Exploring plant nutritional strategies in orchid and arbuscular mycorrhizal associations using stable isotope natural abundance

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Exploring plant nutritional strategies in orchid and arbuscular mycorrhizal associations using stable isotope natural abundance

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ZUSAMMENFASSUNG

Die Mykorrhiza-Symbiose mit verschiedenen Wurzelpilzen ermöglicht Pflanzen ein breites Spektrum an Strategien zur Kohlenstoffgewinnung. Die Ernährungsstrategien reichen von der allgemein bekannten vollständig autotrophen Kohlenstoffgewinnung durch Photosynthese über partielle Mykoheterotrophie bis hin zu einer vollständig mykoheterotrophen Kohlenstoffversorgung ausschließlich durch Mykorrhiza-Pilze. Mykoheterotrophie, die Fähigkeit einer Pflanze, organischen Kohlenstoff von Pilzpartnern zu erhalten, kehrt die ‚klassische' Richtung des Kohlenstoffflusses von Pflanze zu Pilz in der Mykorrhiza-Symbiose um. Das Ausmaß, in dem Pflanzen Kohlenstoff von Pilzen beziehen, ist kontextabhängig und variiert auf evolutionärer, entwicklungsbezogener und ökologischer Ebene. Während das nicht-grüne Erscheinungsbild vollständig mykoheterotropher Pflanzen ein offensichtlicher Hinweis auf ihre Abhängigkeit von der Kohlenstoffversorgung durch Pilze ist, sind die Ernährungsstrategien chlorophyllhaltiger Pflanzen weniger augenscheinlich.

Diese Dissertation befasst sich mit dem Autotrophie-Mykoheterotrophie-Kontinuum bei chlorophyllhaltigen Pflanzen, die Orchideen- oder arbuskuläre Mykorrhiza aufweisen. Um die Kohlenstoffgewinnungsstrategien von Pflanzen in ihrem natürlichen Lebensraum zu bestimmen, ist die Analyse natürlicher stabiler Isotopenhäufigkeiten ($\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$ und $\delta^{18}O$) in Kombination mit der molekularen Bestimmung der Pilzpartner eine etablierte Methode. Mykoheterotrophe Pflanzen weisen charakteristische Isotopensignaturen auf: Partielle und vollständig mykoheterotrophe Pflanzen können im Allgemeinen durch ihre ^{13}C-, ^{15}N- und ^{2}H-Anreicherung von autotrophen Pflanzen unterschieden werden.

Der Schwerpunkt dieser Arbeit liegt auf der Orchideen-Mykorrhiza. Untersucht wurden (i) das Mykoheterotrophie-Autotrophie-Kontinuum und Änderungen von Pilzpartnern auf ontogenetischer und ökologischer Ebene, sowie (ii) die Isotopensignaturen der Pilzstrukturen in den Orchideenwurzeln. Darüber hinaus wurde in dieser Arbeit (iii) erstmalig untersucht, ob partielle Mykoheterotrophie bei Sämlingen von verholzten Pflanzenarten, die arbuskulär mykorrhiziert werden, auftritt und (iv) ein Beitrag zum aktuellen wissenschaftlichen Diskurs über gemeinsame Mykorrhiza-Netzwerke (‚common mycorrhizal networks') geleistet.

In **Manuskript 1** & **2** wurden drei verschiedene Entwicklungsstadien (Protokorme, chlorophyllhaltige Sämlinge, adulte Individuen) von zwei chlorophyllhaltigen Orchideenarten, die unterschiedliche Pilzpartner aufweisen, untersucht. Bei beiden Orchideenarten (*Cremastra appendiculata* und *Neuwiedia malipoensis*) ging eine Änderung der Pilzpartner während der Ontogenese mit einer abnehmenden Kohlenstoffversorgung durch Pilzpartner bis hin zur autotrophen Ernährung einher.

Manuskript 3 befasst sich mit der Änderung von Pilzpartnern und Ernährungsstrategien in der Orchideen-Mykorrhiza auf ökologischer Ebene am Beispiel der partiell mykoheterotrophen Orchidee *Neottia ovata,* die an Wiesen- und Waldstandorten vorkommt. *N. ovata* war an beiden Standorttypen überwiegend mit Rhizoctonia-Pilzen mykorrhiziert, wobei Individuen im Wald zusätzlich Ektomykorrhiza-Pilze rekrutierten. Das Vorhandensein oder Fehlen von Ektomykorrhiza-Pilzen, das Habitat und die Lichtverfügbarkeit hatten keinen wesentlichen Einfluss auf die Isotopensignaturen von *N. ovata*. Diese Ergebnisse deuten darauf hin, dass Rhizoctonia-Pilze eine wichtige funktionelle Rolle bei der Kohlenstoff- und Nährstoffversorgung spielten, während Ektomykorrhiza-Pilze einen vernachlässigbaren Beitrag zum Kohlenstoffhaushalt der Orchideen leisteten, was auf ein frühes evolutionäres Stadium der partiellen Mykoheterotrophie von *N. ovata* schließen lässt.

Manuskript 4 identifizierte die natürlichen Häufigkeitssignaturen stabiler Isotope (^{13}C, ^{15}N) von ‚Pelotons', den charakteristischen Pilzstrukturen in Orchideenwurzeln, und damit die direkte potentielle Pilz-Kohlenstoff-Quelle von Orchideen. Rhizoctonia-Pelotons zeigten eine geringfügige ^{13}C-Anreicherung im Vergleich zu Pflanzengewebe, und ihre ^{13}C-Anreicherung war im Vergleich zu Pelotons von Orchideen, die Ektomykorrhiza-Pilze rekrutierten, signifikant niedriger. Die geringe ^{13}C-Anreicherung von Rhizoctonia-Pelotons erklärt die unauffällige ^{13}C-Anreicherung von mit Rhizoctonia assoziierten Orchideen und erschwert es, einen Kohlenstoffgewinn von Pilzpartnern festzustellen. Die Ergebnisse unterstreichen die Notwendigkeit, bei der Untersuchung chlorophyllhaltiger Orchideen, die hauptsächlich Rhizoctonia-Pilze rekrutieren, ^{2}H Isotopensignatur als Ersatz für ^{13}C hinzuzuziehen.

Manuskript 5 widmet sich dem weniger untersuchten Forschungsbereich von partieller Mykoheterotrophie bei arbuskulär mykorrhizierten Pflanzen. Es wurde der Fragestellung

nachgegangen, ob partielle Mykoheterotrophie bei Sämlingen von tropischen verholzten Pflanzenarten mit arbuskulärer Mykorrhiza des *Paris*-Typs auftritt. Der *Paris*-Typ (einer von zwei Morphologietypen bei arbuskulärer Mykorrhiza) weist morphologisch ähnliche Pilzstrukturen in Pflanzenwurzeln auf wie Orchideenmykorrhiza und wurde als Voraussetzung für einen Kohlenstofftransfer vom Pilz zur Pflanze bei arbuskulärer Mykorrhiza postuliert. Partielle Mykoheterotrophie ließ sich basierend auf natürlichen Isotopenhäufigkeitssignaturen bei Sämlingen von sechs der insgesamt 21 verholzten tropischen Pflanzenarten mit arbuskulärer Mykorrhiza des *Paris*-Typs feststellen.

In Form eines ‚Perspektiven'-Artikels hebt **Manuskript 6** mykoheterotrophe Pflanzen als natürlichen Beleg für gemeinsame Mykorrhiza-Netzwerke hervor und bringt diesen bisher eher vernachlässigten Aspekt in den aktuellen wissenschaftlichen Diskurs über die Verbreitung und die potenziellen Funktionen von gemeinsamen Mykorrhiza-Netzwerken ein.

SUMMARY

The mycorrhizal symbiosis with various root fungi enables plants to pursue a wide spectrum of carbon acquisition strategies. Mycoheterotrophy, i.e. the ability of a plant to obtain organic carbon from fungi, reverses the usual direction of plant-to-fungus carbon flow in mycorrhizae. Nutritional strategies range from an entirely autotrophic carbon gain through photosynthesis *via* partial mycoheterotrophy to a fully mycoheterotrophic carbon supply solely from mycorrhizal fungi. The degree to which plants obtain fungal carbon is highly context-dependent shifting on an evolutionary, ontogenetic and ecological scale. While the non-green appearance of fully mycoheterotrophic plants provides an obvious indication of their dependence on carbon supply from fungi, the nutritional strategies of chlorophyllous plants are less apparent.

This thesis investigated the autotrophy-mycoheterotrophy continuum in chlorophyllous plants forming arbuscular or orchid mycorrhiza. These endo-mycorrhizae are the most common mycorrhizal relationships among flowering plants species-wise and also share similarities in form and possibly function. Multi-element natural abundance stable isotope analysis ($\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$ and $\delta^{18}O$) in combination with molecular determination of fungal associates was used to elucidate nutritional strategies of plants in their natural habitat. Mycoheterotrophs generally exhibit unique stable isotope signatures, enabling the differentiation of partially and fully mycoheterotrophic plants from autotrophic ones through their ^{13}C, ^{15}N, and ^{2}H enrichment.

The thesis was focused on orchid mycorrhiza, examining (i) the mycoheterotrophy-autotrophy continuum and shifts in fungal associates on ontogenetic and environmental scales, and (ii) the stable isotope signatures of pelotons as a direct fungal source of orchid nutrition. This thesis also (iii) initially explored whether partial mycoheterotrophy occurs among seedlings of woody arbuscular mycorrhizal species, and (iv) contributes to the current discussion on common mycorrhizal networks.

Manuscripts 1 & 2 examined three different development stages (protocorms, chlorophyllous seedlings, adults) of two chlorophyllous orchid species, demonstrating a progressive switch of fungal associations with decreasing dependency of orchid species on fungal carbon up to autotrophy during ontogenesis. **Manuscript 1** focused on *Cremastra appendiculata*, an orchid species displaying mycoheterotrophy with saprotrophic Psathyrellaceae fungal associates in early development stages and usually

transitioning to rhizoctonia fungal associates in maturity. The study established that *C. appendiculata* spans the entire mycoheterotrophy-autotrophy continuum during ontogenesis, linking this shift to alterations in subterranean morphology. The results highlight the remarkable flexibility in nutritional strategies and mycobiont associations on ontogenetic and environmental scales. **Manuscript 2** delved into *Neuwiedia malipoensis*, a member of the subfamily Apostasioideae, which comprises only chlorophyllous species from two genera. Unlike *Apostasia nipponica,* which gains carbon from ectomycorrhiza-forming fungi, *N. malipoensis* consistently associated with rhizoctonia fungi throughout its ontogenesis, despite a change in fungal family. This suggests a divergence in mycobionts between *A. nipponica* and *N. malipoensis*, allowing for varied resource acquisition and adaptations to environmental conditions within the earliest-diverging orchid clade.

Manuscript 3 explored ecological factors influencing fungal shifts and nutritional strategies in orchid mycorrhiza associations. As a partially mycoheterotrophic model species, *Neottia ovata* was investigated across habitats ranging from open grassland to shady forests. *N. ovata* predominantly associated with rhizoctonia fungi in both grassland and forest habitats, with forest orchids additionally recruiting ectomycorrhizal fungi. The presence or absence of ectomycorrhizal fungi, habitat type and light availability did not substantially influence the isotope signatures of *N. ovata*. These results indicate that rhizoctonia fungi played a major functional role in carbon and nutrient supply while ectomycorrhizal fungi made a negligible contribution to the orchids' carbon budget suggesting an early evolutionary stage of partial mycoheterotrophy for *N. ovata*.

Manuscript 4 identified the natural abundance stable isotope signatures (^{13}C, ^{15}N) of pelotons, the characteristic hyphal structures formed in orchid roots and thus the direct fungal source in orchid nutrition. Rhizoctonia pelotons showed minor ^{13}C enrichment compared to plant tissues and their ^{13}C enrichment was significantly lower relative to pelotons from orchids recruiting ectomycorrhizal fungi. The low ^{13}C enrichment of rhizoctonia pelotons explains the inconspicuous ^{13}C enrichment of rhizoctonia-associated orchids and hampers the detection of carbon gains from fungal associates. The findings underline the importance of a multi-element approach, including ^{2}H as a substitute for ^{13}C, when investigating chlorophyllous orchids predominately recruiting rhizoctonia fungi.

Manuscript 5 addressed the less explored area of partial mycoheterotrophy in arbuscular mycorrhiza. The occurrence of partial mycoheterotrophy in seedlings of tropical woody plants that exhibit *Paris*-type arbuscular mycorrhiza structures in their roots was investigated. The *Paris*-type, characterised by hyphal coils with morphological similarity to orchid pelotons, is considered a necessary condition for the fungus-to-plant carbon transfer in arbuscular mycorrhiza. Our microscopic observations revealed a greater prevalence of *Paris*-type among tropical plant genera than previously recognised. Natural abundance stable isotope signatures provided evidence for partial mycoheterotrophy in seedlings of six out of the 21 investigated *Paris*-type arbuscular mycorrhizal woody species.

As an outlook, **Manuscript 6** offers a perspective that introduced mycoheterotrophic plants as natural evidence for common mycorrhizal networks to the ongoing discussion about the prevalence and potential functions of these networks.

LIST OF ABBREVIATIONS

AM	arbuscular mycorrhiza(l)
C	carbon
CMN(s)	common mycorrhizal network(s)
ECM	ectomycorrhiza(l)
ERM	ericoid mycorrhiza(l)
H	hydrogen
N	nitrogen
O	oxygen
OM	orchid mycorrhiza(l)
P	phosphorous
SAP	saprotrophic (non-rhizoctonia)
spp.	species

ON THIS THESIS

1 Introduction

1.1 Mycorrhizal interactions: Types and asymmetries in carbon and nutrient exchange

Mycorrhiza is a symbiosis formed between plants and specialised soil fungi inhabiting their roots and/or rhizoids (first described by Frank, 1885). It is among the ecologically most important and widespread biological inter-kingdom interactions (Genre *et al.*, 2020). Mycorrhizal interactions are even considered a key element for the successful establishment of plants in almost all terrestrial ecosystems (Field *et al.*, 2015). All major lineages of land plants, with the exception of mosses (Hoysted *et al.*, 2018), form associations with fungi belonging to the phyla Mucoromycota, Basidiomycota or Ascomycota (van der Heijden *et al.*, 2015; Tedersoo *et al.*, 2020). Mycorrhizal fungi are an important part of the plant microbiome (Tedersoo *et al.*, 2020) and more than 90% of higher land plants are considered mycorrhizal (Smith & Read, 2008).

Based on root-fungi morphological characteristics and different plant identities, four main types of mycorrhizae have been defined (Fig. 1) (Smith & Read, 2008; Brundrett & Tedersoo, 2018): (1) ***Arbuscular mycorrhiza*** *(AM)*, (2) ***Orchid mycorrhiza*** *(OM)*, (3) *Ericoid mycorrhiza (ERM)*, and (4) *Ectomycorrhiza (ECM)*. In ECM, the fungal hyphae grow only within the apoplastic space between the plant root cells typically forming a so-called Hartig net (Smith & Read, 2008). In contrast, AM, OM and ERM are described as endo-mycorrhizae with the fungal hyphae that penetrate the cell wall of root cortical cells and form complex intracellular fungal structures.

AM is considered the most ancestral type of mycorrhizal interactions. It is formed between most vascular plants from multiple plant families and fungi belonging to the basal sub-phylum of Glomeromycotina. In OM, ERM and ECM mycorrhizal fungi belong to the fungal phyla of Ascomycota and Basidiomycota. OM is solely found in the Orchidaceae and ERM is unique to certain Ericales plant partners, while ECM mainly occurs in tree species.

Fungi can occasionally be shared among plant partners with different mycorrhizal types, i.e. certain fungal taxa are involved in OM and are simultaneously ECM in trees.

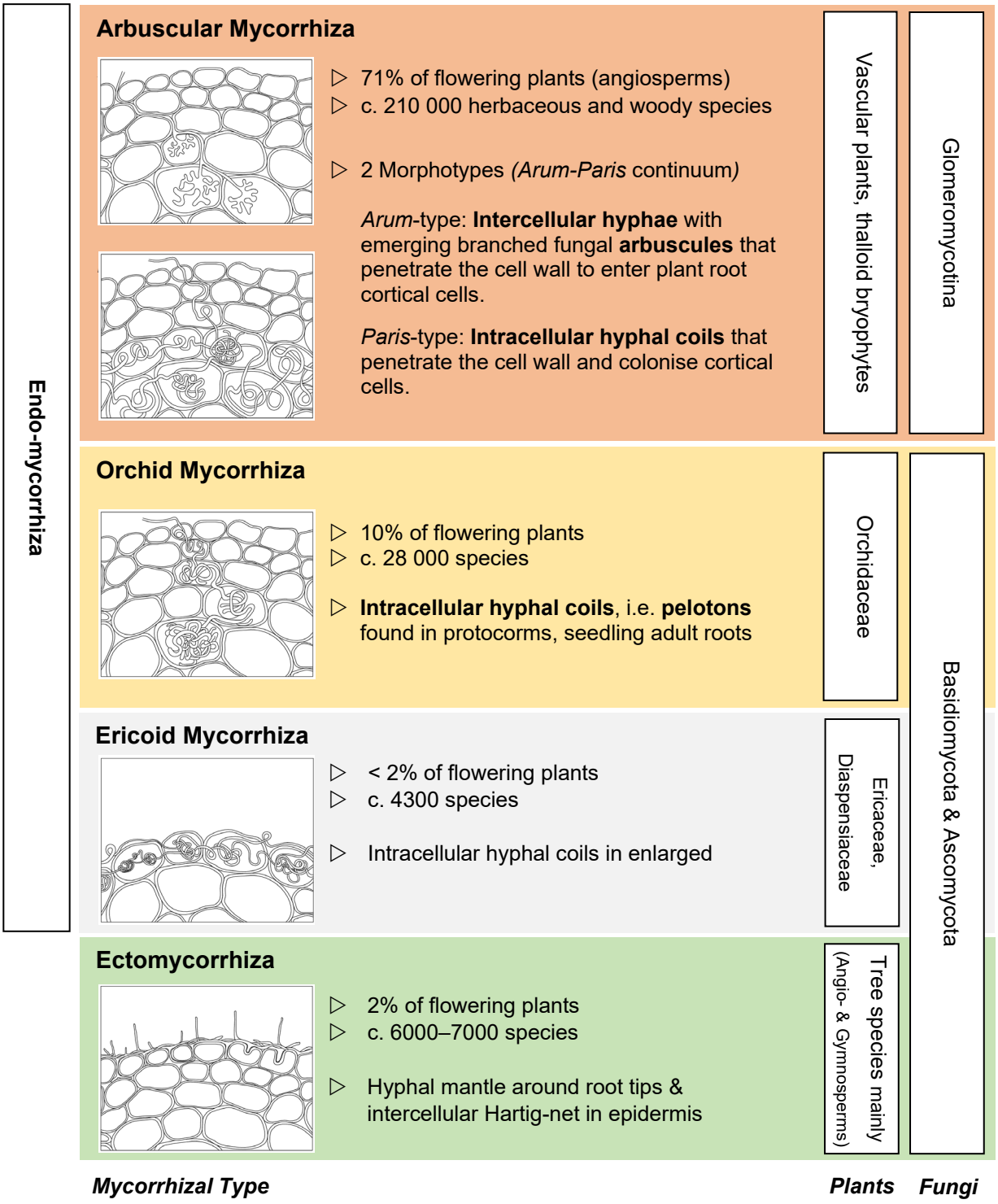


FIG 1 Major types of mycorrhizae with main cellular features and identity of plant and fungal associates. All mycorrhizal types involve fungal hyphae penetrating root cell walls but staying separated from the cell cytoplasma by a plant-derived membrane and interfacial matrix, forming an apoplastic compartment with varying chemical composition. Usually, a particular type of mycorrhiza is specific to a plant species. Some 'dual-mycorrhizal' plants (mostly woody) form both AM and ECM, often depending on temporal and spatial context (Teste *et al.*, 2020). Notably, various endophytic 'non-mycorrhizal' fungi (e.g. fine root endophytes and dark septate endophytes) form a functional symbiosis with plants (Jumpponen, 2001; Sinanaj *et al.*, 2021), including plant taxa classically considered 'non-mycorrhizal' (van Der Heijden *et al.*, 2017; Giesemann *et al.*, 2020a). Figure information is based on Peterson & Massicotte, 2004; Brundrett & Tedersoo, 2018.

