Fungal association and root morphology shift stepwise during ontogenesis of orchid *Cremastra appendiculata* towards autotrophic nutrition

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**Abstract**

The chlorophyllous, terrestrial orchid *Cremastra appendiculata* from East Asia is unique concerning its fungal mycorrhiza partners. The initially mycoheterotrophic protocorms exploit rather specialized non-rhizoctonia saprotrophic Psathyrellaceae. Adult individuals of this orchid species are either linked to Psathyrellaceae being partially mycoheterotrophic or form mycorrhiza with fungi of the ubiquitous saprotrophic rhizoctonia group. This study provides new insights on nutrition mode, subterranean morphology and fungal partners across different life stages of *C. appendiculata*. We compared different development stages of *C. appendiculata* to surrounding autotrophic reference plants based on multi-element natural abundance stable isotope analyses (δ¹³C, δ¹⁵N, δ²H, δ¹⁸O) and total N concentrations. Site- and sampling-time-independent enrichment factors of stable isotopes were used to reveal trophic strategies. We determined mycorhizal fungi of *C. appendiculata* protocorm, seedling and adult samples using high-throughput DNA sequencing. We identified saprotrophic non-rhizoctonia Psathyrellaceae as dominant mycoheterotrophic protocorms were enriched in ¹⁵N, ¹³C and ²H compared to reference plants. Seedlings showed an intermediate isotopic signature, underpinning the differences in the fungal community depending on their subterranean morphology. In contrast to the suggestion that *C. appendiculata* is a partially mycoheterotrophic orchid species, we provide novel evidence that mature *C. appendiculata* with rhizoctonia mycobionts can be entirely autotrophic. Besides an environmentally driven variability among populations, we suggest high within-individual flexibility in nutrition and mycobionts of *C. appendiculata*, which is subject to the ontogenetic development stage.

**Keywords:** *Cremastra appendiculata*; mycoheterotrophy; mycorrhiza; ontogenesis; Orchidaceae; protocorm; rhizoctonia; saprotrophic; seedling; stable isotopes; subterranean morphology; Taiwan.

**Introduction**

The Orchidaceae are one of the largest and most diverse plant families on Earth, containing an estimate of roughly 28 500 species (WCSP 2021). It is particularly fascinating that orchids exhibit different trophic strategies at both phylogenetic and ontogenetic level (Dearnaley et al. 2012; Field et al. 2017). In this sense, probably all representatives within the Orchidaceae rely on carbon (C) gained through the interaction with associated fungi during their early below-ground development stage (Rasmussen 1995; Leake 2004; Rasmussen et al. 2015). This initially mycoheterotrophic nutrition mode may be linked to the ‘dust seeds’ of orchids, which contain an embryo but lack an endosperm, resulting in very limited carbohydrate reserves (Leake 1994; Arditti and Ghani 2000; Eriksson and Kainulainen 2011). Even after ‘symbiotic germination’, orchids remain mycoheterotrophic throughout their non-photosynthetic protocorm phase (Dearnaley et al. 2012). The dependency of orchids on fungal carbon usually decreases towards adulthood (Rasmussen et al. 2015). However, over 200 orchid species from several genera remain achlorophyllous and thus fully mycoheterotrophic for their entire life span.

Achlorophyllous, fully mycoheterotrophic orchid specimens tend to exploit ectomycorrhizal networks (ECM) in temperate latitudes, while in the (sub)tropics additionally non-rhizoctonia litter- and wood-decaying saprotrophic fungi (SAP) seem to be important associating fungi (Martos et al. 2009; Ogura-Tsujita et al. 2009, 2018; Lee et al. 2015).

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Nonetheless, most orchids develop chlorophyll and the ability to photosynthesize, becoming either autotrophic or, when getting additional carbon from fungi, partially mycoheterotrophic (Gebauer and Meyer 2003; Merckx et al. 2009). Amongst autotrophic or partially mycoheterotrophic orchids, saprotrophic fungi are likely to lower extent fungi simultaneously forming ectomycorrhizas with forest trees are fungal partners (Bidartondo et al. 2004). Mycorrhizal associations with the polyphyletic fungal assembly of rhizoctonia, comprising saprotrophic fungi belonging to the Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae within the Basidiomycetes, are thus the most common group involved in orchid mycorrhiza and are unique to Orchidaceae (Rasmussen 2002; Taylor et al. 2002; Dearnaley et al. 2012). Orchid mycorrhizal interactions likely emerged by the evolutionary recruitment of endophytes that became mycorrhizal (Selosse et al. 2021). Generally, shifts of fungal partners among Orchidaceae often co-occur with switches in trophic modes (Wang et al. 2021).

It is understood that conclusions on the evolution from autotrophy to mycoheterotrophy and the relevance of changing mycorrhizal fungi can be drawn by studying leafy and leafless plant relatives as well as ontogenetic changes in fungal mycorrhiza associations within one plant species (Ogura-Tsujita et al. 2021). Covering two achlorophyllous, leafless and three chlorophyllous, leafy species, the orchid genus Cremastra from East Asia is such a prime example of SAP–mycoheterotrophic plant lineages (Ogura-Tsujita et al. 2021; Suetsugu 2021).

