their total area was determined using the free software ImageJ version 1.8.0 (National Institutes of Health, Bethesda, MD, USA). After removing the biggest leaf for the measurement of chlorophyll content, the shoot and leaves were dried at 70 °C for 48 h. Since *G. zollingeri* is an opposite-leaved plant and is considered to be bilaterally symmetrical, the total shoot dry weight (shoot weight) was calculated by doubling the weight of the largest leaf to compensate for the missing leaf that was removed to measure chlorophyll content. Furthermore, the leaves to shoot dry weight ratio (leaf ratio, defined as dry weight of leaves biomass divided by dry weight of shoot biomass) was also calculated. The quantification of root biomass could play a role in assessing the reliance on mycorrhizal symbiosis. Nonetheless, due to root loss during the field sampling process, determining the exact root quantity was not feasible in this study.

Chlorophyll content

The largest leaf from each individual was soaked in 1.0 mL N,N'-dimethylformamide at 4 °C for 24 h, and its absorbance at 646.8, 663.8, and 750 nm was then measured using a spectrophotometer (SP-300; Optima, Tokyo, Japan). The chlorophyll amount (Chl a + b) was determined according to Porra et al. (1989). Chl a + b ($\mu\text{g mL}^{-1}$) was converted to the per unit area ($\mu\text{g mm}^{-2}$) using the leaf area.

Phylogenetic analysis of *Gentiana zollingeri*

Total DNA was extracted from the collected roots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To infer the phylogenetic relationships among the *G. zollingeri* individuals examined, the intron sequence of the *trnL* (UAA) gene in the plant plastid was amplified from the root DNA by polymerase chain reaction (PCR), with primers *trnL*-cF and *trnL*-dR (Taberlet et al. 1991), using TaKaRa Ex Taq Hot Start Version (Takara Bio, Inc., Otsu, Japan).

The PCR mixture contained 1 μL of extracted DNA, 0.075 μL Taq polymerase, 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate, and 1.5 μL of the supplied PCR buffer to make a total volume of 15 μL . The PCR program was performed using the Gene Atlas G02 thermal cycler (Astec, Fukuoka, Japan) as follows: initial denaturation at 94 $^{\circ}\text{C}$ for 2 min, followed by 30 cycles at 94 $^{\circ}\text{C}$ for 20 s, 55 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 1 min, and a final elongation step at 72 $^{\circ}\text{C}$ for 10 min. PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taipei, Taiwan) and cloned using the pGEM-T Easy Vector System I (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA inserts were sequenced using a commercial sequencing service facility (Takara Bio) and then the sequences were deposited in the International Nucleotide Sequence Database Collaboration (INSDC; accession numbers LC746786–LC746808).

The *trnL* (UAA) sequences of *G. zollingeri* were previously obtained from Nariyama, Muzai, and Amakubo by Yamato et al. (2021), and were co-analyzed with those from the section Chondrophyllae in *Gentiana*, and this included the sequences of two closely related taxa, *G. wingecarribiensis* (KT907883) and *G. wissmannii* (= *G. wingecarribiensis* var. *wissmannii*; Adams and Williams 1988; KT907884), which were downloaded from the INSDC database. Multiple sequence alignment was performed using MUSCLE (Edgar 2004) in MEGA X (Kumar et al. 2018) for the sequenced and downloaded data. Maximum likelihood (ML) analysis using MEGA X was performed for the data set in which the Tamura three-parameter (T92) was selected as the best-fit model. The phylogenetic tree was drawn using FigTree version 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Molecular identification of the colonizing AM fungi

The partial nuclear small subunit rDNA gene (SSU rDNA) of the AM fungi was amplified from the root DNA using PCR with the primers AMV4.5NF and AMDGR (Sato et al. 2005). The Nextera Transposase Adapters Reads 1 and 2 were added to the 5'-ends of the primers for sequencing

using the Nextera XT index Kit (Illumina, San Diego, CA, USA). The PCR mixture for the first PCR contained 1 μL of DNA extract (5 $\text{ng } \mu\text{L}^{-1}$), 12.5 μL of 2 \times KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, Woburn, MA, USA), and 0.3 μM of each primer, to make a total volume of 25 μL . The first PCR was performed using the following program: initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, followed by 35 cycles at 98 $^{\circ}\text{C}$ for 20 s, 60 $^{\circ}\text{C}$ for 15 s, and 72 $^{\circ}\text{C}$ for 15 s, and a final elongation at 72 $^{\circ}\text{C}$ for 5 min. The PCR products were purified using AMPure XP (Beckman Coulter, Brea, CA, USA). The purified DNA was diluted to 1 $\text{ng } \mu\text{L}^{-1}$ and used as the DNA template for the second PCR. For the second PCR, the mixture contained 2 μL of the DNA template, 6 μL of 2 \times KAPA HiFi Hot Start Ready Mix, and 2 μL of each Index Primer from the Nextera XT Index Kit. The second PCR was performed using the following program: 95 $^{\circ}\text{C}$ for 3 min, and 12 cycles at 98 $^{\circ}\text{C}$ for 10 s, 55 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 30 s. The PCR product was purified using AMPure XP and was sequenced using NovaSeq 6000 version 1.5 with PE250 via a commercial service (Rhelixa, Tokyo, Japan).

Sequence data was generated in a FASTQ format for each index primer pair, and the dataset was deposited in the DDBJ sequence read archive (DRA) (<https://www.ddbj.nig.ac.jp/index-e.html>) under accession number DRA015507.