The mycorrhizal symbiosis aids a bi-directional resource exchange between associates. Plants are traditionally considered to transfer photosynthetically fixed carbon (C) to their fungal associates, which in turn facilitate the uptake of water and limiting soil nutrients, mainly phosphorus (P) and nitrogen (N) in a mutualistic manner (Smith & Read, 2008). Expanding upon the simplified immediate fungus-plant interaction and adding another level of complexity to the mycorrhizal symbiosis, mycorrhizal fungi can form extensive subterranean networks of hyphae, colonising multiple plant partners and occasionally interconnect the roots of con- or heterospecific individual plants through common mycorrhizal networks (CMNs) (Field *et al.*, 2017). Although CMNs are regarded as important for signalling (Délano-Frier & Tejeda-Sartorius, 2008; Babikova *et al.*, 2013; Gorzelak *et al.*, 2015; Johnson & Gilbert, 2015) and multi-directional resource (e.g. C, nutrients, water) exchange between connected plants *via* shared mycorrhizal fungi with far-reaching ecological implications (Tedersoo *et al.*, 2020), the functioning of the multi-partite CMNs is largely unknown.

Even the bi-directional exchange of resources (e.g. C, N, P) between entangled mycorrhizal partners is highly dynamic and often asymmetrical, i.e. some plant species may benefit much more than others, in terms of C invested and nutrients obtained (e.g. van der Heijden *et al.*, 1998, 2015; Walder *et al.*, 2012; Weremijewicz *et al.*, 2016; Field & Pressel, 2018). Beyond that, an extreme example of non-balanced resource exchange is attributed to so-called mycoheterotrophic plants (often referred to as 'cheaters'/parasites) receiving organic C from mycorrhizal fungi (Leake, 1994), which challenges the classical view of a mycorrhizal interaction being a C-for-nutrient exchange.

1.2 Mycoheterotrophy – a plant nutritional strategy for carbon acquisition

Mycoheterotrophy is strictly defined as the ability of a plant to obtain C from fungi and is thereby a trophic strategy contrasting with autotrophy (Merckx, 2013). Besides, mycoheterotrophic plants may receive nutrients from their mycorrhizal fungi as well. It remains uncertain whether mycoheterotrophic plants have a genuinely harmful impact on their associated fungi or if they may offer benefits, such as vitamins, lipids/fatty acids or shelter, to their symbiont (Selosse & Roy, 2009; Jiang *et al.*, 2017).

Full mycoheterotrophs are achlorophyllous plants entirely dependent on fungal-derived C throughout their whole life, all forming endo-mycorrhizal associations, i.e. OM, AM or

ERM (Merckx, 2013). As they have completely lost their photosynthetic ability, full mycoheterotrophs are likely placed on an evolutionary endpoint (Graham *et al.*, 2017). Full mycoheterotrophy has evolved multiple times independently and occurs in liverworts, lycophytes, ferns, angiosperms, and perhaps gymnosperms (Bidartondo, 2005). Among flowering plants approximately 580 leafless, achlorophyllous species, usually small herbs, are currently known belonging to the plant families Orchidaceae, Burmanniaceae, Thismiaceae, Triuridaceae, Corsiaceae, Petrosaviaceae and Iridaceae (monocots) and Gentianaceae, Polygalaceae, Ericaceae (eudicots) (Jacquemyn & Merckx, 2019). Although locally rare, full mycoheterotrophs have a worldwide distribution, which is closely related to the occurrence of forests (Gomes *et al.*, 2019).

Compared to their chlorophyllous relatives, fully mycoheterotrophic plants often show high specificity in their interactions with mycorrhizal fungi and are often associated with a narrower phylogenetic range of fungi (Bidartondo, 2005; Merckx *et al.*, 2009; Perez-Lamarque *et al.*, 2020; Wang *et al.*, 2021). Full mycoheterotrophs are C supplied by being integrated into CMNs through shared fungi that simultaneously form AM (predominantly in the tropics) or ECM (in temperate and boreal regions) with neighbouring plants (= 'epiparasitism' *sensu* Björkman, 1960). In the latter case, the mycoheterotrophic plants only tap into an ECM network, as they are not ECM themselves but rather form OM or ERM (endo-mycorrhizae) (Bidartondo *et al.*, 2004; Merckx, Bidartondo and Hynson, 2009; Egan and Hynson, 2019). Alternatively, fully mycoheterotrophic orchids gain C from mycorrhizal associations with otherwise free-living usually saprotrophic (SAP) fungi that themselves gain C from organic matter decay (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009, 2012, 2021; Lee *et al.*, 2015; Yamashita *et al.*, 2020). Both ECM and AM fungi are largely considered obligate biotrophic (ECM: Koide *et al.*, 2008 but Lindahl & Tunlid, 2015; AM: Trépanier *et al.*, 2005; Tisserant *et al.*, 2013), i.e. they are (organic) C supplied by mycorrhizal plants. Therefore, the mycoheterotrophic C gain of achlorophyllous plants from ECM or AM fungi requires at least a tripartite association including a photosynthetic 'C donor plant' (Bidartondo, 2005). In this case, mycoheterotrophic plants act as a natural positive proof (cf. manuscript 6) for the currently highly disputed relevance of CMNs in C exchange dynamics between plants (Figueiredo *et al.*, 2021; Henriksson *et al.*, 2023; Karst *et al.*, 2023 Klein *et al.*, 2023; Luo *et al.*, 2023).

As an intermediate trophic strategy between autotrophy and full mycoheterotrophy, *partial mycoheterotrophy* (sometimes also referred to as 'mixotrophy') describes a dual nutritional strategy of chlorophyllous plants that complement C gain *via* their own photosynthesis with variable amounts of C gained from their fungal associates (Merckx *et al.*, 2009). This nutritional strategy has been recognised in herbaceous plants forming OM, ERM and AM, although the latter is the least investigated (e.g. Gebauer & Meyer, 2003; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Cameron & Bolin, 2010; Hynson *et al.*, 2016; Giesemann *et al.*, 2020, 2021). Because partially mycoheterotrophic plants often belong to plant families, in which fully mycoheterotrophic representatives occur, the evolution of full mycoheterotrophy from autotrophy is likely to occur *via* intermediate stages (Merckx & Freudenstein, 2010; Motomura *et al.*, 2010; Jacquemyn & Merckx, 2019).

The extent to which plants rely on fungal C is highly context-dependent in space and time. It varies at developmental (within lifetime), ecological (e.g. between habitats) and evolutionary (between plant species) scales. Thus, the nutrition strategies of plants for C acquisition are considered a continuum between the two endpoints 'autotrophy' and 'full mycoheterotrophy' rather than distinct nutritional modes (Jacquemyn & Merckx, 2019). When referring to plant nutrition strategies, we clearly adopt a rather plant-centric perspective, yet, diversity as well as spatial and temporal dynamics are certainly also impacted by fungal identity and ecology.

1.3 The autotrophy-mycoheterotrophy continuum: nutritional strategies of plants

Being integrated into mycorrhizal interactions enables plants to pursue a wide range of C gain strategies ranging from an entirely autotrophic C gain *via* photosynthesis to a fully mycoheterotrophic C supply solely from mycorrhizal fungi. While the non-green appearance of fully mycoheterotrophic plants provides an initial indication of their dependence on C supply from fungi, the nutritional strategies of chlorophyllous plants are less apparent and explored, which makes them particularly fascinating.

Along the autotrophy-mycoheterotrophy continuum, this thesis investigated nutritional strategies of chlorophyllous plants engaging in OM (Orchidaceae) and AM (diverse plant families). Those endo-mycorrhizae are not only the two most widespread mycorrhizal symbioses species-wise among flowering plants, but also have certain similarities in form and possibly function (Miura *et al.*, 2018; Favre-Godal *et al.*, 2020; Perotto & Balestrini, 2023). While our knowledge about the peculiarities of terrestrial orchid nutritional

strategies is relatively well-established, the exploration of nutritional strategies (including potential partial mycoheterotrophy) of chlorophyllous AM plants has only just begun.

1.3.1 Diversity of nutritional strategies in orchid mycorrhiza

Orchidaceae, as one of the largest and most diverse terrestrial plant families with > 29 000 recognised species (POWO, 2023), demonstrate a unique range of different trophic strategies and fungal associates (Ascomycota and Basidiomycota) on both phylogenetic and ontogenetic level while occurring in almost all terrestrial ecosystems (Field *et al.*, 2017). This renders the OM an extraordinary study system to explore plant trophic strategies and mycorrhizal associations on various scales (Fig. 2). Hereby, it must be acknowledged that our current understanding is largely restricted to terrestrial orchids (25% of Orchidaceae). The functional importance of mycorrhiza for epiphytic (70%) and lithophytic (5%) orchids, which may be distinct from terrestrial ones, is largely unexplored (Field *et al.*, 2017; Favre-Godal *et al.*, 2020). Henceforth, orchids in this thesis are explicitly referring to terrestrial orchids.

More than 250 achlorophyllous orchid species from several genera are fully mycoheterotrophic throughout their entire life cycle (Fig. 2, e.g. *Neottia* spp., *Cremastra* spp., *Corallorhiza* spp., *Gastrodia* spp., *Wullschlaegelia* spp., *Epipogium* spp.) (Jacquemyn & Merckx, 2019). In contrast, most other orchids are chlorophyllous at the maturity stage. Rarely, albino forms can be observed, e.g. in *Cephalanthera* and *Epipactis* spp. (Julou *et al.*, 2005; Suetsugu *et al.*, 2017). Both achlorophyllous and chlorophyllous orchids have minute seeds (dust seeds) without endosperm and thus very limited carbohydrate reserves (Leake, 1994; Arditti & Ghani, 2000; Eriksson & Kainulainen, 2011). Thereby, all orchids are *initially mycoheterotrophic* (Merckx, 2013), i.e. they depend on mycorrhizal fungi for nutrient and C supply during germination (symbiotic germination) and early belowground seedling (achlorophyllous protocorm) development (Rasmussen, 1995; Smith & Read, 2008; Rasmussen & Rasmussen, 2014). Most orchids become capable of photosynthesis when fully established and the dependency on fungal derived C usually decreases as the seedling develops chlorophyllous leaves. Yet, several chlorophyllous orchids have been found to be partially mycoheterotrophic at maturity and obtain C from mycorrhizal fungi to varying extents (e.g. Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Hynson *et al.*, 2013; Jacquemyn *et al.*, 2017, 2023; Schiebold *et al.*, 2018).

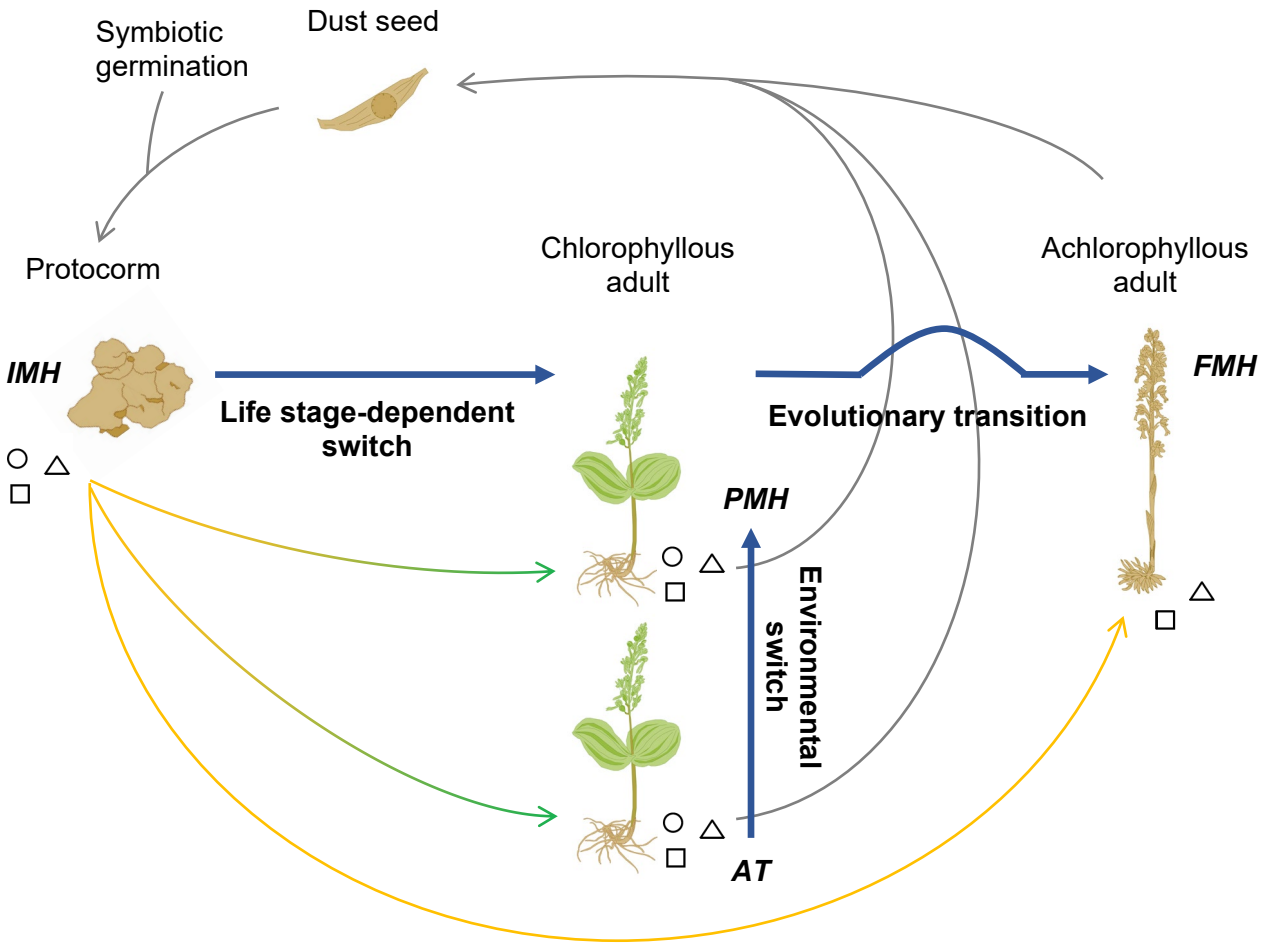


FIG 2 Lifecycle of orchids with dynamic changes in trophic strategies (degree of mycoheterotrophy) at ontogenetic, evolutionary and environmental scale. AT: Autotrophy, IMH: Initial mycoheterotrophy, PMH: Partial mycoheterotrophy, FMH: Fully mycoheterotrophy. Symbols represent potential fungal associates supporting a tropic strategy: rhizoctonia fungi (circles), ectomycorrhizal fungi (triangles), wood- or litter- decaying saprotrophic fungi (squares). Figure based on Field *et al.* 2018, expanded.

The degree of C gain from fungi in chlorophyllous orchids has been linked to plant identity (Zimmer *et al.*, 2007; Leake & Cameron, 2010). In addition, partial mycoheterotrophy is modulated by environmental conditions, e.g. seasonal changes (Ventre Lespiaucq *et al.*, 2021), light availability (Preiss *et al.*, 2010; Gonneau *et al.*, 2014; Liebel *et al.*, 2015) or certain plant traits, like foliar chlorophyll concentration (Stöckel *et al.*, 2011; Suetsugu & Matsubayashi, 2022) or orchids' subterranean morphology (Suetsugu & Matsubayashi, 2021a). For instance, C supply from fungi varied depending on the subterranean morphology of the chlorophyllous orchid *Calypso bulbosa,* which is associated with wood-decaying fungi (Suetsugu & Matsubayashi, 2021a).

Transitions in trophic strategies (autotrophy *via* partial to full mycoheterotrophy) of orchids and shifts in fungal associates often co-occur (Fig. 2) (Wang *et al.*, 2021). The primary fungal associates of most chlorophyllous orchids (autotrophic or partially mycoheterotrophic) are basidiomycetous fungi belonging to the Ceratobasidiaceae, Tulasnellaceae (order Cantharellales) and Serendipitaceae – traditionally categorized as the polyphyletic 'rhizoctonia' group (Rasmussen, 2002; Dearnaley, 2007; Weiß *et al.*, 2011; Dearnaley *et al.*, 2012). Other than being OM mycobionts, rhizoctonia have been reported to be often saprotrophic, plant endophytic or sometimes even ECM (Girlanda *et al.*, 2011; Selosse & Martos, 2014). Yet, the rhizoctonia fungi, which are largely exclusive to OM, remain highly elusive in their ecology and lifestyles. A fully mycoheterotrophic C gain of orchids (with the exception of some albinos) involves non-rhizoctonia associations with simultaneously ECM fungi *via* a CMN (Taylor & Bruns, 1997; Selosse *et al.*, 2004; Bidartondo *et al.*, 2004) and/or free-living, wood- or litter- decaying SAP fungi (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009, 2012, 2021; Lee *et al.*, 2015; Yamashita *et al.*, 2020). The mycorrhizal fungi commonly associated with fully mycoheterotrophic orchids have also been found to contribute to the C supply of chlorophyllous orchids (Jacquemyn & Merckx, 2019). Mycoheterotrophic plants associated with SAP fungi are, according to current knowledge, exclusively detected within the Orchidaceae family, specifically in the Vanillioideae and Epidendroideae subfamilies (Ogura-Tsujita *et al.*, 2021). Orchids associating with SAP fungal associates have been found mainly in tropical (South-)East Asia; yet, several other temperate and boreal orchids may obtain C from SAP fungi (Suetsugu & Matsubayashi, 2021a).

Tightly coiled intracellular fungal hyphae, i.e. pelotons, in the orchid roots are a distinct and constant morphological feature of OM throughout the entire lifetime of orchids (Fig. 1) (Perotto & Balestrini, 2023). Pelotons form independently of the taxonomy or ecology of the multiple fungi involved. As underlying mechanisms for C and nutrient (N, P, S) transfer in OM, two significant processes are recognised: the fungal lysis of pelotons within plant root cells (Burgeff, 1932; Smith & Read, 2008; Bougoure *et al.*, 2014; Kuga *et al.*, 2014) and active transport across intact membranes (Kuga *et al.*, 2014; Dearnaley & Cameron, 2017; Fochi *et al.*, 2017; Ho *et al.*, 2021). According to current knowledge, C is transported from the fungus to the orchid mainly in the form of amino acids (Cameron *et al.*, 2006, 2008; Dearnaley & Cameron, 2017; Fochi *et al.*, 2017; Valadares *et al.*, 2021; Klein *et al.*, 2023) and soluble carbohydrates (i.e. trehalose) (Smith, 1967; Ponert *et al.*,

2021; Li *et al.*, 2022). C and nutrient fluxes are likely dynamic in OM since lab experiments revealed a bi-directional exchange of both C and nutrients at the symbiotic interface (Johnson *et al.*, 1997; Cameron *et al.*, 2006, 2008; Fochi *et al.*, 2017).