Coprinellus spp., rather specialized wood/litter-decaying saprotrophic fungi belonging to the Psathyrellaceae, have been identified as fungal hosts of the fully mycoheterotrophic, leafless orchid Cremastra aphylla (Yagame et al. 2018; Suetsugu et al. 2021a). Consistently, Coprinellus spp. are known to induce seed germination in the protocorm stage of chlorophyllous Cremastra appendiculata (Yagame et al. 2013). Chlorophyllous C. appendiculata with relatively broad green leaves is usually found in light-limited and humid forest ground sites in the (sub)tropics and was therefore considered to be putatively partially mycoheterotrophic as an adult. Recently, mature C. appendiculata var. variabilis individuals sampled in a cool-temperate forest in Japan were recognized to obtain approximately 83.4 ± 0.9 % of their total C demand from wood-decaying Psathyrellaceae (Psathyrella or Coprinellus) (Suetsugu et al. 2021a). Though confirming partial mycoheterotrophy for several individuals with Psathyrellaceae as fungal partners, Yagame et al. (2021) identified rhizoctonia (Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae) as the main mycobionts of C. appendiculata var. variabilis.

Strikingly, Psathyrellaceae fungi were exclusively detected in mature C. appendiculata var. variabilis individuals with coralloid rhizomes (Suetsugu et al. 2021a; Yagame et al. 2021), a morphological root structure typical for mycoheterotrophic plants (Burgeff 1932; Leake 1994), while rhizoctonia were mainly found in individuals without coralloid rhizomes (Yagame et al. 2021). Therefore, Yagame et al. (2021) suggested an environmentally driven link between fungal partners and subterranean morphology.

This study provides new insights on nutrition mode, underground morphology and fungal partners across three different life stages (protocorms, seedlings, adults) of C. appendiculata. We used multi-element natural abundance stable isotope analysis together with high-throughput DNA sequencing to investigate field-collected protocorms, seedlings and adults of a C. appendiculata population (Fig. 1) in a Fagaceous forest habitat in the Taiwanese mountains.

Analysing δ2H and δ18O additionally to δ13C and δ15N enabled us to resolve presumed ‘cryptic partial mycoheterotrophy’ (sensu Hynson et al. 2013) of mature C. appendiculata being mycorrhizal with rhizoctonia. This approach provides specific evidence whether mature C. appendiculata adults with rhizoctonia fungal partners continue to gain some organic matter from a fungal source or whether they are truly autotrophic (Gebauer et al. 2016; Schiebold et al. 2018). We examined whether a change of fungal partners along three different ontogenetic development stages of C. appendiculata is accompanied by an alteration in subterranean root morphology and the mode of nutrition. We assume a change in carbon acquisition to be visualized by gradual changes in stable isotope natural abundance.

**Materials and Methods**

**Sampling sites and experimental design**

Sampling of C. appendiculata var. variabilis at three different development stages and respective autotrophic reference
plants (listed in Supporting Information—Tables S3 and S4) took place in a Fagaceae forest in subtropical Taiwan on the western slope of the Hehuan Mountains at an elevation of 2000 m a.s.l. (24°05′01.4″N, 121°10′10.2″E).

An in situ sowing experiment yielded protocorm samples of C. appendiculata (Fig. 1A). For this purpose, seed packages of C. appendiculata were buried into the soil and covered by litter near the adult at the sampling site. As seeds matured in late October, seeds were collected from 15 capsules, pooled after harvesting and directly put into seed packages. Approximately 300 seeds were placed within a seed package. Seed packages were made of mesh permeable for fungal hyphae but not for plant roots (Rasmussen and Whigham 1998). About 100 seed packages were buried in 2012, 2013, 2014 and 2015 at five plots. As the seed packages were retrieved, they were smoothly rinsed with tap water in the laboratory, then opened and carefully checked under a stereo microscope for examining germination.

The sampling design for stable isotope analysis followed the approach of Gebauer and Meyer (2003), consisting of 1 m² plots with the target orchid species and four to five autotrophic reference plant species. This sampling scheme enables sufficient plots with the target orchid species and four to five autotrophic reference plant species. This sampling scheme enables sufficient plots with the target orchid species and four to five autotrophic reference plant species.

For the high-throughput sequencing experiment, samples were taken from the above-mentioned plots on 19 July 2015, and we differentiated between root morphology types due to variation in the investigated population depending on the development stage. Note that seedlings with very young leaves had both coralloid rhizomes and roots attached, but as seedlings approached maturity, they became detached from rhizomes. Two to three protocorms were collected from each plot (eight protocorms in total from three plots). The protocorms from seed packets in each plot were pooled because of the small amount of tissue. Only three plots with the seedling stage were available, and one coralloid rhizome and one root were collected from each individual in each plot (three coralloid rhizomes and three roots in total from three plots). The colonized samples from coralloid rhizomes were pooled to create a homogeneous mycorrhizal coralloid rhizome sample. The distal 3-cm portions of colonized roots were sectioned into 3-mm fragments, which were then combined to a homogeneous mycorrhizal root sample. For the adult plant sample without coralloid rhizomes, three roots of each individual were collected in each plot (15 roots from five individuals in total from five plots), colonized roots were sectioned and pooled.