The demultiplexed sequences were processed in QIIME 2 version 2021.11 (Bolyen et al. 2019). Paired-end sequences were denoised, dereplicated, and filtered for chimeras using the DADA2 plugin (Callahan et al. 2016). The first 20 and 22 nucleotides from the 5'-ends of the forward and reverse sequences were trimmed, respectively. The 3'-ends of the forward and reverse sequences were truncated at position 140. For amplicon sequence variants (ASVs) made by the DADA2 plugin, taxonomy assignments were performed against SILVA 138 (Quast et al. 2013) by classify-sklearn using the feature-classifier plugin (Bokulich et al. 2018).

After selecting the ASVs that belonged to AM fungi, they were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using their cluster-features-de-novo in the VSEARCH plugin (Rognes et al. 2016). After removing the OTUs with low read numbers (< 10 reads in total), taxonomy was assigned to the remaining OTUs using classify-sklearn in the feature-classifier plugin against MaarjAM-type sequences (Öpik et al. 2010). The read numbers for the OTUs were rarefied to 4538 per sample using the function “rarefy” from the vegan package (Oksanen et al. 2017) for R version 4.2.1 (R Core Team 2022). For the rarefied AM fungal OTU composition data set, rarefaction curves were plotted for each sample to confirm the sampling efficacy using the function “rarecurve” from the vegan package. For the rarefied dataset, the Shannon–Wiener index was calculated as the α -diversity for the

colonizing AM fungi for each sample using the command “diversity” in the vegan package.

Representative DNA sequences of the 13 OTUs with > 1.0% for the total reads were deposited as the Targeted Locus Study in the INSDC database with accession numbers TAAS01000001–TAAS01000013. Sequences from the 10 Glomeromycotina families (Glomeraceae, Gigasporaceae, Pacisporaceae, Diversisporaceae, Acaulosporaceae, Claroideoglomeraceae, Archaeosporaceae, Ambisporaceae, Geosiphonaceae, and Paraglomeraceae) were downloaded from INSDC database as well as those of the mycobionts from the mycoheterotrophic Gentianaceae plants, *Sebaea oligantha*, *Voyriella parvifolia*, *Voyria aphyla*, and *Voyria corymbosa* (Bidartondo et al. 2002; Franke et al. 2006; Merckx et al. 2010). Those from a mycoheterotrophic Triuridaceae plant *Sciaphila tosaensis* (Yamato et al. 2011), that were found to be closely related to OTU1 and OTU2 in this study, were also downloaded. Multiple sequence alignment was performed for the sequenced and downloaded data using MUSCLE in MEGA X for a partial sequence (213–224 bp). ML analysis using MEGA X was then performed, in which Tamura three-parameter (T92) and γ -distribution (+G) were selected as the best-fit model. The phylogenetic tree was drawn using FigTree version 1.4.4.

Stable isotope analysis

The leaves dried at 70 °C for 48 h were collected and cut into fine pieces using small scissors in a small glass bottle. The stable carbon isotope abundance in the samples was measured using Flash EA 1112-ConFlo IV-Delta V Advantage (Thermo Fisher Scientific, Waltham, MA, USA) at the Research Institute for Humanity and Nature (Kyoto, Japan). The relative abundance of the stable isotopes was calculated as $\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$ (‰), where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample and R_{standard} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the Vienna Peedee Belemnite. The carbon isotope ratios were calibrated using two laboratory standards: CERKU-03 (glycine, $\delta^{13}\text{C} = -34.92$ ‰) and CERKU-05 (L-threonine, $\delta^{13}\text{C} = -9.45$ ‰), which can be traced to the international standards (Tayasu et al. 2011). The analytical standard deviations were 0.0370‰ for CERKU-03 and 0.0543‰ for CERKU-05.

Statistical analyses

After testing for normal distribution using the Shapiro-Wilks normality test in R version 4.2.1, Pearson’s or Spearman’s correlations were analyzed among shoot weight, leaves to shoot dry weight ratio (leaf ratio), Chl a + b, Shannon–Wiener index for the α -diversity of colonizing AM fungi (AM fungal diversity), $\delta^{13}\text{C}$ value of the leaf (leaf $\delta^{13}\text{C}$), and the

canopy openness of the sampling position (canopy openness) using the command “corr.test” in the psych package (Revelle 2022) of R. A heat map was then made for the correlation matrix using “corrplot” in the corrplot package (Wei and Simko 2021).

Although normal distribution of the shoot weight was not confirmed by the Shapiro-Wilks normality test, it can be approximated ($W = 0.910$, $p = 0.0409$), thus linear models (LMs) were used to determine the effects of the variables described above, on the shoot weight. In the LM, shoot weight was treated as a response variable, and other variables, such as AM fungal diversity, leaf $\delta^{13}\text{C}$, and canopy openness, that were suggested to have positive influences on shoot weight by the correlation analyses, were used as the explanatory variables. The effects of the explanatory variables were evaluated using type II analysis of variance (ANOVA) in the car package (Fox and Weisberg 2019). Those with significant effects ($p < 0.05$) were selected and reevaluated.

Results

Sampling, light conditions, and shoot characteristics

In total, 23 individuals of *G. zollingeri* were collected from seven habitats. The sampling site, plant number, sampling date, and location are shown in Table S1, and all plant samples collected are shown in Fig. S1. Furthermore, some representatives with diverse morphologies are shown in Fig. 1. The canopy openness values ranged 11.0–78.9%, shoot weights 9.1–249.1 mg, and leaf ratios 9.93–49.3%. Leaf ratios < 30% were mostly obtained at the Amakubo and Muzai sites (Table S2). The Chl a + b values of the largest leaves were 135–547 $\mu\text{g mm}^{-1}$, and leaf $\delta^{13}\text{C}$ values were -34.6 ‰ to -27.4 ‰ (Table S2). The relationships between shoot weight and leaf $\delta^{13}\text{C}$, and between canopy openness and leaf ratio are shown in Fig. 2.