Although OM have been studied for decades, the sheer number and diversity of orchid species leaves many questions unanswered, in particular for chlorophyllous orchids. Currently, little is known about variation in mycorrhizal communities during the ontogenetic transition from initial mycoheterotrophy to autotrophy or partial mycoheterotrophy (addressed in manuscripts 1-2) and differences in nutritional strategies and fungal associates of orchids with changing habitats (addressed in manuscript 3). Furthermore, the most common fungal group involved in OM, namely the polyphyletic rhizoctonia, remains highly elusive (addressed in manuscript 4).

1.3.2 Unexplored nutritional strategies of chlorophyllous arbuscular mycorrhizal plants

In evolutionary terms, arbuscular mycorrhiza (AM) is the oldest mycorrhizal type, attributed to the majority of land plant species ranging from liverworts to angiosperms, which associate with Glomeromycotina mycobionts (Smith & Read, 2008; Brundrett, 2017). CMNs enable a fully or initially mycoheterotrophic nutrition strategy among several AM plant species including liverworts, lycophytes, ferns and angiosperms (Merckx, 2013; Gomes *et al.*, 2022). Yet, investigations on nutritional strategies of chlorophyllous AM plants in terms of their potential for partial mycoheterotrophy in natural ecosystems are generally scarce (Cameron & Bolin, 2010; Merckx *et al.*, 2010; Bolin *et al.*, 2017; Suetsugu *et al.*, 2020a,b; Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022). Recent evidence, however, suggests that various chlorophyllous herbaceous AM plant species in the understory of temperate forests have the ability to gain C from their AM fungal associates (Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022).

While OM exhibits one distinct morphological fungal structure, namely the pelotons, two different morphotypes of AM have been proposed (Fig. 1) (Gallaud, 1905; Smith & Smith, 1997). The *Arum*-type morphology (named after its occurrence in *Arum maculatum)*, is characterised by intercellular hyphae with emerging branched fungal structures (i.e. arbuscules) into the cortical cells, while in the *Paris*-morphotype (named after its occurrence in *Paris quadrifolia*), intracellular hyphal coils, which occasionally form fine arbuscule-like ramifications, colonise the plant root cortical cells (Smith & Smith, 1997).

The *Arum*-type, which is common among cultivated plants, is far more investigated in terms of nutrient-transfer mechanisms than the *Paris*-type (Dickson, 2004). However, the latter is the most common morphological type of AM in natural ecosystems and occurs in a significant proportion (≈ 40%) of all AM plants (Brundrett & Kendrick, 1990; Dickson *et al.*, 2007). It appears that there may be sturdy *Arum*- or *Paris*-type forming plant species. Yet, the occurrence of intermediate structures supports a continuum of mycorrhizal structures ranging from *Arum* to *Paris* influenced by plant and AM fungus identity, and possibly the environment (Dickson, 2004; Karandashov *et al.*, 2004; Muthukumar *et al.*, 2006; Dickson *et al.*, 2007).

The coil structures of the *Paris*-type AM, which resemble the OM pelotons (Perotto & Balestrini, 2023), have been suggested to be one of several pre-requisites for a C gain from fungi in both achlorophyllous (Imhof, 1999; Gomes *et al.*, 2023) and chlorophyllous AM plant species. Yet, not all chlorophyllous *Paris*-type AM plant species seem to gain C from fungi (Giesemann *et al.*, 2021). The exact location and the functioning of AM coil structures for C transfer between plants and fungi remain obscure (Gomes *et al.*, 2020), and our knowledge about the relationship between *Paris*-type AM and mycoheterotrophy is limited to few studies (Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022). However, the similarities in the form and possibly also in the function of the intracellular, endo-mycorrhizal fungal structures in OM (i.e. pelotons) and in AM, particularly the hyphal coils (Rasmussen & Rasmussen, 2014; Miura *et al.*, 2018; Perotto & Balestrini, 2023), make chlorophyllous plants forming the *Paris*-type AM promising candidates for partially mycoheterotrophic capability. In contrast, the *Arum*-type AM has been attributed to an autotrophic nutrition of plants (Giesemann *et al.*, 2020b, 2021) and the typical arbuscles are considered as the major site of C-for-mineral nutrient exchange in the AM symbiosis (Bago *et al.*, 2000; Wipf *et al.*, 2019; Shi *et al.*, 2023).

Considering partial mycoheterotrophy a strategy to gain additional C where photosynthesis is severely light-limited, it should provide a significant ecological and evolutionary advantage not only for various herbaceous AM plant species (Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022) but also for seedlings of woody species in the understory of forests. Fungus-to-plant C exchange in trees and tree seedlings has been studied in ECM systems (e.g. Simard *et al.*, 1997; Avital *et al.*, 2022; Cahanovitc *et al.*, 2022; Klein *et al.*, 2023) and is part of the current debate on the relevance of CMNs in C exchange

dynamics between plants (addressed in manuscript 6). However, woody plant species forming AM have so far been largely overlooked in this context. AM woody plant species, though co-occurring with prevailing ECM trees in temperate forests (Baldrian *et al.*, 2023), dominate in tropical (lowland) forests (e.g. Mangan *et al.*, 2004; Alexander & Lee, 2009). In the shaded understory of tropical forests seedlings encounter severe light limitation, yet, seedlings of many species can survive, i.e. maintain a positive C balance, for many years (Smith *et al.*, 1992; Hubbell *et al.*, 1999; Rüger *et al.*, 2009). In fact, several common tropical woody species are in genera that form *Paris*-type AM (comparison of Dickson *et al.*, 2007 with Condit *et al.*, 2013). Consequently, partial mycoheterotrophy might occur among seedlings of tropical woody species (addressed in manuscript 5).

1.4 Stable isotopes as a tool to confirm trophic strategies of chlorophyllous plants

The complexity of mycorrhizal interactions and the often multi-partite networks obligatory for fungus-to-plant-C-transfer in OM and AM associations require *in situ* investigations in natural ecosystems. Natural stable isotope abundances of bulk or component-specific samples are valuable for tracking the origin and movement of substances in the natural environment. Thereby, natural stable isotope abundances reveal both *process* and *source* information, as physical and chemical processes alter isotope ratios (*isotope fractionation*) within ecosystems. This technique provides long-term flux data, unlike snapshots provided by labelling experiments, and is not prone to experimental artefacts (for possible labelling artefacts, see Karst *et al.*, 2023). Although interpreting natural isotope data can be complex, combined with molecular identification of mycorrhizal fungi, multi-element natural abundance stable isotope analyses (^{13}C, ^{2}H, ^{18}O, ^{15}N) of bulk plant material are suitable state-of-the-art techniques enabling *in situ* evaluation of nutritional lifestyles of plants.

General enrichment of mycoheterotrophs in the heavy isotopes ^{13}C, ^{2}H and ^{15}N

Fundamental to the natural abundance stable isotope approach is the fact that plants entirely relying on C from mycorrhizal fungi mirror the isotope pattern of their heterotrophic fungal 'C suppliers' (= *source*). The heterotrophic fungal source comprises secondary organic compounds that are usually enriched in ^{13}C and ^{2}H relative to autotrophic tissues (Yakir, 1992; Gebauer *et al.*, 2016; Cormier *et al.*, 2018, 2019). To that effect, full mycoheterotrophs have been generally characterised by enrichment in the

heavy isotopes ^{13}C (OM: Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Hynson *et al.*, 2016; AM: Merckx *et al.*, 2010; Courty *et al.*, 2011) and ^{2}H (OM: Gebauer *et al.*, 2016; AM: Gomes *et al.*, 2020) relative to surrounding autotrophic reference plants. Partially mycoheterotrophic chlorophyllous plants acquire C through both photosynthesis and fungal associations. These plants usually exhibit ^{13}C and ^{2}H isotope signatures that fall between those of putatively autotrophic and fully mycoheterotrophic plants (e.g. Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Hynson *et al.*, 2016; Schiebold *et al.*, 2018). A reference plant system approach, i.e. comparing stable isotope signatures of chlorophyllous plants with C3-type of photosynthesis growing in close proximity under identical microclimate and light conditions, allows using ^{13}C and ^{2}H natural abundance as time- and site-independent indicator for a potential fungus-to-plant C transfer. Thereby, several plant-physiological and environmental factors affecting ^{13}C and ^{2}H isotope signatures are ruled out (Gebauer & Meyer, 2003; Gebauer *et al.*, 2016). Furthermore, ^{18}O isotope abundance can be used to separate mycoheterotrophic C gains from a transpiration effect on plant leaf ^{13}C and ^{2}H abundance, caused by variation in stomatal regulation.

An additional ^{15}N enrichment is often observed in mycoheterotrophic plants (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Hynson *et al.*, 2016; Gomes *et al.*, 2020) and described for their fungal source relative to its substrate (Gebauer & Taylor, 1999). Compared to inorganic N, which is primarily accessible for plants, organic compounds containing N (and always C) are ^{15}N-enriched. Thus, N transported from the fungus to the plant incorporated in organic compounds, e.g. amino acids, likely explains ^{15}N enrichment of mycoheterotrophic plants. Yet, organic compounds transported from fungus-to-plant do not ultimately contain N and ^{15}N enrichment is not linearly related to the degree of heterotrophic C gain (Leake & Cameron, 2010; Merckx, 2013). Thus, ^{15}N signature is not necessarily diagnostic for a mycoheterotrophic nutrition.

Despite a general trend of enrichment in the heavy stable isotopes ^{13}C, ^{2}H and ^{15}N among (partially) mycoheterotrophic plants, deviations in their stable isotope signature can occur. These variations result from factors such as the identity and ecology of both plants and mycorrhizal fungi, as well as environmental conditions like nutrient availability. Depending on the nutrient acquisition capabilities of the mycorrhizal fungi (e.g. sources, niches), their isotope signatures, which are mirrored by mycoheterotrophic plants, can

vary. Besides, different ratios of biomolecules (e.g. carbohydrates, amino acids, lipids/fatty acids) with distinct isotope enrichment can be delivered to the plants.

Conspicuous stable isotope signatures of OM and AM mycoheterotrophs

For instance, the usually inconspicuous ^{13}C enrichment of partially mycoheterotrophic orchids associating with rhizoctonia fungi relative to autotrophic reference plants is peculiar (Hynson *et al.*, 2013). However, distinct ^{15}N enrichment (Bidartondo *et al.*, 2004; Hynson *et al.*, 2013; Stöckel *et al.*, 2014) and ^{2}H enrichment, serving as a valuable substituent for ^{13}C (Gebauer *et al.*, 2016; Guidi *et al.*, 2023), have provided evidence for the transfer of organic C from fungi to several chlorophyllous orchids (e.g. Fay *et al.*, 2018; Schiebold *et al.*, 2018). Even initially mycoheterotrophic protocorms of rhizoctonia-associated orchids showed only low ^{13}C enrichment (Stöckel *et al.*, 2014, manuscript 2). Therefore, there is reason to presume that ^{13}C enrichment of rhizoctonia fungi is relatively small (addressed in manuscript 4) resulting in no conspicuous ^{13}C enrichment of their orchid associates' leaves (Selosse & Martos, 2014). Mycoheterotrophic AM plants are generally less enriched in ^{13}C and ^{2}H compared to mycoheterotrophic orchids entangled in an ECM network (Gomes *et al.*, 2020; Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022), although in both cases fungal-derived C mainly stems from 'C donor plants'. A possible cause for a relatively lower ^{13}C and ^{2}H enrichment of the AM fungal source (Klink *et al.*, 2020, 2022; Gomes *et al.*, 2023), which is reflected in the mycoheterotrophs, could be the prevalence of lipids as the primary carbon storage compounds in AM fungi (Gleixner *et al.*, 1993; Cormier *et al.*, 2019). Lipids are ^{13}C- and ^{2}H-depleted relative to carbohydrates (Gleixner *et al.*, 1993; Cormier *et al.*, 2019) and AM fungi receive substantial amounts of lipids (i.e. fatty acids) from their 'C donor plant' partner (Jiang *et al.*, 2017; Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017).

In OM, ^{15}N enrichment is a diagnostic indicator of mycoheterotrophic nutrition due to the contribution of amino acids to the fungus-to-plant C transfer (Cameron *et al.*, 2006; Ponert *et al.*, 2021). Yet, the extent of ^{15}N enrichment in orchids varies depending on the fungal taxonomic and functional groups contributing to orchid nutrition. For instance, orchids associated with ECM *Tuber* species (Ascomycota), like *Epipactis atrorubens*, exhibit substantial ^{15}N enrichment relative to other ECM-integrated orchids, possibly because these fungi possess unique enzymes that access ^{15}N-enriched organic matter (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). AM mycoheterotrophic plants display a

certain variability in ^{15}N isotope signature, with occasionally inconspicuous ^{15}N enrichment possibly attributed to a wide range of plant taxonomy, fungal community and geographical differences in N availability (Courty *et al.*, 2011; Gomes *et al.*, 2020).

Notations for stable isotope signatures

Stable isotope natural abundances are notated as δ-values relative to standards:

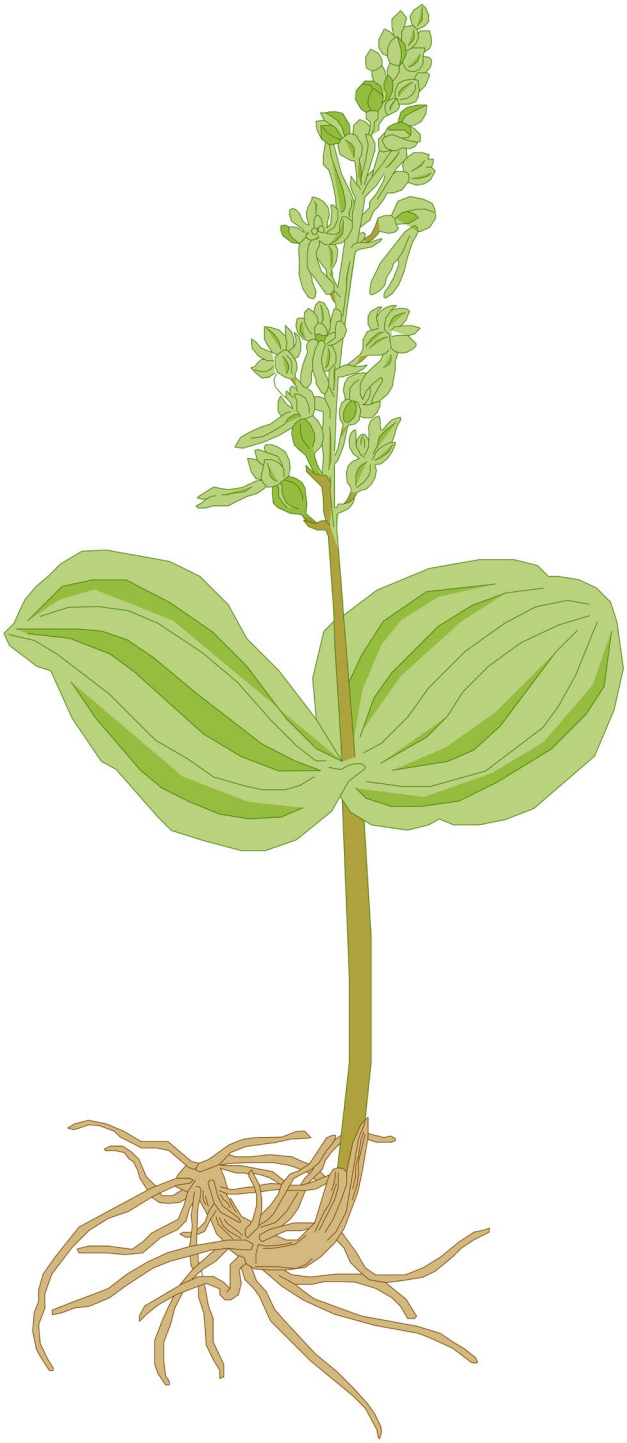
$\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R\text{sample}/R\text{standard} - 1) \times 1000$ [‰],

where R is the ratio of the heavy to the respective light isotope.

When evaluating nutritional strategies of plants using the reference plant system approach, δ-values are usually standardised by calculating time- and site-independent enrichment factors ε according to Preiss & Gebauer (2008):

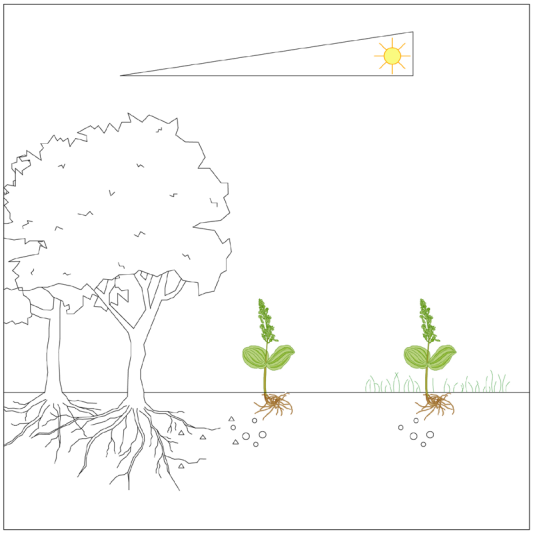
$\varepsilon = \delta S - \delta REF$,

where δS is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O$ value of an individual (target or reference) plant, and δREF is the mean value across all autotrophic reference plants in the close proximity.



Manuscript 1 & 2

Manuscript 3



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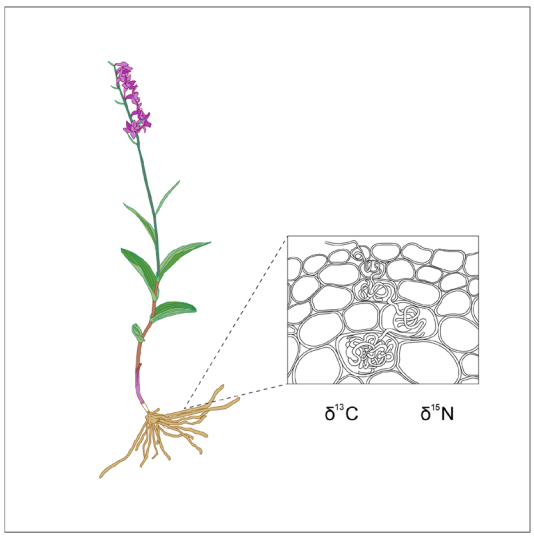

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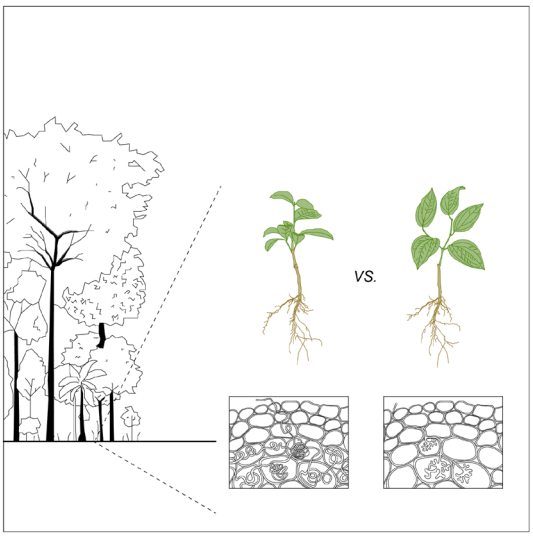

2 Objectives

This thesis focuses on the nutritional strategies of chlorophyllous orchids and arbuscular mycorrhizal plants and aims to provide further puzzle pieces to our overall understanding of

(i) Fungus-to-plant C transfer in *orchid mycorrhiza* by

- ▷ Understanding the mycoheterotrophy-autotrophy continuum and shifts in fungal associates during ontogenetic development of chlorophyllous orchids (manuscripts 1 & 2).
- ▷ Elucidating ecological factors of fungal shifts and nutritional strategies in OM associations (manuscript 3).
- ▷ Identifying the stable isotope signatures of pelotons as a direct fungal source of orchid nutrition (manuscript 4).