**Molecular identification of mycorrhizal fungi**

**High-throughput sequencing.** The surface of roots and protocorms was washed in tap water and subsequently sterilized with a 1 % sodium hypochlorite solution for 60 s, followed by three 60-s rinse steps in sterile distilled water and microscopically checked for mycorrhizal colonization. Afterwards, DNA was extracted from 0.05–0.1 g mycorrhizal samples using the Plant Genomic DNA Purification Kit as described by the manufacturer (GMbiolab Co. Ltd, Taichung, Taiwan).

The internal transcribed spacer 1 (ITS1) region of the nuclear ribosomal RNA genes was amplified using the primer pair ITS1F and ITS2R (see Supporting Information—Table S2; Adams et al. 2013). PCR was carried out in 20 μL reactions containing 10 ng of genomic DNA, 0.8 μL of each primer (5 μM), 2 μL of 2.5 mM dNTPs, 4 μL of 5x TransStart® FastPfu Buffer (TransGen Biotech Co., Ltd, Beijing, China) and 0.4 μL of TransStart® FastPfu Polymerase. The parameters of reactions consisted of an initial denaturation at 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, and 55 °C for 30 s and a final extension of 72 °C for 45 s. PCR products were separated by gel electrophoresis, and amplicons within the appropriate size range were gel purified using the AxyPrep DNA Gel Extraction Kit (GMbiolab Co. Ltd, Taichung, Taiwan) and quantified using QuantiFluor™ Fluorometer (Promega Corporation, Madison, WI, USA). Samples were then pooled in equimolar concentrations and paired-end sequenced (2 × 250 bp) on an Illumina Miseq. Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17) with the following criteria. First, 300-bp reads were truncated at any site receiving an average quality score < 20 over a 50-bp sliding window, discarding the truncated reads shorter than 50 bp. Second, reads with one or two nucleotide mismatches in primer matching measurements and reads containing ambiguous characters were removed. Third, only sequences that had an overlap longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

**Bioinformatics.** Sequences obtained from the Illumina Miseq run were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm implemented in USEARCH version 7 (Edgar 2013). Operational taxonomic units were clustered with 97 % similarity cut-off using UPARSE (version 7.1; http://drive5.com/uparse/) and chimerae were identified and removed using the UNITE UCHIME reference data set. The taxonomy of each ITS1 region was analysed by RDP Classifier (http://rdp.cme.msu.edu/) against the UNITE fungal ITS database using a confidence threshold of 70 %. Remaining OTUs were assigned taxonomic identities based on the top 10 BLAST (megablast algorithm) (Altschul et al. 1990) results of the OTU representative sequences (selected by UPARSE) using the GenBank nucleotide (nt) database (Benson et al. 2012), including uncultured/environmental entries. Operational taxonomic units identified by BLAST were then manually screened for potential orchid-associating mycorrhizal families following a stepwise process. Firstly, OTUs represented by short sequences (<150 bp) or having a low sequence similarity (<90 %) with fungal species across their sequence length were removed. Secondly, only OTUs found on at least one orchid root sample in this study were retained.

**Phylogenetic analysis.** To test phylogenetic hypotheses, we generated complete ITS sequences from the DNA extractions of mycorrhizal samples. The ITS region of the...
fungal nuclear ribosomal RNA gene was amplified with the primer combinations ITS1F/ITS4 (Gardes and Bruns 1993) or ITS1-OF/ITS4-OF (Taylor and McCormick 2008). PCR amplification and sequencing were carried out as described by Yagame et al. (2016). PCR products that were difficult to sequence directly were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). For phylogenetic analysis, ITS sequences of Psathyrellaceae from GenBank were added to the analysis by referring to Yagame et al. (2013) and Suetsugu et al. (2021a, 2021b), and the sequence of Parasola leiocephala was used as the outgroup taxon. ITS sequences of Ceratobasidiaceae from GenBank were added to the analysis by referring to Suetsugu and Matsubayashi (2020), and the sequence of Botryobasidium subcoronatum was used as the outgroup taxon. ITS sequences of Sebacinales from GenBank were added to the analysis by referring to Yagame et al. (2016), and the sequence of Auricularia auricula-judae was used as the outgroup taxon. The sequence data were aligned using CLUSTALX (Thompson et al. 1994), followed by manual adjustment. Phylogenetic analyses were conducted by a model-based Bayesian method using MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). The ‘best-fit’ model of evolution was selected under the Akaike information criterion test (Akaike 1974) as implemented in MrModeltest 2.2 (Nylander 2004), and the general time-reversal plus invariant rates and a gamma distribution (GTR + I + Γ) was selected for the analyses. Two separate runs of four Monte Carlo Markov chains (Yang and Rannala 1997) were performed for 10 000 000 generations until the mean deviation of split frequency dropped below 0.01, and a tree was sampled every 1000th generation. Trees from the first 25% of generations were discarded using the ‘burn-in’ command. The remaining trees were used to calculate a 50% majority-rule consensus topology and determine posterior probabilities for individual branches.