Gentiana zollingeri phylogeny

Sequences of the *trnL* (UAA) gene from the plant plastid DNA were obtained from all *G. zollingeri* individuals examined. The phylogenetic analysis of the *G. zollingeri* sequences, including those obtained by Yamato et al. (2021) and *Gentiana* sequences in the section Chondrophyllae in the INSDC database (Mishiba et al. 2009), showed that all *G. zollingeri* sequences formed a clade in the ML phylogeny (Fig. S2). Although identity with the database sequences KT907887 and AB453056 of this plant obtained from Japan (Favre et al. 2016; Mishiba et al. 2009) was confirmed in the phylogeny, a subclade, including two other species,

Fig. 1 *Gentiana zollingeri* individuals collected in this study with diverse morphologies: (a) Na-2 collected in Nariyama, (b) AmB-3 collected in Amakubo B, (c) Ob-3 collected in Obara, and (d) Su-4 collected in Sumada. Bar, 1 cm

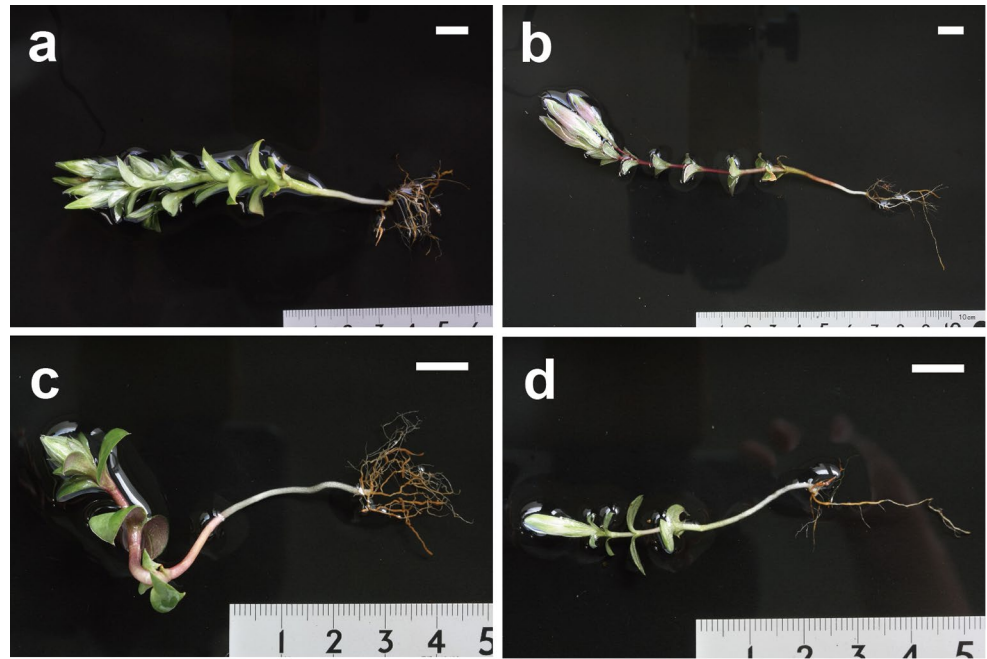
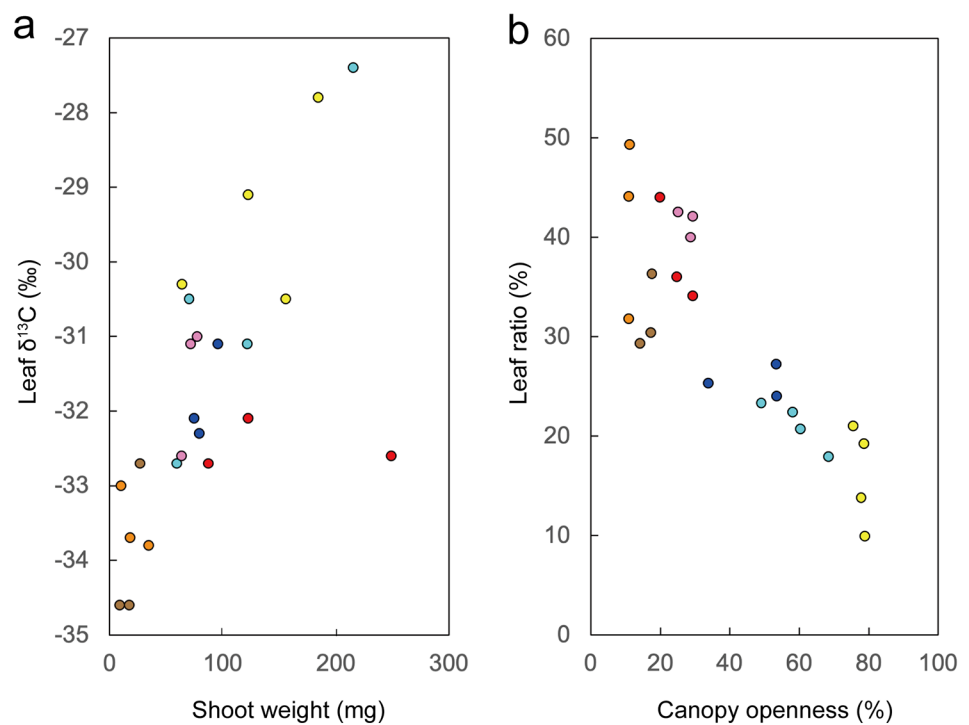


Fig. 2 Scatter plots showing the relationships (a) between shoot weight and leaf $\delta^{13}\text{C}$ in the *Gentiana zollingeri*, and (b) between canopy openness in the sampling position and leaf ratio in the shoot weight obtained from seven sampling sites, Nariyama (red), Muzai (yellow), Amakubo A (blue), Amakubo B (light blue), Obara (pink), Kamihonjo (orange), and Sumada (brown)



G. wingecarribiensis and *G. wissmannii*, were formed by individuals collected from three habitats in Hyogo Prefecture (Obara, Kamihonjo, and Sumada). Meanwhile, two sequences (LC590907 and LC590908) from the same habitat (Muzai) that were obtained by Yamato et al. (2021) are placed inside and outside of the subclade (Fig. S2).