(ii) Fungus-to-plant C transfer in *arbuscular mycorrhizal* associations by

- ▷ Exploring partial mycoheterotrophy in seedlings of woody AM species (manuscript 5).

Specifically, this thesis comprises six manuscripts focusing on the following objectives:

(1) Investigate fungal associates, subterranean root morphology and nutrition strategies across different development stages of the chlorophyllous orchid species *Cremastra appendiculata* as a model species within the SAP-mycoheterotrophic plant-lineage.
(2) Identify fungal associates and nutrition strategies of different development stages of the chlorophyllous orchid species *Neuwiedia malipoensis* within the earliest-diverging orchid subfamily Apostasioideae.
(3) Elucidate the ecological drivers in fungal shifts and ecophysiology of partially mycoheterotrophic *Neottia ovata* in grassland and forest habitats.
(4) Determine the ^{13}C and ^{15}N stable isotope signatures of fungal pelotons as the direct fungal source for rhizoctonia-associated and ECM-network-entangled orchids.
(5) Investigate whether partial mycoheterotrophy occurs in seedlings of woody AM species in a tropical lowland forest in Panama.
(6) Contribute to the current scientific debate on CMNs by addressing mycoheterotrophs as natural evidence for the relevance of CMNs in C transfer among plants (*PERSPECTIVE*).

3 Synopsis

3.1 Nutritional strategies and fungal associations across different development stages of *Cremastra appendiculata* – Manuscript 1

Mycoheterotrophs utilising either specific or a broader range of SAP fungi as a source of C are currently only known within the Orchidaceae, i.e. within the two subfamilies Vanilloideae and Epidendroideae (Ogura-Tsujita *et al.*, 2021). The orchid genus *Cremastra* within the latest diverged subfamily Epidendroideae is one of only two genera among the SAP-mycoheterotrophic plant lineages that comprise both leafless and leafy species. Manuscript 1 focuses on the chlorophyllous, broadleaved orchid *Cremastra appendiculata*, which is distributed in temperate and subtropical regions of East Asia, such as Taiwan (POWO, 2023). SAP Psathyrellaceae and *Coprinus* fungal species are known as mycobionts of *C. appendiculata* protocorms (Yagame *et al.*, 2013; Gao *et al.*, 2022) and attributed to adult individuals that form coralloid rhizomes. The association with these fungi enables mature *C. appendiculata* to meet up to 80% of its total C demand *via* a partially mycoheterotrophic nutrition in shaded forests (Yagame *et al.*, 2021). *C. appendiculata* is mainly associated with rhizoctonia fungi (members of Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae) when mature (Yagame *et al.*, 2021) and also occurs in rather open habitats (Gao *et al.*, 2022). The nutrition strategy of mature *C. appendiculata* individuals when associated with rhizoctonia fungi remained unresolved, opening up the possibility that *C. appendiculata* covers the entire spectrum on the mycoheterotrophy-autotrophy continuum even within its ontogenetic development.

We revealed the nutritional strategies of three different development stages, namely protocorms, seedlings and flowering individuals, of *C. appendiculata* from one population in subtropical Taiwan. In addition, the mycorrhizal fungi of each development stage with differentiation between orchid-root morphology types (i.e. coralloid rhizomes, roots) were determined. Investigating different orchid developmental stages of a single population provides the most accurate means of evaluating ontogenetic changes within a single plant lifetime, a level of detail not attainable with current methods. A gradual shift from SAP Psathyrellaceae, dominant in protocorms and seedling rhizomes, to rhizoctonia fungi (i.e. *Ceratobasidium*, *Thanatephorus* and Serendipitaceae) as main mycobionts in roots of seedling roots and mature *C. appendiculata* coincided with a switch from a fully

mycoheterotrophic to a fully autotrophic nutrition during ontogenesis of *C. appendiculata* (Fig. 3 A).

We provided clear evidence for an autotrophic nutrition of mature *C. appendiculata* when associated with rhizoctonia fungi as none of the stable isotope signatures (^{13}C, ^{2}H, ^{18}O, ^{15}N) of mature *C. appendiculata* differed from reference plants. This contrasts with previous suggestions that adult *C. appendiculata* with rhizoctonia mycobionts is putatively partially mycoheterotrophic due to enrichment in ^{15}N and its occurrence in the light-limited forest understory (Yagame *et al.*, 2021). Thus, our findings extend our knowledge on the plasticity of *C. appendiculata,* being capable of an entirely autotrophic lifestyle when mature. Enrichment in ^{15}N, ^{13}C and ^{2}H of *C. appendiculata* protocorms conforms to the characteristic signature of orchid specimens entirely relying on fungal nutrition, and we presented the first stable isotope signatures obtained from orchid protocorms associated with SAP fungi. Seedlings showed an intermediate ^{15}N and ^{13}C isotopic signature with large variation likely due to differences in the fungal community depending on their subterranean morphology. 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 associates in seedlings.

The gradual changes in nutritional strategy, root morphology and mycobionts of different development stages of *C. appendiculata*, suggest a high within-individual flexibility of *C. appendiculata*, in addition to environmentally driven within-species variability in mycobionts and nutrition among diverse populations (Yagame *et al.*, 2021). Covering the entire range on the mycoheterotrophy-autotrophy continuum during ontogenesis makes *C. appendiculata* an ideal study model to explore unknown spatial and temporal changes in nutritional strategies within the SAP-mycoheterotrophs plant lineages in future.

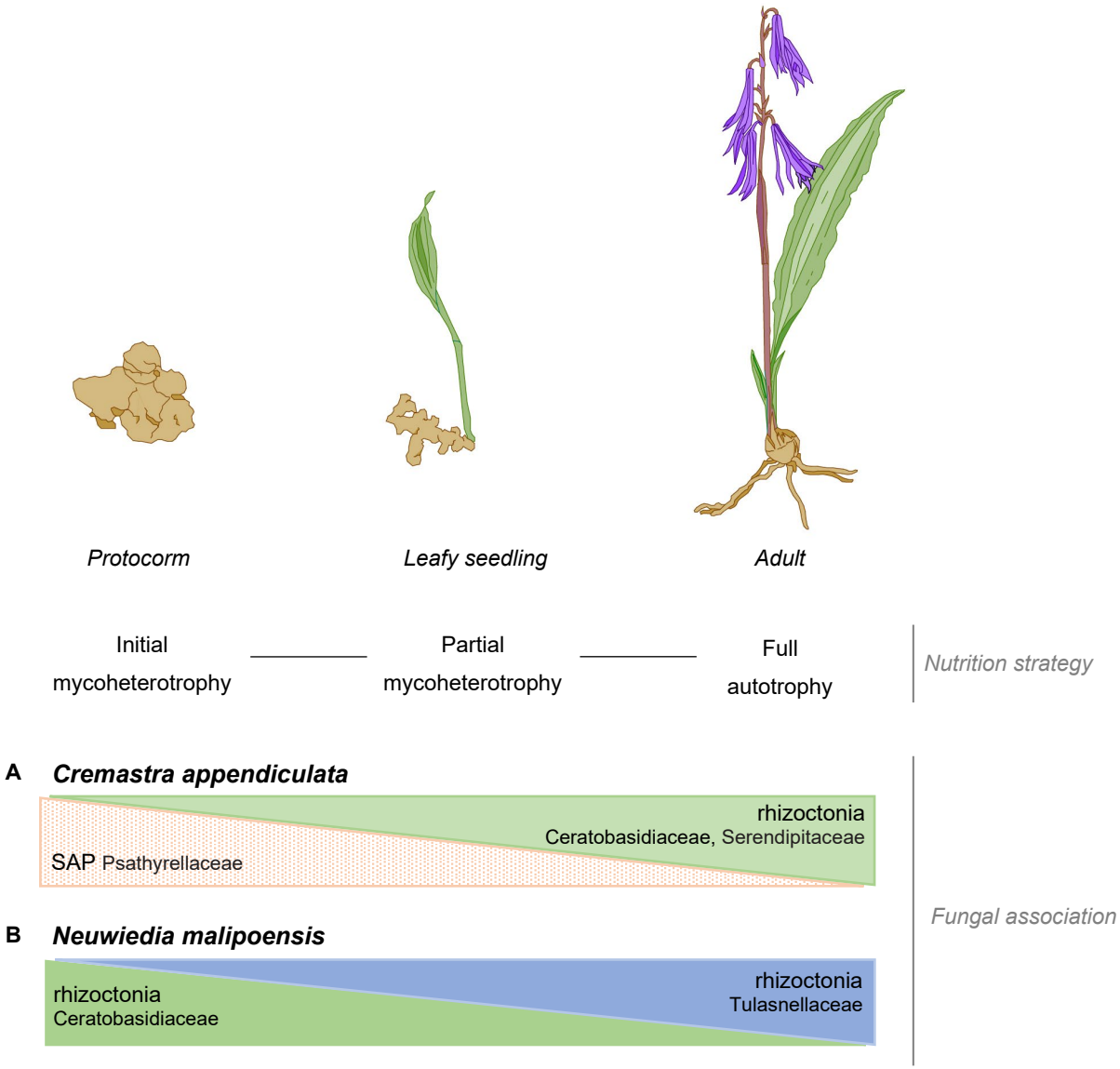

FIG 3 Nutritional strategies and fungal associations across different development stages of the chlorophyllous orchids (A) *Cremastra appendiculata* and (B) *Neuwiedia malipoensis.* During ontogenesis (probably) both orchids span the entire continuum from initial mycoheterotrophy of the protocorm *via* partial mycoheterotrophy of leafy seedlings to fully autotropic adult orchids. *C. appendiculata* recruits saprotrophic (SAP) Psathyrellaceae in its early development and shifts to rhizoctonia fungi (i.e. *Ceratobasidium, Thanatephorus* and Serendipitaceae) towards maturity. *N. malipoensis* associates with rhizoctonia fungi throughout its ontogenesis with dominant fungi switching from Ceratobasidiaceae to Tulasnellaceae.

3.2 Nutritional strategies and fungal associations across different development stages of *Neuwiedia malipoensis* – Manuscript 2

Other than in later diverging Orchidaceae lineages (cf. Epidendroideae, e.g. *Cremastra*) no mycorrhizal interaction with SAP fungi (e.g. *Mycenaceae* and *Psathyrellaceae*) appears in the earliest-diverging small orchid subfamily Apostasioideae (Ogura-Tsujita *et al.*, 2021). The Apostasioideae contain only two genera (*Apostasia* and *Neuwiedia*) with terrestrial species confined to the humid areas of Southeast Asia, southern China, Japan, and northern Australia, and no fully mycoheterotrophic, achlorophyllous species are known (POWO, 2023). Although a partial gain of C from ECM-forming Ceratobasidiaceae has been reported for chlorophyllous mature *Apostasia nipponica* (Suetsugu & Matsubayashi, 2021b), it is unclear whether the strategy of partial mycoheterotrophy in mature *A. nipponica* is a unique example or a common strategy in the early divergent Apostasioideae. Therefore, revealing trophic strategies and fungal associates of *Neuwiedia malipoensis* as a sister group of *Apostasia* adds a missing piece in the early divergent orchid clade.

Assessing nutritional strategies and fungal associates of three different development stages (cf. manuscript 1), we found a progressive switch of fungal associates from rather saprotrophic rhizoctonia Ceratobasidiaceae at the protocorm stage to rhizoctonia Tulasnellaceae at the seedling and adult stage that corresponded with a decreasing dependency on fungal N and C of *N. malipoensis* throughout its ontogenesis (Fig. 3 B). Seedlings of *N. malipoensis* showed indication for partially mycoheterotrophic nutrition being enriched in ^{13}C and ^{2}H. However, the extent of reduced fungal C supply in adults remains unclear due to a transpiration effect on these stable isotope data, implying that mature. *N. malipoensis* could even be fully autotrophic.

The divergence of mycorrhizal fungal associates between *A. nipponica* and *N. malipoensis* enables variation in resource acquisition opening up different adaptions to environmental conditions and habitat options in the earliest-diverging orchid clade. *A. nipponica* limits its distribution range to forests with ECM networks that certainly allow a higher C supply from fungi and even cover the C demand of achlorophyllous orchids (Bidartondo, 2005). By contrast, association with the rhizoctonia fungi, as found for *N. malipoensis*, enables the successful settlement of the Orchidaceae in an extremely wide range of habitats worldwide.

3.3 Level of mycoheterotrophy and variation in fungal communities in grassland and forest populations of *Neottia ovata* – Manuscript 3

Chlorophyllous orchid species within mycoheterotrophic clades often engage in dual mycorrhizal associations with usually rhizoctonia and non-rhizoctonia fungal associates (cf. *C. appendiculata*, manuscript 1) opening up different habitat options (cf. *Neuwiedia* vs. *Apostasia*, manuscript 2). Thus, these orchid species represent a unique study system to investigate the ecological drivers in mycorrhizal shifts and their impact on orchid nutrition. In the ECM-mycoheterotrophic orchid lineage of Neottieae, the partially mycoheterotrophic *Neottia ovata* is such a species (Těšitelová *et al.*, 2015; Schweiger *et al.*, 2019) with a native range from Europe to Himalaya (primary in the temperate biome). In our study, *N. ovata* was used as a model species and investigated for root and soil fungal communities, multi-element stable isotope abundances and microhabitat conditions (light intensity and edaphic factors) across six sites ranging from open grassland to shady forests (Fig.4). *N. ovata* predominantly associated with rhizoctonia fungi of the family Serendipitaceae in both grassland and forest habitats, but orchids in forests also recruited ECM Sebacinaceae fungi, which was in agreement with previous findings (Jacquemyn *et al.*, 2015; Oja *et al.*, 2015; Těšitelová *et al.*, 2015; Schweiger *et al.*, 2019; Vogt-Schilb *et al.*, 2020). Root fungal communities highly resembled soil fungal communities and variation in root fungal communities was significantly related to habitat type and edaphic factors. At each site, significant enrichment in ^{2}H and ^{15}N (except for individuals from one site), but not in ^{13}C relative to surrounding autotrophic reference plants confirmed a partially mycoheterotrophic nutrition of *N. ovata* and resembled the usual isotopic pattern reported for rhizoctonia-associated orchids (Gebauer *et al.*, 2016; Schiebold *et al.*, 2018; Schweiger *et al.*, 2018). The habitat type (forest vs. grassland) with the presence or absence of ECM fungi and the light availability did not substantially influence the isotope signatures of *N. ovata*.

Although we cannot rule out the possibility that ^{13}C and ^{2}H isotope signatures were masked by higher transpiration rates and/or lower water use efficiency of forest orchids compared to reference plants (indicated by negative ^{18}O enrichment), our data suggests that the contribution of ECM fungi to the C nutrition budget of *N. ovata* is probably negligible. Instead, rhizoctonia fungi play a major functional role in nutritional supply of *N. ovata* from fungal resources in both habitat types.

The discrepancy between plant nutrition and symbiotic association hints at an early evolutionary stage of partial mycoheterotrophy for *N. ovata*. By dual symbiotic association in low-light forest habitats, *N. ovata* has already recruited mycorrhizal partners closely related to those of its fully mycoheterotrophic relatives *N. nidus-avis, N. accuminata* and *N. camtschatae.* However, the functional symbiotic switch to new partners, i.e. Sebacinaceae, has probably not yet been achieved (Fig. 4).

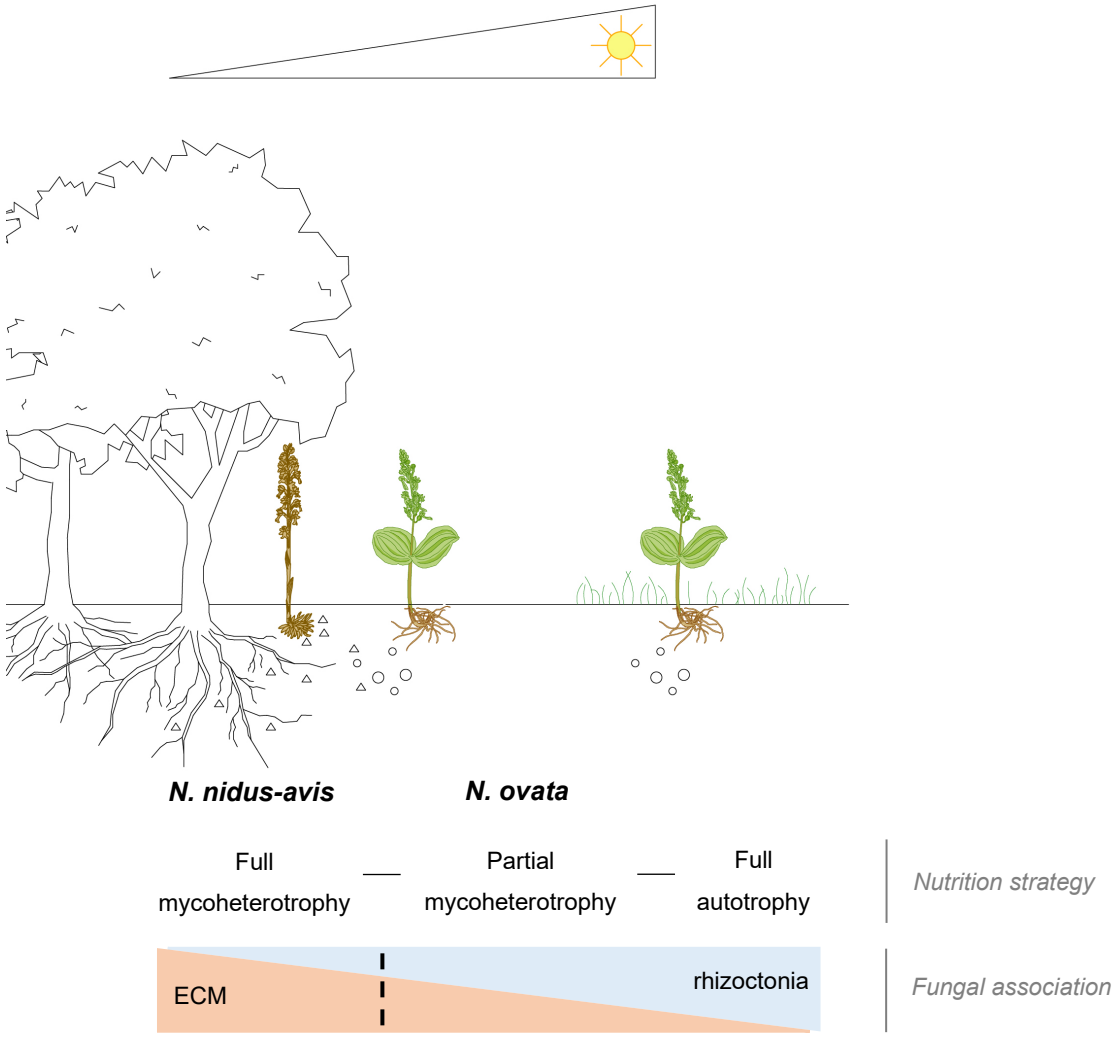

FIG 4 Nutritional strategies, fungal associations and light availability of chlorophyllous orchid *Neottia ovata* in grassland and forest populations. Compared with the fully mycoheterotrophic *N. nidus-avis* that exclusively associates with ectomycorrhizal fungi (ECM, triangles) (e.g. McKendrick *et al.*, 2002), *N. ovata* is mainly associated with rhizoctonia fungi (circles) and likely at an early stage of partial mycoheterotrophy in grassland and forest habitats. (Figure based on Wang *et al.*, 2023, modified).