Multi-element stable isotope analyses

For multi-element stable isotope analysis, sample preparation was according to Supporting Information—Table S1. Relative natural abundance analysis of carbon (13C/12C) and nitrogen (15N/14N) isotopes was determined simultaneously using an EA-IRMS coupling according to Bidartondo et al. (2004) and Supporting Information—Table S1. Relative natural abundances of hydrogen (2H/H) and oxygen isotopes (18O/16O) of each plant sample were measured using a TC-IRMS coupling as described in Supporting Information—Table S1. The oxygen isotope abundances were measured to assess a potential transpiration effect on the relative enrichment in 13C and 2H caused by differences in stomatal regulation and transpiration between orchids and the non-orchid reference plants. Transpiration affects 13C, 2H and 18O isotope abundance in plant tissues simultaneously (da Silveira Lobos Sternberg 1988; Ziegler 1989; Flanagan et al. 1991; Cernusak et al. 2004). A transpiration effect can only be precluded if the 18O isotope abundance of the target species is similarly distributed as the 18O isotope signature of the non-orchid reference plants (Gebauer et al. 2016). Measured relative isotope abundances are denoted as δ values that were calculated according to the following equation: δ13C, δ15N, δ2H or δ18O = (Rsample/Rstandard − 1) × 1000 ‰, where Rsample and Rstandard are the ratios of heavy to the light isotope of the samples and the respective standard. The reference sampling system allowed achieving year- and site-independent and thus comparable stable isotope data of the potentially mycoheterotrophic plants by calculating enrichment factors (ε) from the measured δ values as follows: ε = S – δREF, where δS is a single δ13C, δ15N, δ2H or δ18O value of an orchid individual or an autotrophic reference plant and δREF is the mean value of all autotrophic reference plants by plot (Preiss and Gebauer 2008). The ε approach was essential because sampling of different development stages within 1 year was not feasible. All single values and mean values ± standard deviations of δ13C, δ15N, δ2H and δ18O, of enrichment factors ε13C, ε15N, ε2H and ε18O, and of total N concentrations of the investigated orchid species and their autotrophic references are available in Supporting Information—Tables S2 and S3.

We tested for pairwise differences in the isotopic enrichment factors ε13C, ε15N, ε2H and ε18O between orchid specimens and all autotrophic reference plants applying non-parametric statistical tests because Shapiro–Wilk tests revealed non-normality. Autotrophic reference plants and C. appendiculata protocorm samples from different years, respectively, were treated as one group each due to insignificant differences within groups. After a significant Kruskal–Wallis H-test, pairwise differences between mature C. appendiculata, seedlings, protocorms and autotrophic references were assessed performing a post hoc Mann–Whitney U-test. Because of the relatively small sample size and few groups, we assumed a low risk of false positives and did not correct for multiple testing. We compared N concentrations of different groups using the Student’s t-test since Shapiro–Wilk test and Bartlett’s test confirmed normal distribution and homogeneity of variance, respectively. Statistical analyses and plotting were carried out in R Version 4.0.3 (R Core Team 2020) with a significance level of α = 0.05.

Results

Molecular identification of mycorrhizal fungi and germination mycobiots

The quality-filtered and normalized Miseq data set contained samples from protocorms, coralloid rhizomes of seedlings, roots of seedlings and roots of adult plants. The protocorm sample comprised 9 OTUs (41 093 sequences), the coralloid rhizomes of the seedling sample comprised 9 OTUs (41 078 sequences), the roots of the seedling sample comprised 35 OTUs (41 005 sequences) and the roots of the adult plant sample comprised 21 OTUs (41 127 sequences) (excluding OTUs with <200 total sequences; see Supporting Information—Table S2). The rarefaction curve analysis indicated that the numbers of OTUs were close to saturation after 20000 sequence counts [see Supporting Information—Fig. S1].