Molecular identification of AM fungi

Illumina NovaSeq 6000 sequencing yielded 1,270,185 high-quality AM fungal DNA sequences, which were classified into 166 ASVs and 58 OTUs. After removing the rare OTUs and rarefaction analyses, 51 OTUs remained, which were numbered in decreasing order of their read numbers, and then used for subsequent analyses.

The rarefaction curves of the AM fungal OTUs plotted for each sample are presented in Fig. S3. The 51 OTUs included members of the Glomeraceae (42 OTUs), Claroideoglomeraceae (3 OTUs), Diversisporaceae (3 OTUs), Gigasporaceae (2 OTUs), and Ambisporaceae (1 OTU). The

Shannon diversity indices for the AM fungi are shown in Table S2. The distribution of the 13 dominant AM fungal OTUs, each of which constituted > 1.0% of the total rarefied reads, is shown in Fig. 3. The most dominant OTU (OTU1) constituted 50.2% of the total rarefied reads. The second

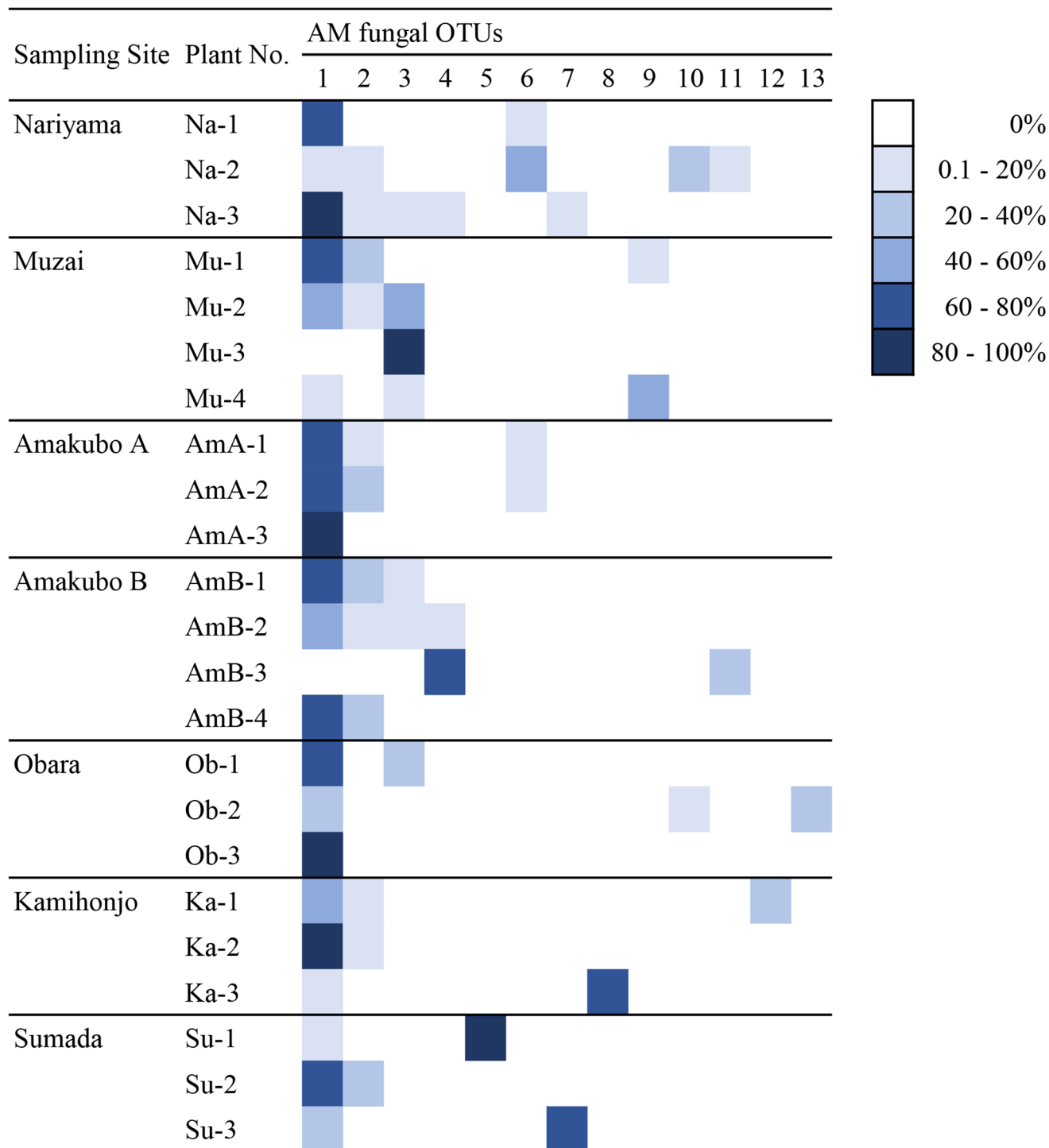


Fig. 3 Heat map showing the read ratio for the nuclear SSU rDNA of the AM fungal OTUs retained after rarefactions to 4538 reads per sample. DNA was obtained from the roots of *Gentiana zollingeri* individu-

als collected from seven sampling sites (Nariyama, Muzai, Amakubo A, Amakubo B, Obara, Kamihonjo, and Sumada). The 13 OTUs with > 1.0% of the total reads were selected and shown

most dominant OTU (OTU2) constituted 8.8%. OTU1 was detected in most individuals, except Mu-3 in Muzai and AmB-3 in Amakubo B.