3.4 Stable isotope signatures of fungal pelotons as the direct fungal source in orchid nutrition – Manuscript 4

The natural abundance stable isotope method is a powerful tool for studying the nutritional strategies of orchids directly in natural ecosystems. However, when investigating chlorophyllous orchids, a multi-element approach including ^{2}H is indispensable, because of their usually inconspicuous ^{13}C enrichment when associated with rhizoctonia fungi (cf. manuscript 1-3). It has been speculated that the ^{13}C isotope signature of the rhizoctonia fungal source is too close to the ^{13}C isotope abundance of autotrophic plants, hampering the detection of C gains from fungi (Selosse & Martos, 2014; Stöckel *et al.*, 2014). For orchid mycobionts simultaneously forming ECM with woody plants natural abundance stable isotope data of the fruiting bodies of mycorrhizal fungi exist (Trudell *et al.*, 2003; Julou *et al.*, 2005; Ogura-Tsujita *et al.*, 2009; Lee *et al.*, 2015; Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). Yet, as rhizoctonia fungi do not typically produce macroscopic aboveground fruiting bodies, no such proxy for the fungal source of rhizoctonia-associated orchids is available. As a direct fungal source, we instead extracted intracellular hyphae, so-called pelotons, directly from orchid roots *via* a mechanical approach adapted from Klink *et al.* (2020). ^{13}C and ^{15}N stable isotope signatures of fungal pelotons were obtained from (i) orchid species associated with either specific or a set of rhizoctonia fungi (*Anoectochilus sandvicensis* and *Epipactis palustris*), and (ii) orchid species that are part of tripartite ECM networks (*Epipactis atrorubens* and *Epipactis leptochila*) with different preferences for Basidio- and Ascomycota (Schiebold *et al.*, 2017; Swift *et al.*, 2019).

Remarkably, pelotons of all investigated orchid species (and additionally *Ophrys insectifera* and *Orchis militaris*, cf. companion paper Gomes *et al.*, 2023) displayed c. 3‰ higher mean $\varepsilon^{13}C$ values than leaves of respective orchids (Fig. 5). ^{13}C enrichment of pelotons extracted from *A. sandvicensis* and *E. palustris* was similar, irrespective of their specificity to certain rhizoctonia fungi, and considerably smaller than $\varepsilon^{13}C$ of pelotons isolated from the forest-dwelling ECM-fungi-associated *E. leptochila* and *E. atrorubens.* Our results suggest an explicit fungal nutrition source of orchids associated with ECM fungi. Yet, the relatively small ^{13}C enrichment (<3‰) of rhizoctonia pelotons extracted from *A. sandvicensis* and *E. palustris* explains the inconspicuous ^{13}C enrichment of

rhizoctonia-associated orchids and limits the suitability of leaf ^{13}C signatures as a nutrition indicator for mycoheterotrophic C gain of rhizoctonia-associated orchids.

In addition, distinct patterns were observed in the ^{15}N signatures of pelotons depending on the fungal phyla. For instance, pelotons of *E. leptochila* with Tuberaceae as dominant mycobionts displayed high ^{15}N enrichment, also known for *Tuber* spp. sporocarps (Fig. 5). The distinct isotope signatures observed in pelotons were reflected in the ^{15}N patterns of orchid leaves, highlighting the informative nature of these signatures in elucidating the functional role of fungal associates in plant nutrition. ^{15}N signatures of orchid leaves may even allow narrowing down the variety of fungi present in orchid roots to a selection of fungi with relevance for the orchid nutrition. We observed relatively smaller ^{15}N enrichment of pelotons than of orchid (leaf) tissues that may result *inter alia* from a known preferential transfer of C in form of ^{15}N-enriched amino-acids from (mycorrhizal) fungi to the orchid (Cameron *et al.*, 2006, 2008; Dearnaley & Cameron, 2017; Fochi *et al.*, 2017; Favre-Godal *et al.*, 2020; Valadares *et al.*, 2021). Enhanced extraction of fungal pelotons from orchid roots for simultaneous multi-element stable isotope analysis (including ^{2}H) and fungal sequencing could directly link isotope data of pelotons with fungal taxa that form them. Yet, challenges persist in separating diverse fungal taxa co-occurring in roots, including other endophytic fungi with largely unexplored ecology, e.g. Helotiaceae (also detected in this study). Nevertheless, the natural abundance stable isotope analysis of orchid pelotons and other fungal hyphae (cf. companion paper Gomes *et al.*, 2023) obtained from *in situ* investigations in natural habitats promises valuable insights into the intricate relationship between plants and their fungal symbionts.

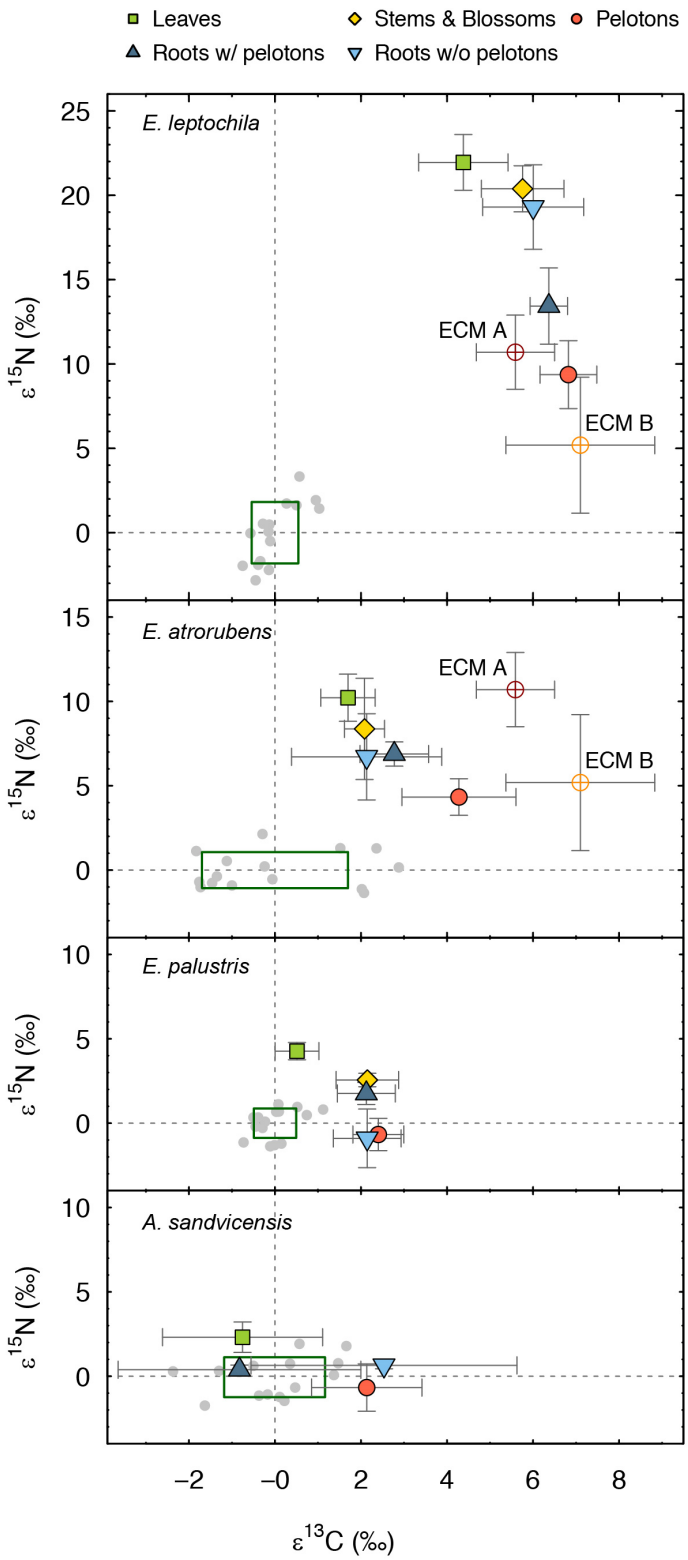

FIG 5 Carbon and nitrogen stable isotope enrichment factors ε (mean ± SD) of orchid parts ('leaves', 'stems & blossoms', 'roots with pelotons', 'roots without pelotons') and isolated pelotons for *Epipactis leptochila, Epipactis atrorubens, Epipactis palustris and Anoectochilus sandvicensis.* Enrichment factors ε of autotrophic reference plants are indicated by the green frame (standard deviation around a mean enrichment factor of zero, by definition) and grey dots (single values). For comparison, carbon and nitrogen stable isotope enrichment factors ε (mean ± SD) of sporocarps of four ECM ascomycete *Tuber* species (ECM A) from Schiebold *et al.* (2017), and 11 ECM basidiomycete species (ECM B) from (Gebauer *et al.*, 2016). (Figure from Zahn *et al.*, 2023).

3.5 Partial mycoheterotrophy in seedlings of tropical woody species forming arbuscular mycorrhiza – Manuscript 5

Whereas the coil structure of pelotons is a unique feature in OM, which enables fungus-to-plant C transfer in achlorophyllous and chlorophyllous orchids (cf. manuscripts 1-4), comparable suggestions have been made for the similar appearing fungal coils related to the *Paris*-morphotype in AM (Imhof, 1999; Giesemann *et al.*, 2021). As quite a number of common tropical woody species are in genera that form *Paris*-type AM (comparison of Dickson *et al.*, 2007 with Condit *et al.*, 2013), their seedlings in the light-limited forest understory are potential candidates for a partially mycoheterotrophic nutrition. We evaluated for the first time whether seedlings of common tropical woody species (i.e. trees or shrubs) in the understory of tropical forests gain organic C from their fungal associates in addition to their own photosynthesis. To this end, we assessed the AM morphological type (*Paris*- or *Arum*-type) for tropical woody species from genera that include species with known AM-morphotype (based on Dickson *et al.,* 2007), and compared natural abundances of stable isotope (^{13}C, ^{2}H, ^{18}O, ^{15}N) of potentially mycoheterotrophic (i.e. *Paris*-type) target woody seedlings with neighbouring autotrophic 'non *Paris*-type' (i.e. *Arum*-type or non-mycorrhizal) reference plants to determine if they are partially mycoheterotrophic.

Our microscopic root assessments of tropical woody species in lowland moist forests in Panama did not always conform to our *a priori* literature-based classification on plant genus level (Dickson *et al.*, 2007). We revealed that there are more tropical genera with *Paris*-type AM than previously known. Our observations demonstrated that *in situ* AM morphotype evaluation is pivotal, as it remains poorly understood what exactly determines the appearance of specific AM morphotype structures (Dickson *et al.*, 2007; Bennett & Groten, 2022), and consequently if a plant individual, species or genus may be capable of partial mycoheterotrophy. *Paris*-type AM occurred in 50% of the investigated species (21 of 42) and we considered them as potentially partially mycoheterotrophic '*Paris*-type' target species. Twelve species were regarded as autotrophic 'non *Paris*-type' reference plants and 8 species were excluded from further analysis as the AM morphotype could not be conclusively determined.

Seedlings of 6 species with *Paris*-type AM (*Anacardium excelsum, Annona spraguei, Cecropia insignis, Croton billbergianus, Acalypha macrostachya* and *Trichilia pallida*) displayed significant ^{13}C enrichment compared to the surrounding autotrophic 'non *Paris*-type' reference plants, without a parallel enrichment of ^{18}O (Fig. 5). For all ^{13}C-enriched '*Paris*-type' target species, we can exclude that enrichment in ^{13}C was caused by lower transpiration and/or a higher water use efficiency (no enrichment in ^{18}O) or by differences in plant phenology of target relative to reference plants. Thus, the ^{13}C enrichment provides a clear indication for fungus-to-plant organic C transfer for these species.

Only seedlings of one '*Paris*-type' species, namely *Anacardium excelsum,* showed a simultaneous ^{2}H enrichment relative to 'non *Paris*-type' reference plants providing an additionally independent indication for biogenic derived C (and H) from fungi. Five out of the six ^{13}C-enriched '*Paris*-type' species exhibited significant ^{2}H depletion (Fig. 6). A transpiration effect (indicated by ^{18}O depeletion) might have caused an underestimation of a heterotrophic part of nutrition and may partly explain ^{2}H depletion for two of these species. The reasons for ^{2}H depletion with simultaneous ^{13}C enrichment of three '*Paris*-type' target species remain subject to speculations because a transpiration effect can be excluded. A major fungus-to-seedling C supply in form of ^{2}H-depleted lipids (relative to carbohydrates) might potentially explain ^{2}H-depleted leaf tissues for these species (Cormier *et al.*, 2018).

Seedlings of nine out of 21 of the '*Paris*-type' AM species were not enriched in ^{13}C and ^{2}H, and indistinct in ^{18}O from reference plants (Fig. 6), displaying isotopic signatures that clearly do not support a partially mycoheterotrophic nutrition.

Manuscript 5 provides novel evidence that partially mycoheterotrophic nutrition among AM plants is not limited to herbaceous temperate forest understory species, but also occurs within seedlings of woody species. Specifically, we detected partially mycoheterotrophic nutrition among seedlings of almost 30% of the investigated woody species with *Paris*-type AM in the understory of neo-tropical lowland forests. This has possibly far-reaching implications for seedling recruitment and subsequent ecosystem community structures and functioning.

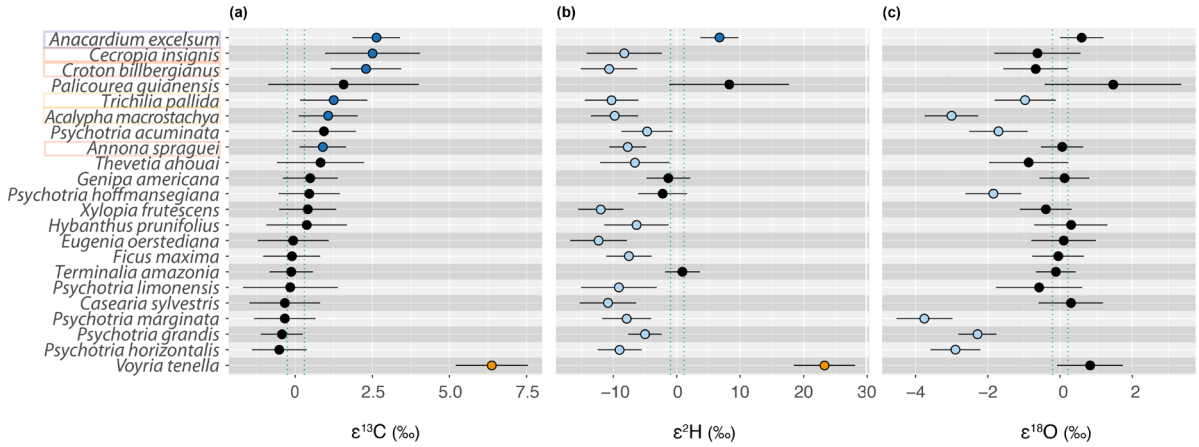

FIG 6 Estimated enrichment factors ε^{13}C (a), ε^{2}H (b) and ε^{18}O (c) and 95% CI of individual '*Paris*-type' species in comparison to pooled 'non *Paris*-type' reference plant species (95% CI displayed by dotted vertical lines) based on linear mixed models. Dark and light blue symbols indicate significant enrichment or depletion of '*Paris*-type' species in comparison to 'non *Paris*-type' reference plants, respectively. Orange symbols indicate significant enrichment for FMH *Voyria.*

3.6 Mycoheterotrophs as positive controls for common mycorrhizal networks – Manuscript 6

The mycoheterotrophic C gain of plants from AM (cf. manuscript 5) or ECM fungi (e.g. orchids, cf. manuscripts 3 & 4) requires CMNs with surrounding photosynthetic 'C donor plants' (Bidartondo, 2005) because both ECM and AM fungi are largely considered obligate biotrophic (ECM: Koide *et al.*, 2008 but Lindahl & Tunlid, 2015; AM: Trépanier *et al.*, 2005; Tisserant *et al.*, 2013). Fully and partially mycoheterotrophic plants associated with these fungi are thus natural evidence for the relevance of CMNs in C exchange dynamics between plants. Manuscript 6 offers a perspective that brings mycoheterotrophic plants into the current debate about the prevalence and potential functions of CMNs, where they have received little attention so far. Mycoheterotrophy and its widespread prevalence challenges the dogma of C-for-nutrients transfer in the mycorrhizal symbiosis and the assumption that all green plants are strict autotrophs (cf. Manuscripts 1-5). This opens exciting prospects for investigating CMNs and their function.

4 Main conclusions and outlook

This doctoral thesis contributes to our understanding of the mycoheterotrophy-autotrophy continuum in OM on ontogenetic, phylogenetic and environmental scales (manuscript 1-3). Shifts in nutritional strategies and fungal associates were elucidated for the chlorophyllous orchids *Cremastra appendiculata,* belonging to the SAP-mycoheterotrophic plant-lineage, and *Neuwiedia malipoensis,* belonging to the earliest-diverging orchid subfamily Apostasioideae. Ecological drivers in fungal shifts and ecophysiology of partially mycoheterotrophic *Neottia ovata* in grassland and forest habitats were studied. Further, identifying the stable isotope signatures of pelotons as a direct fungal source of orchid nutrition *in situ* resolved the inconspicuous ^{13}C enrichment of rhizoctonia-associated orchids and enabled a better functional understanding of the mycobiont (manuscript 4). Lastly, exploring partial mycoheterotrophy in seedlings of woody AM species (manuscript 5) contributes to a so far largely unexplored research area and tackles a highly topical and contentious issue: the relevance of fungus-to-plant C transfer in CMNs, where mycoheterotrophs provide natural evidence (manuscript 6).

Further research could include:

- ▷ Explore the spatial and temporal changes in nutritional strategies within SAP-mycoheterotrophic plant lineages using *C. appendiculata* as a study model.
- ▷ Isolate of larger quantities of fungal pelotons from orchid roots enabling simultaneous use for multi-element stable isotope analysis (including ^{2}H).
- ▷ Consider the largely unexplored ecological role of multiple root endophytic fungi, such as dark septate endophytes (e.g. Helotiaceae), in OM and beyond.
- ▷ Apply within-cell spatial microanalysis of natural stable isotope abundance using ablation-isotope ratio mass spectrometry for higher resolution data.
- ▷ Evaluate the possible link between AM morphology and mycoheterotrophy, determine root fungal communities in AM.
- ▷ Combine natural abundance stable isotope analyses of fungal source (hyphae extraction) and *in situ* labelling to substantiate mycoheterotrophy in AM woody species.
- ▷ Gain a better understanding of nutritional strategies and ecological niches of fungi.

5 List of author contributions to the manuscripts

– MANUSCRIPT 1–

Zahn FE, Lee YI, Gebauer G. 2022. **Fungal association and root morphology shift stepwise during ontogenesis of orchid *Cremastra appendiculata* towards autotrophic nutrition**.

Own contribution:

Concepts and planning:	5%	Data analysis:	60%
Field and laboratory work:	40%	Manuscript preparation:	90%

FEZ analysed the isotope abundance data and wrote the first manuscript draft. YIL carried out the sampling and the molecular analyses. GG 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. The manuscript contains partial data from FEZs master thesis to a limited extent.

– MANUSCRIPT 2 –

Zahn FE, Jiang H, Lee Y-I, Gebauer G. **Mode of carbon gain and fungal associations of *Neuwiedia malipoensis* within the evolutionary early diverging orchid subfamily Apostasioideae**.

Own contribution:

Concepts and planning:	5%	Data analysis:	50%
Field and laboratory work:	30%	Manuscript preparation:	50%

YIL and GG had the idea for the research project. FEZ analysed the isotope abundance data. HJ carried out the sampling. YIL carried out the molecular and phylogenetic analyses. FEZ, YIL and GG wrote the manuscript. FEZ and HJ contributed equally to this study. All co-authors approved the final version.

– MANUSCRIPT 3 –

Wang, D, Gebauer, G, Jacquemyn, H, **Zahn, FE**, Gomes, SIF, Lorenz, J, van der Hagen, H, Schilthuizen, M, & Merckx, VSFT. 2023. **Variation in mycorrhizal communities and the level of mycoheterotrophy in grassland and Forest populations of *Neottia ovata* (Orchidaceae)**.