In the protocorm sample, the OTU with the highest number of reads belonged to Psathyrellaceae (OTU1—31 108 sequences, 75.7 %), while the typical orchid mycorrhizal families, Ceratobasidiaceae (OTU2—285 sequences, 0.69 %) and Sebacinales (OTU4—370 sequences, 0.9 %) were less abundant. In the coralloid rhizome sample of seedlings, the OTU with the highest number of reads also belonged to Psathyrellaceae (OTU1—23 767 sequences, 57.86 %), whereas the typical orchid mycorrhizal families, Ceratobasidiaceae (OTU2—188 sequences, 0.46 %), Ceratobasidiaceae (OTU6—2 sequences, 0.005 %), Sebacinales (OTU4—914 sequences, 2.23 %) had lower proportions (Fig. 2). On the contrary, in the root samples
of seedlings and adult plants, the typical orchid mycorrhizal fungi, i.e. Ceratobasidiaceae and Sebacinales, were the most dominant fungal partners (Fig. 2). In the root sample of seedlings, the proportions of two OTUs of Ceratobasidiaceae were higher than in adult root samples, i.e. OTU2 (9708 sequences, 23.68 %) and OTU6 (3306 sequences, 8.06 %). The proportion of Sebacinales also increased from seedling to adult root samples (OTU4—2039 sequences, 4.97 %). In the root sample of adult plants, the OTU with the highest number of reads also belonged to Sebacinales (OTU4—7592 sequences, 18.45 %), followed by two OTUs of Ceratobasidiaceae, i.e. OTU2 (1439 sequences, 3.51 %) and OTU6 (1360 sequences, 3.31 %).

Phylogenetic analyses revealed that the fungal sequence from protocorm and rhizome samples demonstrated a high DNA sequence homology with the fungal genus Coprinellus (Psathyrellaceae) [see Supporting Information—Fig. S2]. In root samples, two fungal sequences were related to the genera Ceratobasidium and Thanatephorus but not grouped with the ECM-forming clade of Ceratobasidiaceae [see Supporting Information—Fig. S4], suggesting the role of saprotrophic fungi. Besides, another fungal sequence from root samples belonged to Serendipitaceae [see Supporting Information—Fig. S3].

**Stable isotope natural abundances and total N concentrations**

Significant patterns emerged when examining stable isotope natural abundances and N concentrations of investigated orchid specimens and autotrophic reference plants (Table 1).

Mycoheterotrophic protocorms of *C. appendiculata* sampled in 2019 and 2015 exhibited similar enrichment factors on average with ε\(^{15}\)N of 3.16 ± 1.77 ‰ and 2.68 ± 0.12 ‰, respectively, and ε\(^{13}\)C of 8.01 ± 0.76 ‰ and 7.98 ± 0.34 ‰, respectively (Fig. 3A). Protocorm stage of *C. appendiculata* showed significant enrichment in \(^{15}\)N and \(^{13}\)C compared to autotrophic reference plants (U = 281, P < 0.001 and U = 298, P < 0.001). While protocorms of *C. appendiculata* from 2019 were on average 28.83 ± 5.80 ‰ significantly enriched in \(^{2}\)H (U = 125, P < 0.001), they were depleted in \(^{18}\)O relative to the reference plants (U = 15, P = 0.006) (Fig. 3B; Table 1).

Conversely, none of the stable isotope signatures of mature *C. appendiculata* differed statistically from autotrophic reference plants (Table 1; Fig. 3A and B).

*Cremas tra appendiculata* seedlings had on average an ε\(^{15}\)N of 4.15 ± 1.57 ‰, being significantly enriched relative to autotrophic reference plants (U = 108, P = 0.008). Therefore, \(^{15}\)N enrichment of seedlings was in a similar range as \(^{15}\)N enrichment of protocorms (Fig. 3A). Seedling samples were non-significantly enriched in \(^{13}\)C relative to reference plants (U = 82, P = 0.182), displaying an intermediate position between protocorms and mature *C. appendiculata* (Fig. 3A). Enrichment factor ε\(^{13}\)C of seedlings showed a rather considerable variation (3.06 ± 3.59 ‰) and was only statistically distinct from protocorms (U = 24, P = 0.012).

Total N concentration was on average highest in *C. appendiculata* protocorms (3.26 ± 0.28 mmol per g dry weight), followed by leaf N concentrations of seedlings with the largest variation (2.76 ± 1.23 mmol per g dry weight). Total N concentration of adult *C. appendiculata* (2.52 ± 0.18 mmol per g dry weight) and autotrophic reference plants (2.53 ± 0.49 mmol per g dry weight) were on average similar. They were statistically distinct from total N concentrations in protocorms (Fig. 4; see Supporting Information—Table S3).

**Discussion**

In this study, we assessed for the first time fungal associates, subterranean morphology and nutrition mode of three different ontogenetic development stages of *C. appendiculata* within one population: protocorms, seedlings as an intermediate stage and adults.