The ML phylogeny for the representative sequences of these 13 AM fungal OTUs, some species-identified AM fungi, and the mycorrhizal fungi of *G. zollingeri* and some mycoheterotrophic plants is shown in Fig. 4. The two most dominant OTUs (OTU1 and OTU2) formed clades with the mycorrhizal fungi obtained from the mycoheterotrophic seedlings of *G. zollingeri* (Yamato et al. 2021). Two sequences, LC723719, and LC723720, of an AM fungus CII701 that was recently isolated and identified as conspecific or closely related to *Dominikia aurea* (Oehl & Sieverd.) Błaszk, Chwat, G.A. Silva & Oehl in Glomeraceae (Kusakabe and Yamato 2023), which is specifically associated with the mycoheterotrophic seedlings of this plant, also formed clades with OTU1 and OTU2, respectively.

Statistical analyses

Normal distributions were confirmed for the leaf ratio, Chl a + b, AM fungal diversity, and leaf $\delta^{13}\text{C}$, but not for the shoot weight and canopy openness. Accordingly, correlations between the normal distributions were examined by Pearson's tests, and other correlations including shoot weight or canopy openness were examined using Spearman's tests (Fig. 5). Leaf ratio was negatively correlated with canopy openness ($R = -0.848$, $p < 0.01$, adjusted for multiple tests; same below). Shoot weight was positively correlated with leaf $\delta^{13}\text{C}$ ($R = 0.719$, $p < 0.01$) and negatively correlated with Chl a + b ($R = -0.661$, $p < 0.01$). Furthermore, leaf $\delta^{13}\text{C}$ was found to be positively correlated with canopy openness ($R = 0.844$, $p < 0.01$).

A LM, in which shoot weight was treated as the response variable, showed the significant effects of leaf $\delta^{13}\text{C}$ ($F = 18.8$, $p < 0.01$) and AM fungal diversity ($F = 5.21$, $p < 0.05$), which were confirmed using a type II ANOVA.

Discussion

Phylogeny of *Gentiana zollingeri*

To infer the phylogenetic relationships among the *G. zollingeri* individuals in this study, a phylogeny based on the *trnL* (UAA) gene from the plant plastid DNA was constructed. *G. zollingeri* sequences formed a clade in the ML phylogeny (Fig. S2); however, a subclade, including two Australian annual *Gentiana* taxa, *G. wingecarribiensis* and *G. wissmannii* (= *G. wingecarribiensis* var. *wissmannii*; Adams and Williams 1988), was formed by all individuals collected from Obara, Kamihonjo, and Sumada in the

Hyogo Prefecture. Although two sequences (LC590907 and LC590908) from the same habitat (Muzai) in a previous study (Yamato et al. 2021) were placed inside and outside of the subclade, a significant genetic differentiation may have occurred within this species. Unfortunately, it was not possible to distinguish the effects of the putative genotype and environmental factors on the morphology and ^{13}C abundance in this study, as all subclade individuals were collected from forest environments. In addition, the *trnL* (UAA) gene examined in this study is probably not sufficient for the examination of species delimitation. A future study based on high-throughput sequencing platforms (e.g., Suyama and Matsuki 2015) might thus be required to examine the potential genetic differentiation within this plant group.

AM fungal symbioses of *Gentiana zollingeri*

Recently, an AM fungus specifically associated with mycoheterotrophic seedlings of *G. zollingeri* was identified as conspecific or closely related to *D. aurea* (Kusakabe and Yamato 2023). In the phylogenetic analysis based on partial SSU rDNA sequences (Fig. 4), the OTU1 and OTU2 fungi in this study formed a clade with sequences of this fungus, LC723719 and LC723720, respectively, as well as the mycorrhizal fungi of *G. zollingeri* that were identified in a previous study (Yamato et al. 2021). LC723719 and LC723720 sequences were obtained from a cultured AM fungus originating from a single isolate. The sequence differences between the two OTUs, which were classified by a 97% similarity threshold, probably originated from a polymorphism in the genome of the AM fungus. Thus, the OTU1 and OTU2 fungi in this study may have originated from a conspecific fungus.