Own contribution:

Concepts and planning:	30%	Data analysis:	20%
Field and laboratory work:	50%	Manuscript preparation:	20%

VSFTM, DW, GG and HJ designed the experiment. GG, FEZ, HJ and HVDH contributed to the selection of the sampling site. DW, VSFTM, HJ, FEZ and JL collected and processed the samples. DW analysed data with input from VSFTM, GG, HJ, FEZ and SIFG. DW wrote the first manuscript draft. All authors commented and approved the final version of the manuscript.

– MANUSCRIPT 4 –

Zahn FE, Söll E, Chapin TK, Wang D, Gomes SIF, Hynson NA, Pausch J & Gebauer G. 2023. **Novel insights into orchid mycorrhiza functioning from stable isotope signatures of fungal pelotons**.

Own contribution:

Concepts and planning:	60%	Data analysis:	90%
Field and laboratory work:	40%	Manuscript preparation:	90%

ES, FEZ, TKC and NAH collected and processed the samples. FEZ analysed the isotope abundance data and wrote the first manuscript draft. DW conducted the molecular analyses, and together with SIFG and FEZ processed the data. GG developed the idea for the project and supervised the isotope abundance analyses. NAH, TKC, ES and FEZ contributed to the research design. JP provided essential equipment and contributed to the manuscript. All authors commented and approved the final version of the manuscript.

– MANUSCRIPT 5 –

Zahn FE, Contreras B, Engelbrecht B, Gebauer G. **Stable isotope analysis indicates partial mycoheterotrophy in arbuscular mycorrhizal woody seedlings in tropical forests**.

Own contribution:

Concepts and planning:	60%	Data analysis:	95%
Field and laboratory work:	70%	Manuscript preparation:	80%

BMJE conceived the idea for the study. BMJE, GG and FEZ developed the research design. All authors were involved in the sample collection. BC identified plant species. FEZ conducted the sample preparation and analyses. GG supervised and quality-controlled the isotope abundance analyses. FEZ analysed the data and wrote the first manuscript draft with inputs from BMJE and GG. All authors commented and approved the final version of the manuscript.

– MANUSCRIPT 6 –

Merckx, VSFT, Gomes SIF, Wang D, Verbeek C, Jacquemyn H, **Zahn FE**, Gebauer G, Bidartondo MI. **Mycoheterotrophy in the wood-wide web.**

The focus of this Perspective was conceived by all of the authors. V.S.F.T.M. led the writing, with contributions from S.I.F.G., D.W., C.V., H.J., F.E.Z., G.G., and M.I.B.

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Zahn FE, Lee YI, Gebauer G. 2022. Fungal association and root morphology shift stepwise during ontogenesis of orchid *Cremastra appendiculata* towards autotrophic nutrition. *AoB PLANTS* 14(3): plac021. doi: 10.1093/aobpla/plac021.

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MANUSCRIPTS

MANUSCRIPT 1

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 myco-heterotrophic protocorms exploit rather specialised 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 ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$) and total N concentrations. Site- and sampling-time-independent enrichment factors of stable isotopes were used to reveal trophic strategies. We determined mycorrhizal fungi of *C. appendiculata* protocorm, seedling, and adult samples using high-throughput DNA sequencing.

We identified saprotrophic non-rhizoctonia Psathyrellaceae as dominant mycorrhizal fungi in protocorm and seedling rhizomes. In contrast, the roots of seedlings and mature *C. appendiculata* were mainly colonised with fungi belonging to the polyphyletic assembly of rhizoctonia (*Ceratobasidium*, *Thanatephorus* and Serendipitaceae). Mature *C. appendiculata* did not differ in isotopic signature from autotrophic reference plants suggesting a fully autotrophic nutrition mode. Characteristic of orchid specimens entirely relying on fungal nutrition, *C. appendiculata* protocorms were enriched in ^{15}N, ^{13}C and ^{2}H 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.

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 (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 belowground 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 & Ghani, 2000; Eriksson & 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; Egan & Hynson, 2019).

Nonetheless, most orchids develop chlorophyll and the ability to photosynthesise, becoming either autotrophic or, when getting additional carbon from fungi, partially mycoheterotrophic (Gebauer & Meyer, 2003; Merckx *et al.*, 2009). Amongst autotrophic or partially mycoheterotrophic orchids, saprotrophic rhizoctonia and to a 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 specialised 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 recognised to obtain approximately 83.4 $\pm$ 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 Fagaceae forest habitat in the Taiwanese mountains.

Analysing $\delta^{2}H$ and $\delta^{18}O$ additionally to $\delta^{13}C$ and $\delta^{15}N$, 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 visualised by gradual changes in stable isotope natural abundance.

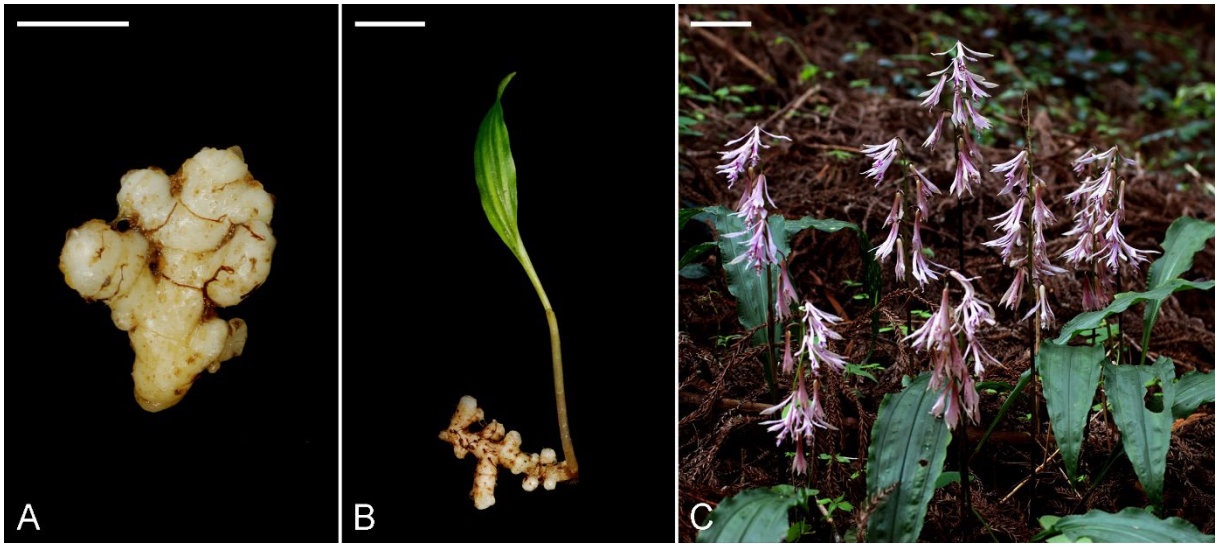

Fig. 1 Development stages of *Cremastra appendiculata*. (A) Protocorm. (B) Seedling with very early leaf. (C) Flowering mature individuals; Scale: A = 5 mm; B = 5 cm; C = 5 cm.

MATERIALS AND METHODS

Sampling sites and experimental design

Sampling of *Cremastra appendiculata* var. *variabilis* at three different development stages and respective autotrophic reference plants (listed in Supporting Information Table S3 & Table S4) took place in a Fagaceae forest in subtropical Taiwan on the western slope of the Hehuan Mountains at an elevation of 2000 m asl (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 & Whigham, 1998). About one hundred 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 stereomicroscope for examining germination.

The sampling design for stable isotope analysis followed the approach of Gebauer and Meyer (2003), consisting of 1 m^2 plots with the target orchid species and four to five autotrophic reference plant species. This sampling scheme enables sufficient replicates of the target orchid. It ensures almost identical microclimatic conditions, soil properties and light availability of the target orchids and the respective reference plants to avoid bias on ^{2}H, ^{13}C and ^{15}N relative abundance due to microsite differences (Dawson *et al.*, 2002). Autotrophic non-orchid reference plants were chosen according to the criteria discussed by Gebauer and Meyer (2003) and Hynson *et al.* (2013). We strived to cover a broad spectrum of growth habits, life forms, and taxonomy. We took leaf samples from five flowering mature *C. appendiculata* individuals in 2011/06/02 (Fig. 1C) and from three *C. appendiculata* seedlings with very early green leaves in 2015/07/19 (Fig. 1B), respectively. The seven analysed protocorms originate from seed packages buried *in situ* for at least three years (harvested in October of 2015 and 2019).

For the high-throughput sequencing experiment, samples were taken from the above mentioned plots on 2015/07/19, 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 colonised samples from coralloid rhizomes were pooled to create a homogeneous mycorrhizal coralloid rhizome sample. The distal 3-cm portions of colonised 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 (fifteen roots from five individuals in total from five plots), colonised 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 sterilised 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 colonisation. 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 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 cut and purified using the AxyPrep DNA Gel Extraction Kit (GMbiolab Co. Ltd., Taichung, Taiwan)

and quantified using QuantiFluor™ Fluorometer (Promega Corporation, Madison, WI). Samples were then pooled in equimolar concentrations and paired-end sequenced (2 x 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). OTUs were clustered with 97 % similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimaeras 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. OTUs identified by BLAST were then manually screened for potential orchid-associating mycorrhizal families following a step-wise 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 internal transcribed spacer (ITS) sequences from the DNA extractions of mycorrhizal samples. The internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene was amplified with the primer combinations ITS1F/ITS4 (Gardes & Bruns, 1993) or ITS1-OF/ITS4-OF (Taylor & 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.* (2021), 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.*, 1997), 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 (MCMC; 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 (PP) for individual branches.

Multi-element stable isotope analyses

For multi-element stable isotope analysis, sample preparation was according to Table S1. Relative natural abundance analysis of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) 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 ($^{2}H/^{1}H$) and oxygen isotopes ($^{18}O/^{16}O$) 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 ^{13}C and ^{2}H caused by differences in stomatal regulation and transpiration between orchids and the non-orchid reference plants. Transpiration affects ^{13}C, ^{2}H and

^{18}O isotope abundance in plant tissues simultaneously (da Silveira Lobo Sternberg, 1988; Ziegler, 1989; Flanagan *et al.*, 1991; Cernusak *et al.*, 2004). A transpiration effect can only be precluded if the ^{18}O isotope abundance of the target species is similarly distributed as the ^{18}O 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: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000$ ‰, where R_{sample} and $R_{standard}$ 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: $\varepsilon = \delta S - \delta REF$, where δS is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O$ value of an orchid individual or an autotrophic reference plant and δREF is the mean value of all autotrophic reference plants by plot (Preiss & Gebauer, 2008). The ε approach was essential because sampling of different development stages within one year was not feasible. All single values and mean values ± standard deviations of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$, of enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and $\varepsilon^{18}O$, 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 $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and $\varepsilon^{18}O$ 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 $\alpha = 0.05$.

RESULTS

Molecular identification of mycorrhizal fungi and germination mycobionts

The quality-filtered and normalized Miseq dataset contained samples from protocorms, coralloid rhizomes of seedlings, roots of seedlings and roots of adult plants. The protocorm sample comprised 9 OTUs (41093 sequences), the coralloid rhizomes of the seedling sample comprised 9 OTUs (41078 sequences), the roots of the seedling sample comprised 35 OTUs (41005 sequences), and the roots of the adult plant sample comprised 21 OTUs (41127 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 - 31108 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 - 23767 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 was 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).

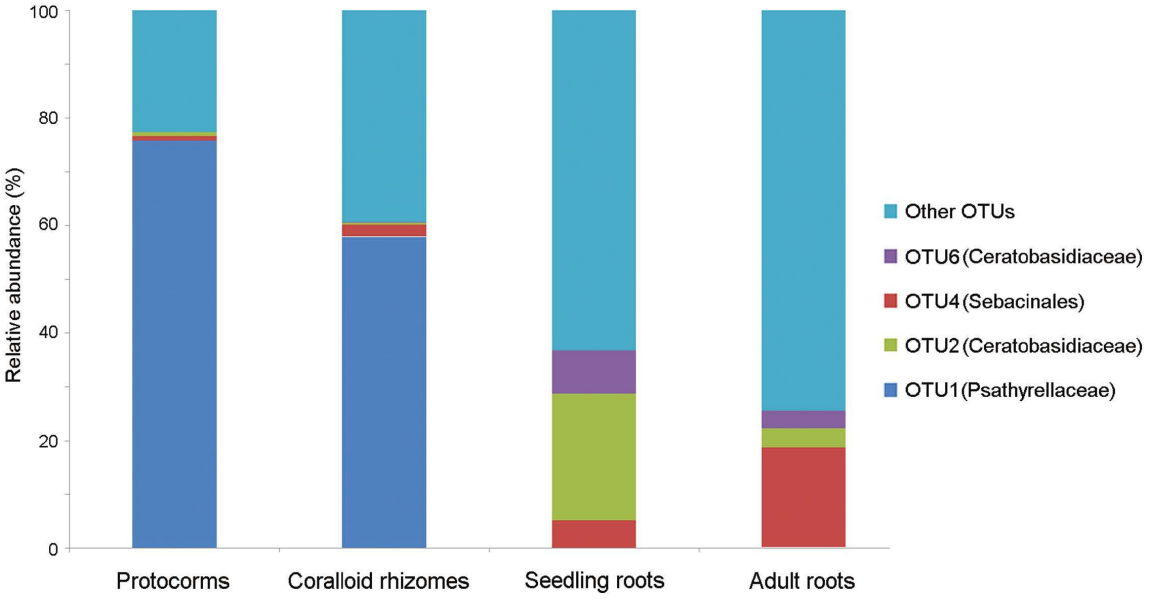

Fig. 2 The proportion of putative orchid mycorrhizal fungi, including OTU1 (Psathyrellaceae), OTU2 (Ceratobasidiaceae), OTU4 (Sebacinales), and OTU6 (Ceratobasidiaceae) detected in protocorms, seedling rhizomes, seedling roots and adult roots of *Cremastra appendiculata* in central Taiwan. Most of other OTUs are Ascomycota, not related to the fungal taxa in orchid mycorrhizal association in the published database.

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 $\varepsilon^{15}N$ of 3.16 ±1.77 ‰ and 2.68 ± 0.12 ‰, respectively, and $\varepsilon^{13}C$ 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. 3 A, B).

C. appendiculata seedlings had on average an $\varepsilon^{15}N$ 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 $\varepsilon^{13}C$ of seedlings showed a rather considerable variation (3.06 ± 3.59 ‰) and was only statistically distinct from protocorms ($U = 24$, $P = 0.012$).

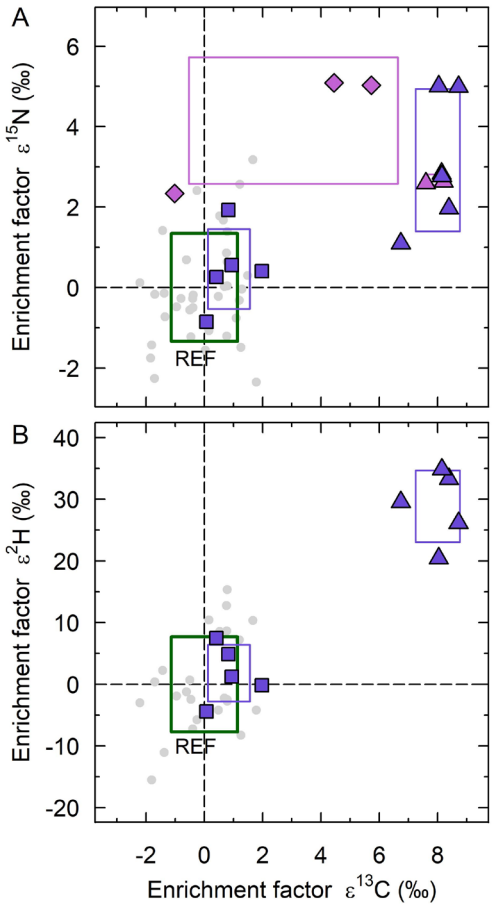

Fig. 3 Carbon and nitrogen (A) and carbon and hydrogen (B) stable isotope enrichment factors ε of *Cremastra appendiculata* protocorms (triangle), seedlings (diamonds) and adults (squares), and respective reference plants (REF, dots). Frames represent the standard deviation from the mean enrichment factors ε of each group, while each symbol denotes a single plant individual. Identical colours represent same sampling plot scheme (dark purple: 2011/2019, light purple: 2015). The green frame represents the standard deviation of autotrophic reference plants around a mean enrichment factor of zero, by definition. Data on hydrogen stable isotope enrichment factors ε of seedlings and of protocorms from 2015 are not available due to material limitation of these samples.

Table 1 Pairwise comparisons of $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and $\varepsilon^{18}O$ 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 ($\varepsilon^{13}C$: H = 22.821, df = 3, P < 0.001; $\varepsilon^{15}N$:H = 22.354 , df = 3, P < 0.001; $\varepsilon^{2}H$: H = 12.802, df = 2, P = 0.002; $\varepsilon^{18}O$: H = 9.6663, df = 2, P = 0.008).

		$\varepsilon^{15}N$		$\varepsilon^{13}C$		$\varepsilon^{2}H$		$\varepsilon^{18}O$	
		U	*P*	*U*	*P*	*U*	*P*	*U*	*P*
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	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	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	NA	NA

Significances are highlighted in bold.

Total N concentration was on average highest in *C. appendiculata* protocorms (3.26 ± 0.28 mmol g d.wt $^{-1}$), followed by leaf N concentrations of seedlings with the largest variation (2.76 ± 1.23 mmol g d.wt $^{-1}$). Total N concentration of adult *C. appendiculata* (2.52 ± 0.18 23 mmol g d.wt $^{-1}$) and autotrophic reference plants (2.53 ± 0.49 mmol g d.wt $^{-1}$) were on average similar. They were statistically distinct from total N concentrations in protocorms (Fig. 4, see Supporting Information Table S3).

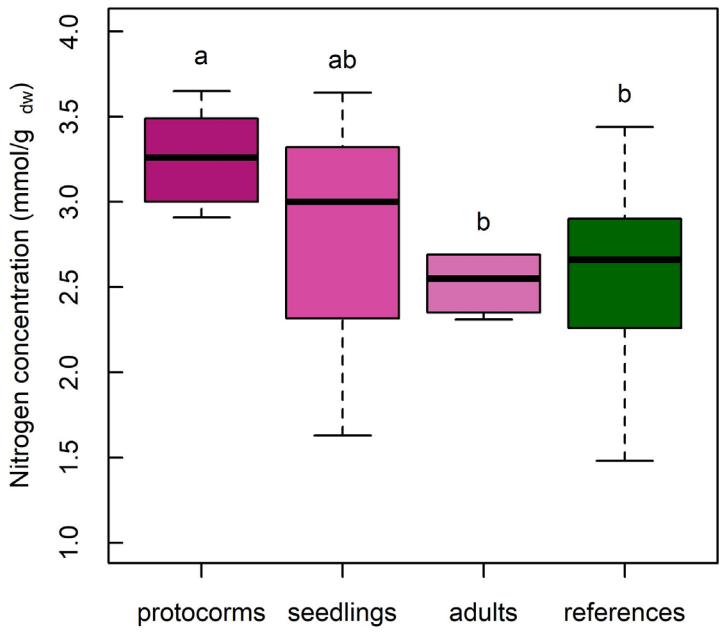

Fig. 4 Nitrogen concentration (total N) for *Cremastra appendiculata* protocorms, seedlings, adults and reference plants. The box spans the first and third quartile, while the horizontal line in the box represents the median; whiskers extend to 1.5*interquartile range. Different letters indicate statistically significant differences (Student´s t-test) between groups.