Initially mycoheterotrophic protocorm stage in the natural environment

Saprotrophic *Coprinellus* spp. seem to be substantial mycobionts involved in the mycoheterotrophic nutrition mode within the *Cremas tra* genus. They are fungal hosts of fully mycoheterotrophic orchid *C. aphylla* (Yagame et al. 2018) and partially mycoheterotrophic mature *C. appendiculata* (Suetsugu et al. 2021a; Yagame et al. 2021). Identifying wood/litter-decaying *Coprinellus* fungi as dominant mycobionts in
Table 1. Pairwise comparisons of $\delta^{15}$C, $\delta^{15}$N, $\delta^2$H and $\delta^{18}$O between the three development stages of Cremastra appendiculata (protocorms, seedlings, adults) and autotrophic reference plants using the Mann–Whitney U-test after a significant Kruskal–Wallis H-test ($\delta^{15}$C: $H = 22.821$, df = 3, $P < 0.001$; $\delta^{15}$N: $H = 22.354$, df = 3, $P < 0.001$; $\delta^2$H: $H = 12.802$, df = 2, $P = 0.002$; $\delta^{18}$O: $H = 9.0663$, df = 2, $P = 0.008$).

|                | $\epsilon^{15}$N | $\epsilon^{13}$C | $\epsilon^2$H | $\epsilon^{18}$O |
|----------------|------------------|------------------|---------------|-----------------|
| Protocorm vs. reference | 281 < 0.001     | 281 < 0.001     | 281 < 0.001   | 281 < 0.001     |
| Seedling vs. reference  | 108 0.008       | 82 0.182        | NA            | NA              |
| Adult vs. reference    | 119 0.313       | 132 0.130       | 74 0.552      | 33 0.108        |
| Protocorm vs. seedling  | 6 0.279         | 24 0.012        | NA            | NA              |
| Protocorm vs. adult     | 39 0.003        | 40 0.002        | 25 0.008      | 0 0.008         |
| Seedling vs. adult      | 15 0.036        | 10 0.571        | NA            | NA              |

Significances are highlighted in bold.

$C. appendiculata$ protocorm–rhizomes directly from an in situ experimental set-up is new to science but in accordance with earlier findings from symbiotic laboratory cultivation (Yagame et al. 2013).

Characteristic for mycoheterotrophic orchid specimens, which gain carbohydrates additionally to other nutrients from a fungal partner, $C. appendiculata$ protocorns showed enrichment in $^{13}$N, $^{13}$C and $^2$H and elevated leaf N concentrations. Here we present the first stable isotope patterns of protocorns related to non-rhizoctonia saprotrophic fungal partners, which are distinct from those of protocorns and fully mycoheterotrophic (FMH) adult orchids with rhizoctonia and ECM fungal partners (Table 2). $^{15}$N and $^{13}$C signatures of $C. appendiculata$ protocorns were rather within the range of FMH orchids with non-rhizoctonia SAP fungal partners; however, the substrate of the fungal partner (wood or litter) cannot clearly be distinguished by stable isotopes pattern hitherto (Table 2). Future radiocarbon measurements may reveal whether $C. appendiculata$ protocorns gain carbon indirectly from litter or dead wood (Hatté et al. 2020; Suetsugu et al. 2020), with the latter being likely because dead wood was already shown as the source for Psathyrella and Coprinellus fungal partners of mature, partially mycoheterotrophic $C. appendiculata$ and fully mycoheterotrophic $C. aphylla$ from Japan (Suetsugu et al. 2021a).

Seedlings—intermediate stage ‘mirroring the variability of adults’

During seedling growth of orchids, there can be narrow checkpoints for mycorrhizal range relative to the more promiscuous germination and mature stages, e.g. for Epipactis helleanorine (Bidartondo and Read 2008). However, partial replacement of mycobions in the seedling stage likely reduces the risk of failing to find new mycobions during ontogenesis (McCormick et al. 2006) and is the most frequent scenario of temporal turnover in orchid–mycorrhizal relationships (Ventre Lespiauq et al. 2021). In this sense, seedlings exhibited two groups of fungal associations depending on their subterranean morphology: Coprinellus sp. (Psathyrellaceae) in rhizomes, which were closely related to those isolated of mature $C. appendiculata$ in Japan (Yagame et al. 2013), and rhizoctonia (Ceratosobasidiaceae and Serendipitaceae) in the seedling roots. Analogously to mature $C. appendiculata$ from different populations (Yagame et al. 2021), we can confirm a link between subterranean root system morphology and the type of fungal partners in seedlings.

Overall, $^{15}$N enrichments of seedlings and protocorns were alike, implying the presence of some fungal derived organic matter in $C. appendiculata$ seedlings. Investigated seedlings possessed an intermediate position in $^{13}$C relative abundances between protocorns and adults, but with a considerable variation. We, therefore, suppose the seedling stage of $C. appendiculata$ to be a transition phase between the protocorn stage and adulthood regarding nutrition with increasing independence on fungal derived carbon.

The degree of mycoheterotrophic nutrition in green orchids is apparently modulated by the morphology of subterranean

![Figure 3](image-url)
organs, like in the case of *Calypso bulbosa* mycorrhizal with wood-decaying fungi (Suetsugu and Matsubayashi 2021a). Further, different types of fungal partners enable a more or less pronounced carbon flux from fungus to orchid reflected by different $^{13}$C enrichments (Martos et al. 2009; Stöckel et al. 2014; Schweiger et al. 2018). Thus, the large variance in the $^{13}$C signature of seedlings could be explained by the combination of rhizoctonia in roots and *Coprinellus* sp. in rhizomes. Depending on the seedling’s age and its need for fungal carbon supply, either rhizoctonia or *Coprinellus* sp. may be more critical fungal partners and be mirrored in the stable isotope signature. While *Coprinellus* sp. mycobionts may enable a distinct partially mycoheterotrophic nutrition of younger seedlings, more developed seedlings might have already become detached from rhizomes and switched to rhizoctonia fungal partners being less or even independent on fungal carbon. In future, this tendency could be reinforced by additional seedling samples at different ages and $^2$H analysis.