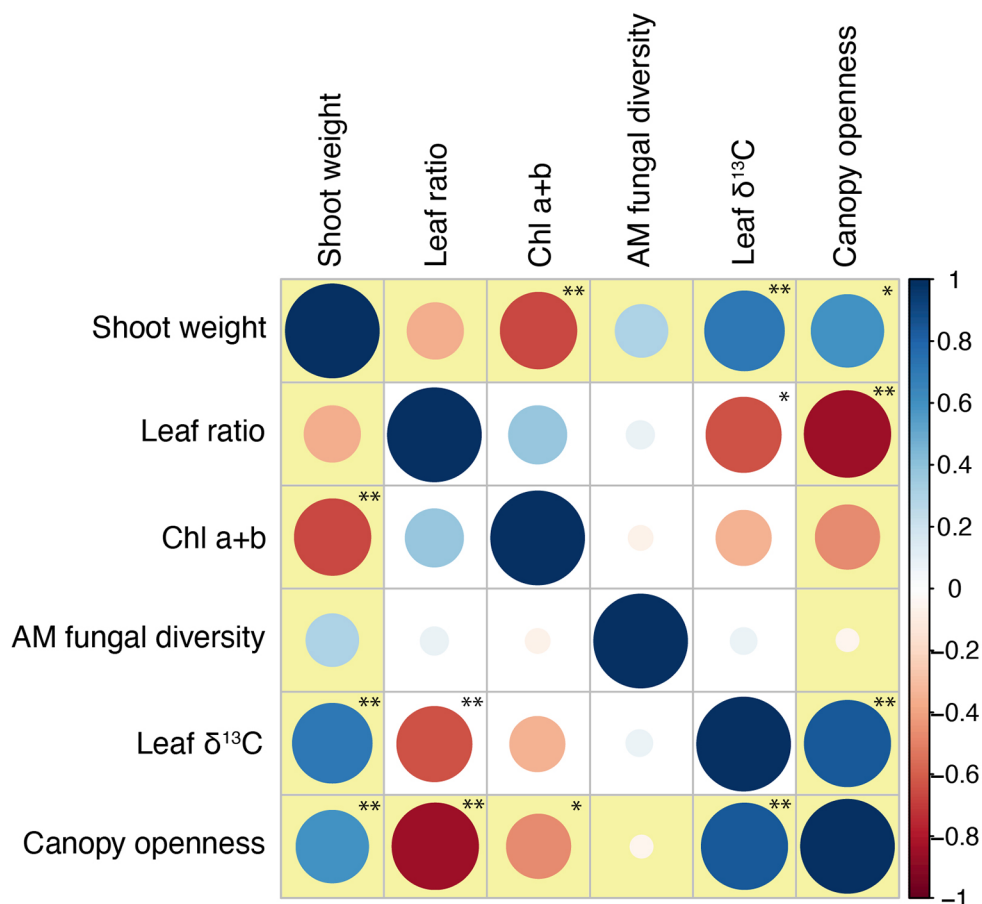
Most *G. zollingeri* individuals examined in this study were colonized by OTU1 fungi, which confirmed the high affinity of *G. zollingeri* to the AM fungi (Fig. 3). This can be caused by the specific colonization of the fungus on the mycoheterotrophic seedling (Yamato et al. 2021). The other AM fungi were found to be phylogenetically diverse (Fig. 4). Overall, the results suggest that the high level of mycorrhizal specificity in *G. zollingeri* seedlings may be somewhat alleviated in the adult stage, and this is likely due to its photosynthetic capabilities; given that mycorrhizal specialization is often considered as a crucial strategy in mycoheterotrophy that enables plants to effectively acquire resources from their fungal partners (e.g., Suetsugu et al. 2021).



Fig. 4 ML phylogenetic tree based on the partial SSU rDNA sequences (213–224 bp) of the Glomeromycotina fungi, including the sequences obtained from *Gentiana zollingeri* in this study (red) and a previous study (purple; Yamato et al. 2021) along with the mycorrhizal fungal sequences from some mycoheterotrophic plants and some species-identified AM fungi. Two sequences from an AM fungus recently identified as conspecific or closely related to *Dominikia aurea* in Glomeraceae (blue; Kusakabe and Yamato 2023), specifically associ-

ated with the mycoheterotrophic seedlings of *G. zollingeri*, are also included. Representative sequences are shown for these 13 AM fungal OTUs. INSDC accession numbers are given for all sequences. The tree is rooted with *Paraglomus occultum* (NG017179). Bootstrap values (BS) with 1000 replications are shown at each node (only BS > 60% are shown). The scale bar indicates the number of substitutions per site. The bar indicates nucleotide substitutions per site

Fig. 5 Heat map showing correlations between the shoot weight, leaf ratio, Chl a + b, AM fungal diversity, leaf $\delta^{13}\text{C}$, and canopy openness for the *Gentiana zollingeri* individuals examined in this study. Pearson's correlations are shown with a white background, while Spearman's correlations have a yellow background. The significance of the correlation coefficients is shown at the top right of each circle ($*p < 0.05$; $**p < 0.01$). Those above the diagonal are adjusted for multiple tests



Morphological and physiological diversity of *Gentiana zollingeri*

Gentiana zollingeri individuals in this study were morphologically diverse. The leaves to shoot dry weight ratio was variable (Fig. 1, Table S2) and negatively correlated with canopy openness (Figs. 2a and 5), which indicated that leaf development is impeded in sunny conditions.

Although AM fungal diversity was not significantly correlated with the variables examined, its effect on shoot weight was confirmed using the LM. Diverse AM fungi colonizing *G. zollingeri* roots may enhance plant growth after the formation of aboveground shoots. However, this result may also have been caused by an encounter with various AM fungi in larger individuals that may have longer roots.

Shoot weight was also variable and positively correlated with leaf $\delta^{13}\text{C}$ values (Figs. 2b and 5), suggesting the major contribution of mycoheterotrophy for the seedling growth (Cameron and Bolin 2010; Giesemann et al. 2020, 2021). Meanwhile, shoot weight was negatively correlated with Chl a + b (Fig. 5), indicating that individuals that grow well via mycoheterotrophy during the underground seedling stage exhibit reduced dependency on photosynthesis, although

chlorophyll digestion to transfer nutrients for shoot growth may also have some effect. In perennial plants, a small shoot for the next year is usually prepared at the flowering stage. However, these shoots have not been observed in *G. zollingeri*, as shown in the collected samples (Fig. 1 and Fig. S1). Thus, this plant is regarded as an annual, although several years may be required until an aboveground shoot is formed. Thus, mycoheterotrophic seedling growth before forming an aboveground shoot (initial mycoheterotrophy) would be important for the growth of this plant.

The positive correlation between canopy openness and leaf $\delta^{13}\text{C}$ may be caused by the lower leaf ratio in sunny conditions, as this could disrupt photosynthesis. However, a positive correlation between irradiance and the leaf $\delta^{13}\text{C}$ value has also been observed in autotrophic plants due to stomatal regulation (Preiss et al. 2010), which may have some effect on the positive correlation in *G. zollingeri*. Furthermore, even if mycoheterotrophy is vital for plant growth, it remains uncertain as to whether this primarily occurs through prolonged mycoheterotrophy at the adult stage, as a partial mycoheterotrophy, or through the utilization of carbon obtained during the underground seedling stage as the initial mycoheterotrophy.