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 *Cremastra* genus. They are fungal hosts of fully mycoheterotrophic orchid *Cremastra 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 *C. appendiculata* protocorm-rhizomes directly from an *in situ* experimental setup 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* protocorms showed enrichment in ^{15}N, ^{13}C and ^{2}H and elevated leaf N concentrations. Here we present the first stable isotope patterns of protocorms related to non-rhizoctonia saprotrophic fungal partners, which are distinct from those of protocorms and FMH adult orchids with rhizoctonia and ECM fungal partners (Table 2). ^{15}N and ^{13}C signatures of *C. appendiculata* protocorms 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* protocorms 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).

Table 2 Mean enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$ and $\varepsilon^{2}H$ $\pm$ *s.d.* 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.

			n_s	$n_{spp.}$	$\varepsilon^{15}N$		$\varepsilon^{13}C$		n_s	$n_{spp.}$	$\varepsilon^{2}H$	
C. appendiculata		protocorm	*8*	*1*	**2.98**	*± 1.36*	**8.00**	*± 0.60*	5	*1*	**28.83**	*± 5.8*
C. appendiculata		adult	*5*	*1*	**0.46**	*± 0.99*	**0.84**	*± 0.99*	5	*1*	**1.80**	*± 4.59*
SAP wood	FMH	adult	*43*	*9*	**4.29**	*± 2.09*	**10.98**	± 2.30	*NA*	*NA*	*NA*	
SAP litter	FMH	adult	*15*	*3*	**4.99**	*± 0.53*	**8.24**	± 0.49	*NA*	*NA*	*NA*	
ECM	IMH	protocorm	*46*	*2*	**7.48**	*± 2.26*	**7.55**	± 0.62	*NA*	*NA*	*NA*	
	FMH	adult	*163*	*15*	**11.78**	*± 3.22*	**7.82**	± 1.57	*10*	*1*	**57.30**	*± 13.83*
Rhizoctonia	IMH	protocorm	*49*	*6*	**5.77**	*± 2.73*	**6.12**	± 2.70	*5*	*1*	**95.78**	*± 6.22*
	FMH	adult	*9*	*2*	**2.20**	*± 2.46*	**9.27**	± 0.75	*NA*	*NA*	*NA*	
	Green	adult	*674*	*65*	**2.94**	*± 2.30*	**-0.48**	± 1.90	136	*17*	**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).

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 helleborine* (Bidartondo & Read, 2008). However, partial replacement of mycobionts in the seedling stage likely reduces the risk of failing to find new mycobionts during ontogenesis (McCormick *et al.*, 2006) and is the most frequent scenario of temporal turnover in orchid-mycorrhizal relationships (Ventre Lespiaucq *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 for a symbiotic seed experiment (Yagame *et al.*, 2013), and rhizoctonia (Ceratobasidiaceae 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 protocorms 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 protocorms and adults, but with a considerable variation. We, therefore, suppose the seedling stage of *C. appendiculata* to be a transition phase between the protocorm 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 organs, like in the case of *Calypso bulbosa* mycorrhizal with wood-decaying fungi (Suetsugu and Matsubayashi 2021). 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 in-dependent 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 discolour* 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 & 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 & 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 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 during adulthood, has been attributed high flexibility regarding adaption to diverse environmental conditions because it can gain carbon from various fungal partners (Yagame *et al.*, 2021). Providing evidence that *C. appendiculata* is capable of a genuinely autotrophic nutrition 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, *Cremastra appendiculata* develops relatively large green leaves. We provide novel, explicit evidence that mature *C. appendiculata* individuals with rhizoctonia mycobionts can be entirely autotrophic. *C. 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 saprotroph-mycoheterotrophic plant-fungus interactions.

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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.

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CONFLICT OF INTEREST

None declared.

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.

SUPPORTING INFORMATION

The following additional information is available in the online version of this article (at https://academic.oup.com/aobpla/article/14/3/plac021/6582690#supplementary-data):

Table S1. Equipment and conditions as used for stable isotope abundance analysis.

Table S2. Summary of fungal operational taxonomic units (OTUs)[a] and their frequencies[b] detected in protocorms, seedling rhizomes, seedling roots and adult roots of *Cremastra appendiculata* using the Illumina Miseq platform. [a]OTUs 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. [b]Normalized 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 $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ 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 $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ 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.).

Fig. S1. OTU rarefaction curves of protocorms, seedling rhizomes, seedling roots and adult roots samples by randomly selecting smaller fractions of reads 100 times.

Fig. 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 %).

Fig. 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%).

Fig. S4. The Bayesian tree based on the sequences of internal transcribed spacer (ITS) nuclear ribosomal DNA of Ceratobasidiaceae 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%).

MANUSCRIPT 2

Mode of carbon gain and fungal associations of *Neuwiedia malipoensis* within the evolutionary early diverging orchid subfamily Apostasioideae.

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Key words: mycoheterotrophy, mycorrhiza, Orchidaceae, Apostasioideae, *Neuwiedia*, stable isotopes, carbon, nitrogen, hydrogen, fungal association

ABSTRACT

Background and Aims

The earliest diverging orchid lineage Apostasioideae consists only of two genera: *Apostasia* and *Neuwiedia*. Previous report of *Apostasia nipponica* indicated a symbiotic association with an ectomycorrhiza-forming Ceratobasidiaceae clade and partial utilization of fungal carbon during the adult stage. However, the trophic strategy of *Neuwiedia* throughout its development remains unidentified. To further improve our understanding of mycoheterotrophy in the Apostasioideae, this study focused on *Neuwiedia malipoensis* examining both the mycorrhizal association and the physiological ecology of this orchid species across various development stages.

Methods

We identified the major mycorrhizal fungi of *N. malipoensis* protocorm, leafy seedling and adult stages using molecular barcoding. To reveal nutritional resources utilized by *N. malipoensis*, we compared stable isotope natural abundance ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$) of different developmental stages to autotrophic reference plants.

Key Results

Protocorms exhibited an association with saprotrophic Ceratobasidiaceae rather than ectomycorrhiza-forming Ceratobasidiaceae and ^{13}C signature was characteristic of their fully mycoheterotrophic nutrition. Seedlings and adults predominantly associated with saprotrophic fungi belonging to the Tulasnellaceae. While ^{13}C and ^{2}H stable isotope data revealed partial mycoheterotrophy of seedlings, it is unclear to what extent the fungal carbon supply is reduced in adult *N. malipoensis*. However, the ^{15}N enrichment of mature *N. malipoensis* suggests partially mycoheterotrophic nutrition. Our data indicated a transition in mycorrhizal partners during ontogenetic development with decreasing dependency of *N. malipoensis* on fungal nitrogen and carbon.

Conclusions

The divergence in mycorrhizal partners between *N. malipoensis* and *A. nipponica* indicates different resource acquisition strategies and allows for various habitat options in the earliest diverging orchid lineage Apostasioideae. While *A. nipponica* relies on the heterotrophic C gain from its ectomycorrhizal fungal partner and thus on forest habitats, *N. malipoensis* rather relies on own photosynthetic C gain as adult allowing it to establish in habitats as widely distributed as those where Rhizoctonia fungi occur.

INTRODUCTION

Orchidaceae is considered the most diverse plant family with > 29 000 species occurring widely over most terrestrial ecosystems (WFO, 2023), and they form distinct mycorrhizal relationships with fungi from both the Basidiomycota and a limited number of Ascomycota, known as orchid mycorrhizas (OrM) (Merckx, 2013). All orchids produce a lot of dust-like seeds that depend on nutrient supply from mycorrhizal association for germination and during the early seedling development stage (Rasmussen, 1995; Smith and Read, 2008). This phenomenon is called 'initial mycoheterotrophy' (Merckx, 2013). Most orchids become photosynthetic when fully established, while more than 250 achlorophyllous orchid species have completely replaced their photosynthetic ability by fungus-supplied carbon and are known as full mycoheterotrophs (Jacquemyn and Merckx, 2019). In addition, several chlorophyllous orchids have been found to obtain carbon from both autotrophic photosynthates and OrM association. This mode of mixotrophic carbon gain is known as 'partial mycoheterotrophy' (Gebauer and Meyer, 2003; Merckx, 2013). Though these trophic modes are evolutionarily widespread among plants, no other group of plants like Orchidaceae demonstrates such a high frequency of trophic mode shift (Jacquemyn and Merckx, 2019; Wang et al., 2021).

The subfamily Apostasioideae is the earliest diverging orchid lineage and consists of only two genera, namely, *Apostasia* and *Neuwiedia* (Kocyan and Endress, 2001; Kocyan et al., 2004), with about 20 species distributed in southeastern Asia, Japan, and northern Australia. Apostasioid orchids possess several unique characters, including nearly actinomorphic flowers with a simple labellum resembling the other two petals, two or three fertile stamens per flower, the presence of loose pollens without the formation of pollinia, and partially free styles and stigma (Kocyan and Endress, 2001). Like most orchids, Apostasioid orchids have tiny seeds without endosperm, and their early stages of development (e.g. seed germination and protocorm stage) are initially mycoheterotrophic, and then become photosynthetic as adults (Merckx, 2013). The potential fungal partners of Apostasioid orchids include three basidiomycetous families: Botryobasidiaceae, Ceratobasidiaceae, and Tulasnellaceae within the order Cantharellales (Yukawa et al., 2009; Rasmussen and Rasmussen, 2014). The mycobionts of *Neuwiedia veratrifolia* seedlings have been identified as members of Ceratobasidiaceae and Tulasnellaceae (Kristiansen et al., 2001; Kristiansen et al., 2004), and adult *Apostasia*

species form mycorrhizal symbiosis with the genera *Ceratobasidium* and *Botryobasidium* (Yukawa et al., 2009). Since Ceratobasidiaceae is the only mycobiont shared by *Apostasia*, *Neuwiedia* and the rest lineages of orchid family, it is likely to represent the plesiomorphic orchid fungal partner (Yukawa et al., 2009).

Recently, *Apostasia nipponica* was found to associate with a specific *Ceratobasidium* species belonging to ectomycorrhizas (ECM)-forming clades and to gain carbon from the mycorrhizal association to compensate for photosynthesis at the adult stage (Suetsugu and Matsubayashi, 2021). It is still unclear whether the strategy of mycorrhizal cheating in *A. nipponica* is a distinct case, or it is a regular strategy present in Apostasioid orchids and also occurs in the genus *Neuwiedia*. This investigation extends our knowledge on fungal associations and trophic modes in the early divergent orchid clade. We investigated *N. malipoensis* with regards to its mycorrhizal associates using high-throughput DNA sequencing and its trophic modes using natural stable isotope signatures as indicator.

Carbon gain from the mycorrhizal associate can be identified by comparing natural stable isotope signatures of the orchid tissues to autotrophic plants co-occurring under the same environmental conditions. A mycoheterotrophic trophic mode of orchids associating with otherwise ECM fungi is generally characterised by an enrichment in ^{13}C, ^{2}H and ^{15}N relative to the reference plants (Gebauer and Meyer, 2003; Gebauer et al., 2016; Hynson et al., 2016), as they mirror the isotope signature of their fungal source (Zahn et al., 2023). Yet, the mycorrhizal associates of most green orchids belong to the polyphyletic Rhizoctonia group, which includes members of the Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae (Taylor et al., 2002; Dearnaley et al., 2012). In this case, a low ^{13}C enrichment of the Rhizoctonia fungal associates, which is mirrored by the orchids, impede the detection of carbon gains from fungal partners (Gomes et al., 2023; Zahn et al., 2023). For orchids receiving C from Rhizoctonia fungi enrichment in ^{2}H is a valid and necessary substituent for ^{13}C to indicate a heterotrophic part of nutrition (Gebauer et al., 2016) and partially mycoheterotrophic orchids often also display ^{15}N enrichment (Bidartondo et al., 2004; Hynson et al., 2013; Stöckel et al., 2014). Leaf ^{18}O isotope abundances, which are affected by transpiration but not by mycoheterotrophic C exchange, can be used to separate the effect of mycoheterotrophic C gains on ^{13}C or ^{2}H abundance from a transpiration effect (Farquhar et al., 1982; da Silveira Lobo Sternberg, 1988; Cernusak et al., 2004; Barbour, 2007): ^{13}C enrichment compared to co-occuring autotrophic plants

without parallel ^{18}O enrichment is indicative of heterotrophic carbon gain rather than higher transpiration and lower water use efficiency.

In a tropical forest at Malipo County, Yunnan Province, China, we found a few protocorms and young seedlings with leaves of *N. malipoensis* around the adults. The presence of naturally occurring protocorms provides an opportunity to investigate the mycorrhizal association and the nutrient gain across different developmental stages. The goals of the present study were to determine (1) fungal partners across different life stages, i.e., protocorms, young seedlings and adults of *N. malipoensis* and (2) whether *N. malipoensis* as a representative of the sister group to *Apostasia nipponica* within the Apostasioideae associate with the ECM-forming Ceratobasidiaceae fungi to obtain carbon from fungal cheating.

MATERIALS AND METHODS

Sample collection and locations

N. malipoensis (Fig. 1), a highly autogamous species, is found in a broadleaf forest situated at an elevation of 1102 m above sea level (23°0'43" N, 104°49'45" E) in Malipo County, Yunnan Province, China. The specimens were sampled on 12th July 2016 for microscopic studies, metabarcoding of mycorrhizal fungi and stable isotope analyses. Voucher specimens of *N. malipoensis* (YAF06702) were deposited in the herbarium of Yunnan Academy of Forestry and Grassland, Kunming. For the high-throughput sequencing experiment, the mycorrhizal roots from one mature individual and one seedling (three roots of each individual were collected from each plot), and protocorms (two protocorms were collected from each plot) were collected within three 1 m^2 plots (six protocorms in total from three plots). The plots were at least 10 m apart from each other. For multielement natural abundance stable isotope analyses ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$), we collected leaf samples from one mature orchid individual, from one orchid seedling and from three autotrophic reference plant species (*Dendrocalamus latiflorus*, *Elatostema multicanaliculatum* and *Selaginella decipiens*) in each of the three plots mentioned above. Among the three plots, protocorm samples of *N. malipoensis* were retrieved from only two plots due to their limited quantity. Protocorm samples could not be separated into infected and non-infected tissues due to their tiny size. Thus, a certain contamination with fungal hyphae tissue could not be excluded. The light climate data at the study site was

collected using a LM-8000 Lux meter positioned 10 cm above the ground. The average light intensity recorded within the forest was 6370 lux, compared to an average of 62400 lux measured in open areas outside the forest at the same time.

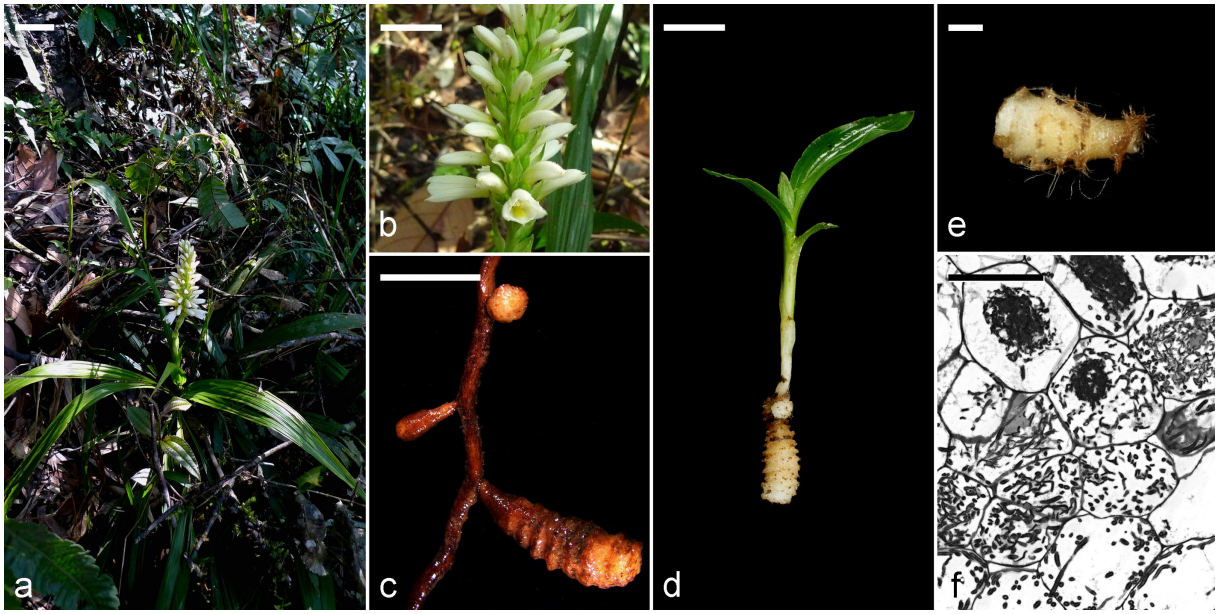

Fig. 1 (a) Plants of *Neuwiedia malipoensis* in their natural habitat. Scale bar = 5 cm; (b) Flower morphology. Scale bar = 2 cm; (c) Root morphology, showing the formation of root tubercles. Scale bar = 1 cm; (d) A young seedling with the leafy shoot. Scale bar = 1 cm; (e) Protocorm morphology. Scale bar = 1 mm; (f) Light micrograph showing a transverse section of a root tubercle. Fungal colonization in the middle cortex cells could be observed. Scale bar = 50 μm.

Microscopy

Mycorrhizal samples of *N. malipoensis* were cut into slices 1 mm thick and promptly immersed in a 2.5 % glutaraldehyde solution (0.1 M phosphate buffer at pH of 6.8), for at least 2 h at 4 ºC. After fixation, the samples were washed in the same buffer three times for 10 min each at room temperature, then subsequently dehydrated in a graded ethanol series. The dehydrated samples were embedded in Technovit 7100 resin (Kulzer & Co., Wehrheim, Germany) according to the method described by Yeung and Chan (2015). Resin sections with a thickness of 3 μm were obtained using a glass knife on a Leica RM2125 rotary microtome and were stained using 0.05 % (w/v) toluidine blue O dissolved in 1 % sodium tetraborate, following the general histological staining procedure detailed by Lee et al. (2015). The stained sections were examined with a Carl Zeiss Axioskop microscope with a digital camera (Carl Zeiss AG, Jena, Germany) for capturing images.

Fungal identification and phylogenetic analyses

Following the examination of fungal colonization in the protocorms and roots through free-hand sectioning under a microscope, mycorrhizal samples of adult roots (Fig. 1c), seedling roots (Fig. 1d) and protocorms (Fig. 1e) were subjected to surface sterilization using a 1% solution of sodium hypochlorite for 60 s. Afterward, the samples were rinsed three times in sterile water for 60 s each and then stored at -80 ºC for future analysis. DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). We used the primer pair ITS1F and ITS2R to amplify the ITS1 region of the nuclear ribosomal RNA genes of mycorrhizal fungi (Adams et al., 2013). Polymerase chain reaction (PCR) was performed in 20 µl reactions, comprising 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 PCR conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s. After PCR amplification, the products were analyzed using gel electrophoresis. Amplicons of the desired size range were then excised from the gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA). Quantification was performed using QuantiFluor™ Fluorometer (Promega Corporation, Madison, WI). After quantification, the samples were pooled at equimolar concentrations and subjected to paired-end sequencing (2 x 250 bp) using the Illumina Miseq sequencing system at Genomics BioSci & Tech Co., Ltd. (Taipei, Taiwan).

The initial processing of raw fastq files involved demultiplexin and quality-filtering using QIIME (version 1.17), applying specific criteria. Initially, reads of 300 bp were subjected to truncation at sites where the average quality score dipped below 20 over a 50 bp sliding window. Following this, reads exhibiting one or two nucleotide mismatches in primer matching measurements, as well as those including uncertain characters, were filtered out. Subsequently, sequences with an overlap longer than 10 bp were assembled based on their overlapping regions, while reads failing to assemble were eliminated from further analysis.