**Adults**

Assessing three different ontogenetic development stages of one *C. appendiculata* population, we found a step-by-step mycobiont turnover from *Psathyrellaceae* at protocorm stage to rhizoctonia fungi because *Serendipitaceae* were dominant mycobionts in roots of adults. Investigated *C. appendiculata* did not feature coralloid rhizomes; thus, the overall change in mycobionts was concomitant with a change in subterranean morphology during the ontogenetic development of studied *C. appendiculata*. Similar ontogenetic changes in fungal community have been reported before, e.g. for Tipularia discolor with wood-decomposing Auriculariales as mycobiont of protocorms and *Tulasnella* as fungal partners at adulthood (McCormick et al. 2004). Among achlorophyllous orchid *Gastrodia elata*, *Mycena* were identified in protocorms, while *Armillaria* were detected in tubers of adults (Park and Lee 2013; Chen et al. 2019).

Yet, investigated mature *C. appendiculata* individuals were truly autotrophic and had no additional fungal N source, as they did not differ from autotrophic reference plants in enrichment factors $\varepsilon^{13}$C, $\varepsilon^2$H and $\varepsilon^{15}$N, and leaf N concentration. In this respect, *C. appendiculata* adults stand out against the majority of the so far investigated orchids with rhizoctonia fungal partners that showed distinct enrichment, particularly in $^2$H (Table 2). Because fungus-to-orchid organic carbon transfer is—to current knowledge—a relatively widespread phenomenon among terrestrial chlorophyllous orchids (Schweiger et al. 2019; Gebauer and Clemens 2021), *C. appendiculata* belongs thereby to a minority of orchids that can be truly autotrophic as a mature plant.

However, our results contrast to findings on *C. appendiculata* var. *variabilis* individuals from Japan, which showed the same subterranean morphology and type of mycobiont but have been proposed as ‘cryptic’ mycoheterotrophic (*sensu* Hynson et al. 2013) due to enrichment in $^{15}$N relative to autotrophic reference plants (Yagame et al. 2021). Yet, mature *C. appendiculata* can be partially mycoheterotrophic.

**Table 2.** Mean enrichment factors $\varepsilon^{15}$N, $\varepsilon^{13}$C and $\varepsilon^2$H (bold) ± SD (italic) of adult leaves and protocorms of here investigated *Cremastra appendiculata* and Orchidaceae specimens extracted from published literature until November 2021 for comparison. Comparative values of orchid specimens are grouped by their type of fungal partner: Association with saprotrophic wood- or litter-decomposing fungi (SAP wood/litter), association with ectomycorrhizal fungi of trees (ECM), association with rhizoctonia. FMH indicate fully mycoheterotrophic, achlorophyllous orchid species. IMH indicate initially mycoheterotrophic protocorms.

| Specimen Type | $n_s$ | $n_{app}$ | $\varepsilon^{15}$N (‰) | $\varepsilon^{13}$C (‰) | $\varepsilon^2$H (‰) |
|---------------|-------|-----------|--------------------------|--------------------------|----------------------|
| *C. appendiculata* protocorm | 8     | 1         | 2.98 ± 1.36              | 8.00 ± 0.60              | 28.83 ± 5.8          |
| *C. appendiculata* adult | 5     | 1         | 0.46 ± 0.99              | 0.84 ± 0.99              | 1.80 ± 4.59          |
| SAP wood     | FMH adult | 43      | 9                        | 4.29 ± 2.09              | 10.98 ± 2.30         |
| SAP litter   | FMH adult | 15      | 3                        | 4.99 ± 0.53              | 8.24 ± 0.49          |
| ECM          | IMH protocorm | 46    | 2                        | 7.48 ± 2.26              | 7.55 ± 0.62          |
| Rhizoctonia  | IMH protocorm | 49    | 6                        | 5.77 ± 2.73              | 6.12 ± 2.70          |
| Green adult  | 674  | 65        | 2.94 ± 2.30              | -0.48 ± 1.90             | 18.71 ± 12.95        |

Data source is according to Schweiger (2018) and extended by data from Suetsugu et al. (2019), Suetsugu et al. (2020), Jacquemyn et al. (2021), Suetsugu and Matsubayashi (2021b), Suetsugu et al. (2021a) and Suetsugu et al. (2021b).
when having already accomplished the switch from rhizoctonia fungal partners to saprotrophic non-rhizoctonia Psathyrellaceae fungi and forming coralloid rhizomes (Suetsugu et al. 2021a; Yagame et al. 2021), traits similar to fully mycoheterotrophic C. aphylla (Yagame et al. 2018; Suetsugu et al. 2021a). Therefore, mature C. appendiculata, hitherto referred to as a partially mycoheterotrophic species, is capable of gaining carbon from various fungal partners (Yagame et al. 2021). Providing evidence that C. appendiculata is fully mycoheterotrophic plant–fungus interactions during adulthood extends our knowledge of C. appendiculata's plasticity by adding another important puzzle piece.