Giesemann et al. (2020, 2021) suggested that partial mycoheterotrophy may be common among green plants forming Paris-type AM from the higher ^{13}C enrichment. However, Murata-Kato et al. (2022) found a positive correlation between light availability and $\delta^{13}\text{C}$ in an understory plant *Trillium camschatcense* along with autotrophic nonmycorrhizal plants. Notably, the study also found that there was ^{13}C enrichment in some understory Arum-type AM plants. Further investigations, using strategies such as ^{14}C labeling, are required to determine the effects of mycoheterotrophy on the growth of *G. zollingeri* at each stage of development and to assess the extent of the partial mycoheterotrophy in other green plants forming Paris-type AM associations.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10265-023-01496-y>.

Acknowledgements This study was supported by JSPS KAKENHI grant number 19K06095. This study was conducted with the support of the Joint Research Grant for the Environmental Isotope Study of the Research Institute for Humanity and Nature. We thank Tomohisa Yukawa, Ayako Shimono, Mari Yano, and Shoei Tohmi for their help in plant sampling and Ryuta Yagi for his help in stable isotope analysis. We would like to thank Enago (www.enago.jp) for the English language review.

Data Availability Sequence data generated in FASTQ format were deposited in the DDBJ sequence read archive (DRA) under accession number DRA015507.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors guarantee compliance with ethical standards.

References

- Adams LG, Williams JB (1988) *Gentiana* sect. *Chondrophyllae* (Gentianaceae) in Australia. *Telopea* 3:167–176. <https://doi.org/10.7751/telopea19884805>
- Bidartondo MI, Redecker D, Hijri I, Wiemken A, Bruns TD, Domínguez L, Sérsic A, Leake JR, Read DJ (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature* 419:389–392. <https://doi.org/10.1038/nature01054>
- Bokulich NA, Kaehler BD, Rideout JR et al (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6:90. <https://doi.org/10.1186/s40168-018-0470-z>
- Bolyen E, Rideout JR, Dillon MR et al (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Callahan B, McMurdie P, Rosen M et al (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 13:581–583. <https://doi.org/10.1038/nmeth.3869>
- Cameron DD, Bolin JF (2010) Isotopic evidence of partial mycoheterotrophy in the Gentianaceae: *Bartonia virginica* and *Obolaria virginica* as case studies. *Ame J Bot* 97:1272–1277. <https://doi.org/10.3732/ajb.0900292>
- Courty PE, Walder F, Boller T, Ineichen K, Wiemken A, Rousteau A, Selosse MA (2011) Carbon and nitrogen metabolism in mycorrhizal networks and mycoheterotrophic plants of tropical forests: a stable isotope analysis. *Plant Physiol* 156:952–961
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. <https://doi.org/10.1093/nar/gkh340>
- Favre A, Michalak I, Chen CH, Wang JC, Pringle JS, Matsuzak S, Sun H, Yuan YM, Struwe L, Muellner-Riehl AN (2016) Out-of-Tibet: the spatio-temporal evolution of *Gentiana* (Gentianaceae). *J Biogeogr* 43:1967–1978. <https://doi.org/10.1111/jbi.12840>
- Fox J, Weisberg S (2019). An R companion to applied regression, 3rd Edn. Thousand Oaks CA: Sage. <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>
- Franke T, Beenken L, Döring M, Kocyan A, Agerer R (2006) Arbuscular mycorrhizal fungi of the *Glomus*-group A lineage (Glomerales; Glomeromycota) detected in myco-heterotrophic plants from tropical Africa. *Mycol Prog* 5:24–31. <https://doi.org/10.1007/s11557-006-0500-2>
- Frazer GW, Canham CD, Lertzman KP (1999) Gap light Analyzer (GLA), Version 2.0: imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation. Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York
- Giesemann P, Rasmussen HN, Liebel HT, Gebauer G (2020) Discreet heterotrophs: green plants that receive fungal carbon through Paris-type arbuscular mycorrhiza. *New Phytol* 226:960–966. <https://doi.org/10.1111/nph.16367>
- Giesemann P, Rasmussen HN, Gebauer G (2021) Partial mycoheterotrophy is common among chlorophyllous plants with Paris-type arbuscular mycorrhiza. *Ann Bot* 127:645–653
- Gomes SIF, Merckx VSFT, Kehl J, Gebauer G (2020) Mycoheterotrophic plants living on arbuscular mycorrhizal fungi are generally enriched in ^{13}C , ^{15}N , and ^2H isotopes. *J Ecol* 108:1250–1261. <https://doi.org/10.1111/1365-2745.13381>
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol* 35:1547–1549. <https://doi.org/10.1093/molbev/msy096>
- Kusakabe R, Yamato M (2023) Isolation and identification of an arbuscular mycorrhizal fungus specifically associated with mycoheterotrophic seedlings of *Gentiana zollingeri* (Gentianaceae). *Mycoscience* 64:55–62. <https://doi.org/10.47371/mycosci.2023.01.001>
- Leake JR (1994) The biology of myco-heterotrophic ('saprophytic') plants. *New Phytol* 127:171–216. <https://doi.org/10.1111/j.1469-8137.1994.tb04272.x>
- Merckx V, Stöckel M, Fleischmann A, Bruns TD, Gebauer G (2010) ^{15}N and ^{13}C natural abundance of two mycoheterotrophic and putative partially mycoheterotrophic species associated with arbuscular mycorrhizal fungi. *New Phytol* 188:590–596. <https://doi.org/10.1111/j.1469-8137.2010.03365.x>
- Merckx V, Freudenstein JV, Kissling J, Christenhusz MJM, Stotler RE, Crandall-Stotler B, Wickett N, van de Rudall PJ (2013a) Kamer H, Mass PJM Taxonomy and Classification. In: Merckx V (ed) Mycoheterotrophy. Springer, New York, pp 19–101
- Merckx V, Kissling J, Hentrich H, Janssens SB, Mennes CB, Specht CD, Smet EF (2013b) Phylogenetic relationships of the