Conclusions
Though usually found in light-limited forests grounds, C. appendiculata develops relatively large green leaves. We provide novel, explicit evidence that mature C. appendiculata individuals with rhizoctonia mycobionts can be entirely autotrophic. Cremastra appendiculata is, therefore, a notable species among the Orchidaceae as it incorporates the entire spectrum from mycoheterotrophy to autotrophy.

Further, this is the first study to demonstrate dramatic, rather gradual than sudden changes in nutritional mode, root morphology and mycobionts throughout the development of C. appendiculata considering the transitional seedling stage. This illustrates how dynamic these aspects can be within orchid individuals.

Besides high within-species variability in fungal partners and nutrition of C. appendiculata depending on environmental conditions (Yagame et al. 2021), we highlight the importance of developmentally driven changes in mycobionts according to the orchid's physiological needs, notably ensuring carbon supply with fungal support or independently. Therefore, we suggest high within-individual flexibility of C. appendiculata regarding its mycobionts and carbon acquisition, which challenges our current, relatively static view of orchid–fungi interactions on species level.

Illuminating under which spatial and temporal environmental conditions C. appendiculata features which nutrition mode could help to deepen our understanding of the mechanisms that contributed to the evolution of saprotrophic–mycoheterotrophic plant–fungus interactions.

Supporting Information
The following additional information is available in the online version of this article—

Table S1. Equipment and conditions as used for stable isotope abundance analysis.
Table S2. Summary of fungal operational taxonomic units (OTUs) and their frequencies detected in protocorms, seedling rhizomes, seedling roots and adult roots of Cremastra appendiculata using the Illumina MiSeq platform. 9OTUs were defined at 3 % sequence dissimilarity using the UPARSE pipeline described in Edgar (2013). Only OTUs representing fungal taxa were retained during analysis, and only those OTUs with >100 total sequences are included here. 10Normalized OTU frequencies are indicated by total number of sequences obtained and the percentage of total sequences that each OTU was detected upon.

Table S3. Mean and single δ15N, δ13C, δ2H, δ18O values, enrichment factors ε15N, ε13C, ε2H, ε18O and total nitrogen concentration data of Cremastra appendiculata adults, protocorms and seedlings from Fagaceae forest site at Mei Feng, Nantou County, Taiwan (24°05′01.4″N, 121°10′10.2″E, 2000 m a.s.l.).
Table S4. Mean and single δ15N, δ13C, δ2H, δ18O values, enrichment factors ε15N, ε13C, ε2H, ε18O and total nitrogen concentration data of autotrophic reference species from Fagaceae forest site at Mei Feng, Nantou County, Taiwan (24°05′01.4″N, 121°10′10.2″E, 2000 m a.s.l.).

Figure S1. Operational taxonomic unit (OTU) rarefaction curves of protocorms, seedling rhizomes, seedling roots and adult roots samples by randomly selecting smaller fractions of reads 100 times.
Figure S2. The Bayesian tree based on the sequences of internal transcribed spacer (ITS) nuclear ribosomal DNA of Psathyrellaceae fungi (OL449677) obtained from protocorms and seedling rhizomes of Cremastra appendiculata and GenBank database (Yagame et al. 2013; Suetsugu et al. 2021a). The values above branches are Bayesian posterior probabilities (>70 %).
Figure S3. The Bayesian tree based on the sequences of internal transcribed spacer (ITS) nuclear ribosomal DNA of Sebacinales fungi (OL449680) obtained from seedling roots and adult roots of Cremastra appendiculata and GenBank database (Yagame et al. 2016). The values above branches are Bayesian posterior probabilities (>70 %).
Figure S4. The Bayesian tree based on the sequences of internal transcribed spacer (ITS) nuclear ribosomal DNA of Ceratobasidiae fungi (OL449678 and OL449679) obtained from seedling roots and adult roots of Cremastra appendiculata and GenBank database (Suetsugu et al. 2020). The values above branches are Bayesian posterior probabilities (>70 %).

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Conflict of Interest
None declared.

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Contributions by the Authors
F.E.Z. analysed the isotope abundance data and wrote the first manuscript draft. Y.-I.L. carried out the sampling and the molecular analyses. G.G. had the idea for the research project and supervised the isotope abundance analyses. All
co-authors contributed critically to the manuscript and approved the final version.

**Data Availability**

For all metagenome data of this investigation, the accession on NCBI Sequence Read Archive is: PRJNA824353. For all single isotope abundance data, see Supporting Information—Tables S3 and S4.

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