- mycoheterotrophic genus *Voyria* and the implications for the biogeographic history of Gentianaceae. *Am J Bot* 100:712–721. <https://doi.org/10.3732/ajb.1200330>
- Merckx V, Mennes CB, Peay KG, Geml J (2013c) Evolution and diversification. In: Merckx V (ed) *Mycoheterotrophy*. Springer, New York, pp 215–244
- Mishiba K, Yamane K, Nakatsuka T, Nakano Y, Yamamura S, Abe J, Kawamura H, Takahata Y, Nishihara M (2009) Genetic relationships in the genus *Gentiana* based on chloroplast DNA sequence data and nuclear DNA content. *Breed Sci* 59:119–127. <https://doi.org/10.1270/jsbbs.59.119>
- Murata-Kato S, Sato R, Abe S, Hashimoto Y, Yamagishi H, Yokoyama J, Tomimatsu H (2022) Partial mycoheterotrophy in green plants forming *Paristype* arbuscular mycorrhiza requires a thorough investigation. *New Phytol* 234:1112–1118. <https://doi.org/10.1111/nph.18049>
- Nakano A, Takahashi K, Kimura M (1999) The carbon origin of arbuscular mycorrhizal fungi estimated from $\delta^{13}\text{C}$ values of individual spores. *Mycorrhiza* 9:41–47. <https://doi.org/10.1007/s005720050261>
- Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlenn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens HH, Szoecs E, Wagner H (2017) Package 'vegan' community ecology package version 2.4-4. <https://cran.ism.ac.jp/web/packages/vegan/vegan.pdf>
- Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier Ü, Zobel M (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytol* 188:223–241. <https://doi.org/10.1111/j.1469-8137.2010.03334.x>
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA). Bioenergetics* 975:384–394. [https://doi.org/10.1016/S0005-2728\(89\)80347-0](https://doi.org/10.1016/S0005-2728(89)80347-0)
- Preiss K, Adam IKU, Gebauer G (2010) Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially mycoheterotrophic orchids. *Proc R Soc B* 277:1333–1336. <https://doi.org/10.1098/rspb.2009.1966>
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596. <https://doi.org/10.1093/nar/gks1219>
- R Core Team (2022) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Revelle W (2022) R package 'psych': Procedures for Psychological, Psychometric, and Personality Research, Northwestern University, Evanston, Illinois, USA. <https://personality-project.org/r/psych-manual.pdf>
- Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. <https://doi.org/10.7717/peerj.2584>
- Sato K, Suyama Y, Saito M, Sugawara K (2005) A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. *Grassl Sci* 51:179–181. <https://doi.org/10.1038/363067a0>
- Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James TY, O'Donnell K, Roberson RW, Taylor TN, Uehling J, Vilgalys R, White MM (2016) A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108:1028–1046. <https://doi.org/10.3852/16-042>
- Stevens PF (2017) Angiosperm Phylogeny Website. Version 14. [WWW document] URL <http://www.mobot.org/MOBOT/research/APweb/welcome.html>
- Suetsugu K, Matsubayashi J, Ogawa NO, Murata S, Sato R, Tomimatsu H (2020) Isotopic evidence of arbuscular mycorrhizal cheating in a grassland gentian species. *Oecologia* 192:929–937. <https://doi.org/10.1007/s00442-020-04631-x>
- Suetsugu K, Matsuoka S, Shutoh K, Okada H, Taketomi S, Onimaru K, Tanabe AS, Yamanaka H (2021) Mycorrhizal communities of two closely related species, *Pyrola subaphylla* and *P. japonica*, with contrasting degrees of mycoheterotrophy in a sympatric habitat. *Mycorrhiza* 31:219–229. <https://doi.org/10.1007/s00572-020-01002-5>
- Suyama Y, Matsuki Y (2015) MIG-seq: an effective PCR-based method for genome-wide single-nucleotide polymorphism genotyping using the next-generation sequencing platform. *Sci Rep* 5:16963. <https://doi.org/10.1038/srep16963>
- Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17:1105–1109. <https://doi.org/10.1007/BF00037152>
- Tayasu I, Hirasawa R, Ogawa NO et al (2011) New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. *Limnology* 12:261–266. <https://doi.org/10.1007/s10201-011-0345-5>
- Wei T, Simko V (2021) R package 'corrplot': Visualization of a Correlation Matrix (Version 0.92). <https://github.com/taiyun/corrplot>
- Yamato M, Yagame T, Iwase K (2011) Arbuscular mycorrhizal fungi in roots of non-photosynthetic plants, *Sciaphila japonica* and *Sciaphila tosaensis* (Triuridaceae). *Mycoscience* 52:217–223. <https://doi.org/10.1007/s10267-010-0084-1>
- Yamato M, Suzuki T, Matsumoto M, Shiraishi T, Yukawa T (2021) Mycoheterotrophic seedling growth of *Gentiana zollingeri*, a photosynthetic Gentianaceae plant species, in symbioses with arbuscular mycorrhizal fungi. *J Plant Res* 134:921–931. <https://doi.org/10.1007/s10265-021-01311-6>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.