The sequences derived from the Illumina Miseq platform were clustered into operational taxonomic units (OTUs) utilizing the UPARSE algorithm within the framework of USEARCH version 7 (Edgar, 2013). OTUs were clustered with 97 % similarity cutoff

employing UPARSE (version 7.1 http://drive5.com/uparse/), with subsequent removal of chimeras identified using the UNITE UCHIME reference data set. Taxonomic classification of each ITS1 region was carried out using the RDP Classifier (http://rdp.cme.msu.edu/) against the UNITE fungal ITS database, setting a confidence threshold of 70 %. The remaining OTUs were taxonomically assigned identities based on the top 10 results from a BLAST search using the megablast algorithm (Altschul et al., 1990) of the OTU representative sequences, which were selected by UPARSE. This assignment utilized the GenBank nucleotide database (Benson et al., 2009). The identified OTUs were further subjected to manual screening to identify potential orchid-associating mycorrhizal families, employing a stepwise approach. Initially, Sequences of representative OTUs that were shorter than 150 bp or showed less than 90% sequence similarity to fungal species across their entire length were eliminated. Next, OTUs with only one sequence per individual orchid root sample were removed from the analysis. Finally, the results were organized into an OTU table where each cell contained read numbers (Table S1).

The fungal specific primer sets ITS1OF/ITS4OF were utilized to amplify the nuclear internal transcribed spacer (ITS) region of the mycorrhizal fungi (Taylor and McCormick, 2008). PCR amplification was conducted following the procedure described by Suetsugu and Matsubayashi (2021). GenBank accession numbers of ITS sequences were OR123806-OR123807. In *N. malipoensis*, since the dominant OTUs belonged to Ceratobasidiaceae and Tulasnellaceae, sequences of the two fungal families were downloaded from the NCBI sequence database (National Center for Biotechnology Information, GenBank) as referenced in Bidartondo et al. (2003), Tedersoo et al. (2008), Nontachaiyapoom et al. (2010), Freitas et al. (2020), and Suetsugu and Matsubayashi (2021). Phylogenetic relationships were examined through a model-based Bayesian approach using MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003). The 'best-fit' evolutionary model was identified via Akaike information criterion test (Akaike, 1974), determined through MrModeltest 2.2 (Nylander, 2004). The general time reversal plus invariant rates and a gamma distribution (GTR + I +Γ) was selected for analysis. Two independent runs consisting of four Monte Carlo Markov chains were conducted for 10,000,000 generations, terminating when the average deviation of split frequency fell below 0.01. Trees were sampled every 1000th generation. The initial 25 % of generated

trees were unused as the burn-in, and the remaining trees were used to make a majority-rule (> 50 %) consensus tree and assess posterior probabilities (PP).

Stable isotope natural abundance and nitrogen concentration analyses

Leaf and protocorm samples were dried to a consistent weight at 105 °C in an oven, subsequently ground into a fine powder using a ball mill (Retsch Schwingmühle MM2, Haan, Germany), and then preserved in a desiccator until being measured. For the analyses, the samples were placed into tin (plant sample weight: 1-2 mg) or annealed silver (plant sample weight: 0.5-1 mg) capsules (micro balances: Sartorius CPA2P & MSE3.6P-000-DM, Göttingen, Germany & Mettler AT21, Gießen, Germany). To determine relative natural abundance analysis of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes as well as N concentrations simultaneously we used an EA-IRMS coupling, which links an elemental analyser (CE Instruments 1108, Milano Italy) with a continuous flow isotope ratio mass spectrometer (delta S, Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Finnigan MAT, Bremen, Germany). Relative natural abundances of hydrogen ($^{2}H/^{1}H$) and oxygen isotopes ($^{18}O/^{16}O$) were measured separately using a TC-IRMS coupling, which consists of a thermal conversion through pyrolysis unit (HTO, HEKAtech, Wegberg, Germany), a continuous flow isotope ratio mass spectrometer (delta V advantage, Thermo Fisher Scientific, Bremen, Germany) and a ConFlo IV open-split interface (Thermo Fisher Scientific, Bremen, Germany). To determine H isotope abundance, each sample was measured four times in a row with the first three measures being neglected to avoid a memory bias of the previous sample owing to the extraordinarily high frequency difference of the isotopes ^{1}H and ^{2}H. Moreover, we analysed H isotope abundance of target orchid samples and respective reference plant samples together in identical sample batches to account for a bias of post-sampling H atom exchange between organically bound hydroxyl groups in our samples and H_2O in ambient air (Gebauer et al., 2016).

Measured relative isotope abundances were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1{,}000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. Standard gases were calibrated in relation to international standards (CO_2 vs. V-PDB (R=0.0111802), N_2 vs. N_2 in air (R=0.0036765), H_2 vs. V-SMOW (R = 0.00015575), CO vs. V-SMOW (R = 0.0020052)). Reference substances were ANU sucrose and NBS19 for C

isotopes, N1 and N2 for the N isotopes, IAEA-CH7, V-SMOW and SLAP for H isotopes, and IAEA601 and IAEA602 for O isotopes, all provided by the International Atomic Energy Agency, Vienna, Austria. Acetanilide and Benzoic acid severed as quality control for C and N isotope and H and O isotope abundance measurements, respectively, and for element concentration calculations.

To enable site- and time-independent stable isotope data, we calculated enrichment factors (ε) from measured δ values using the equation $\varepsilon = \delta_S - \delta_{REF,}$ where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ or $\delta^{2}H$ value of an orchid individual and δ_{REF} is the mean value of all autotrophic reference plants within the same plot (Preiss and Gebauer, 2008).

Mean and single $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ and total nitrogen concentration data of *N. malipoensis* adults, protocorms and seedlings are displayed in Table S2. Analysis of variance (ANOVA) followed by a *post-hoc* Tukey HSD or Kruskal-Wallis rank-sum tests followed by a post-hoc Mann Whitney *U*-test was used to evaluate differences of leaf isotope signatures and total N concentrations among sample types (references, mature, seedling) (Table S3). Protocorm samples were not included in the statistical tests due to $n = 2$.

RESULTS

Fungal associates change across different development stages of *N. malipoensis*

Our high-throughput sequencing data demonstrate the dynamics of fungal community associated with *N. malipoensis* in different developmental stages (Fig. 2). The protocorms were predominately colonized by an OTU (operational taxonomic unit) belonging to Ceratobasidiaceae (OTU1 – 106,195 sequences, 96.11 %), only a few reads belonging to Tulasnellaceae (OTU2 – 4,303 sequences, 3.89 %) were detected. In the seedling sample, the proportion of Tulasnellaceae (OTU2 – 80,359 sequences, 75.89 %) increased obviously, while those of Ceratobasidiaceae (OTU1 – 25,529 sequences, 24.11 %) decreased. In the adult sample, the OTU with the highest number of reads was identified as belonging to Tulasnellaceae (OTU2 – 70,928 sequences, 89.09 %), while a small proportion of Ceratobasidiaceae (OTU1 – 8,687 sequences, 10.91 %) was recorded.

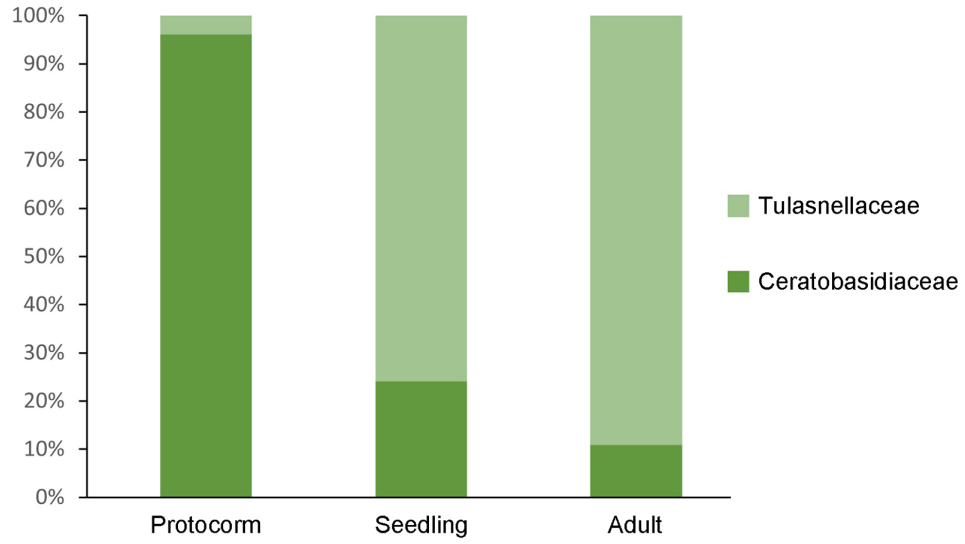

Fig. 2 Frequency distribution of identified operational taxonomic units (OTUs) in the protocorms, young seedling roots and adult roots of *Neuwiedia malipoensis*.

Ceratobasidiaceae and Tulasnellaceae fungi detected in *N. malipoensis* are phylogenetically not clustered with ECM clades

Fungal ITS derived from 18 root samples of 6 individual adult plants/seedlings, and 6 protocorms showed a high level of DNA similarity with species of Ceratobasidiaceae or Tulasnellaceae by BLAST analyses. Additionally, a predominant OTU from either Ceratobasidiaceae or Tulasnellaceae was identified in each case. Bayesian phylogenetic analyses were conducted on the representative sequences of Ceratobasidiaceae or Tulasnellaceae, respectively (Fig. 3 a and b). The dominant Ceratobasidiaceae OTU1 formed a clade with mycobionts previously detected in *Goodyera* species (Shefferson et al., 2010). The dominant Tulasnellaceae OTU2 was closely related to the mycobiont of *Paphiopedilum* species (98 % identity; GU166424; Nontachaiyapoom et al., 2010). Both OTU1 and OTU2 were not grouped with ECM-forming clades of Ceratobasidiaceae or Tulasnellaceae, supporting their putatively saprotrophic mode of life.

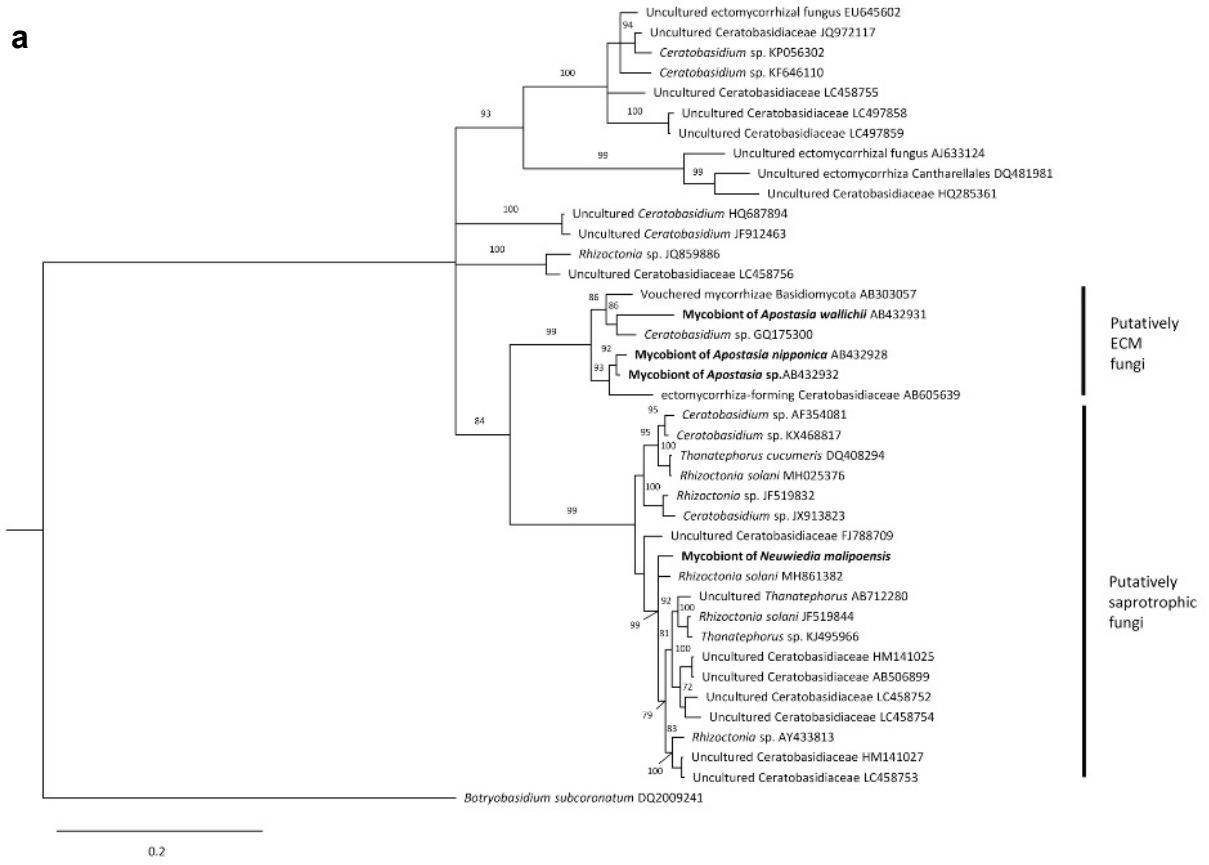

Fig. 3 Phylogenetic relationships of the mycorrhizal fungi of *Neuwiedia malipoensis* based on the Bayesian analysis of partial ITS ribosomal DNA sequences of (**a**) Ceratobasidiaceae (Suetsugu and Matsubayashi, 2021) and (b) Tulasnellaceae (Bidartondo et al., 2003, Tedersoo et al., 2008, Nontachaiyapoom et al., 2010 and Freitas et al., 2020) available in GenBank. Only Bayesian posterior probabilities greater than 70 % are shown.

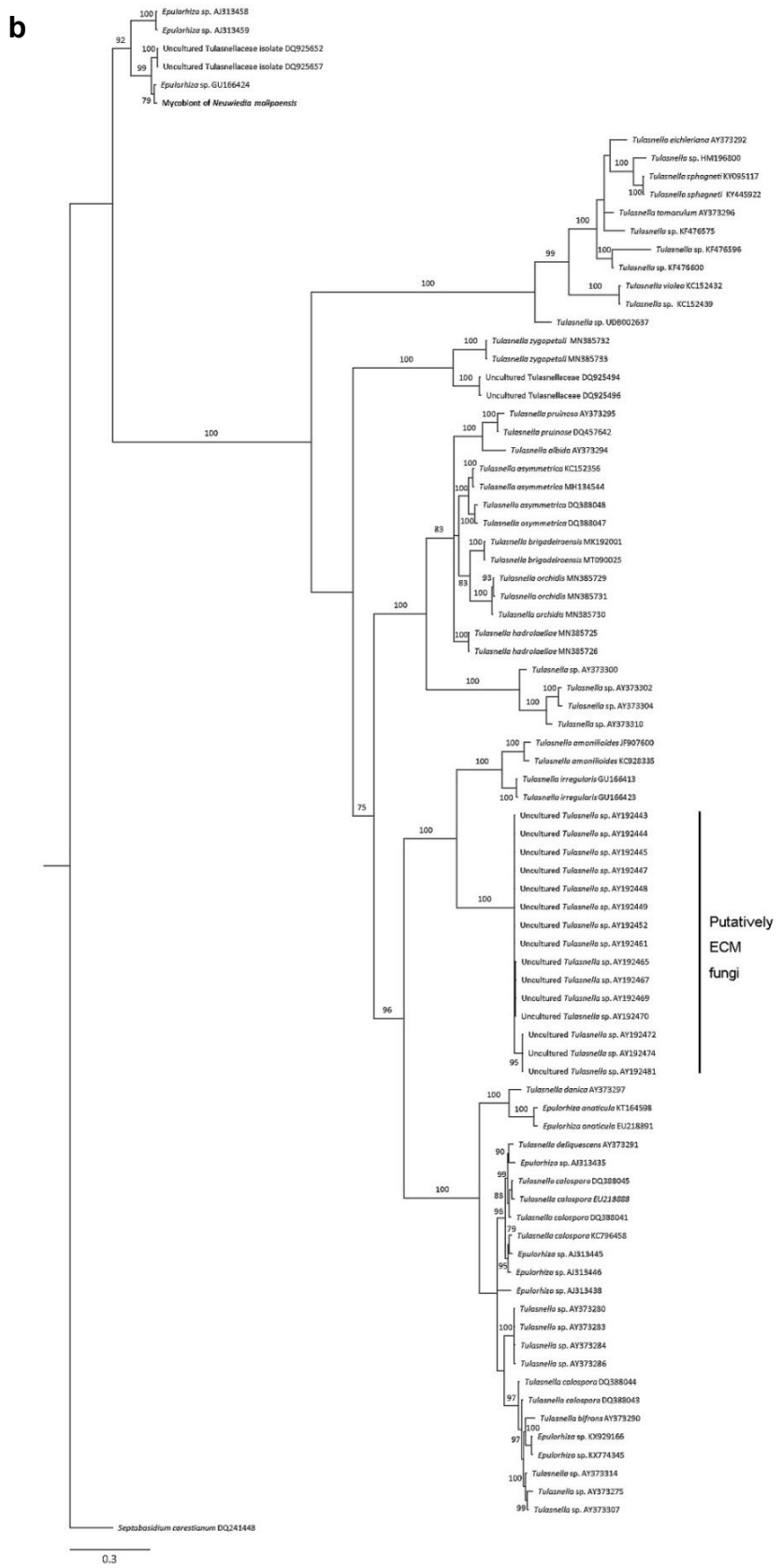

Fig. 3 Phylogenetic relationships of the mycorrhizal fungi of *Neuwiedia malipoensis* based on the Bayesian analysis of partial ITS ribosomal DNA sequences of (a) Ceratobasidiaceae (Suetsugu and Matsubayashi, 2021) and (**b**) Tulasnellaceae (Bidartondo et al., 2003, Tedersoo et al., 2008, Nontachaiyapoom et al., 2010 and Freitas et al., 2020) available in GenBank. Only Bayesian posterior probabilities greater than 70 % are shown.

C gains from fungi decreases during ontogenesis of *N. malipoensis*

The stable isotope data indicate that the degree of mycoheterotrophic nutrition of *N. malipoensis* decreased during ontogenesis. Protocorms and seedlings of *N. malipoensis* were similarly enriched in ^{13}C relative to the reference plants hinting at a fully and partially mycoheterotrophic C gain during these development stages (Fig. 4a). For seedlings a significant enrichment in ^{2}H (Fig. 4b), without difference in ^{18}O signature from reference plants (Fig. S1b), additionally provides evidence for their partially mycoheterotrophic mode of nutrition.

Leaves of the investigated adults of *N. malipoensis* were not enriched in ^{13}C and enrichment in ^{2}H was smaller than that of seedling leaves (Fig. 4). ^{18}O enrichment of adult *N. malipoensis* leaves relative to reference plants (Fig. S1b) indicates that a lower transpiration and a higher water use efficiency of the orchid relative to its reference plants probably contributes to the ^{2}H enrichment. Thus, the suitability of ^{2}H to indicate a heterotrophic part of nutrition (Gebauer et al., 2016) is not conclusive in this case. Based on the ^{2}H signature it remains unclear whether the investigated adult individuals of *N. malipoensis* are partially mycoheterotrophic or entirely autotrophic. However, all three investigated development stages were significantly enriched in ^{15}N relative to the reference plants, which indicates a continuing certain reliance on an organic matter gain from the fungal source even in the adult stage. A decreasing ^{15}N enrichment from the seedling to the adult stage (Fig. 4a) accompanied by decreasing total N concentrations in the orchid tissues (Fig. S1a) of the three development stages hints towards a decrease in reliance on the fungal hosts during the ontogenetic development of *N. malipoensis*.

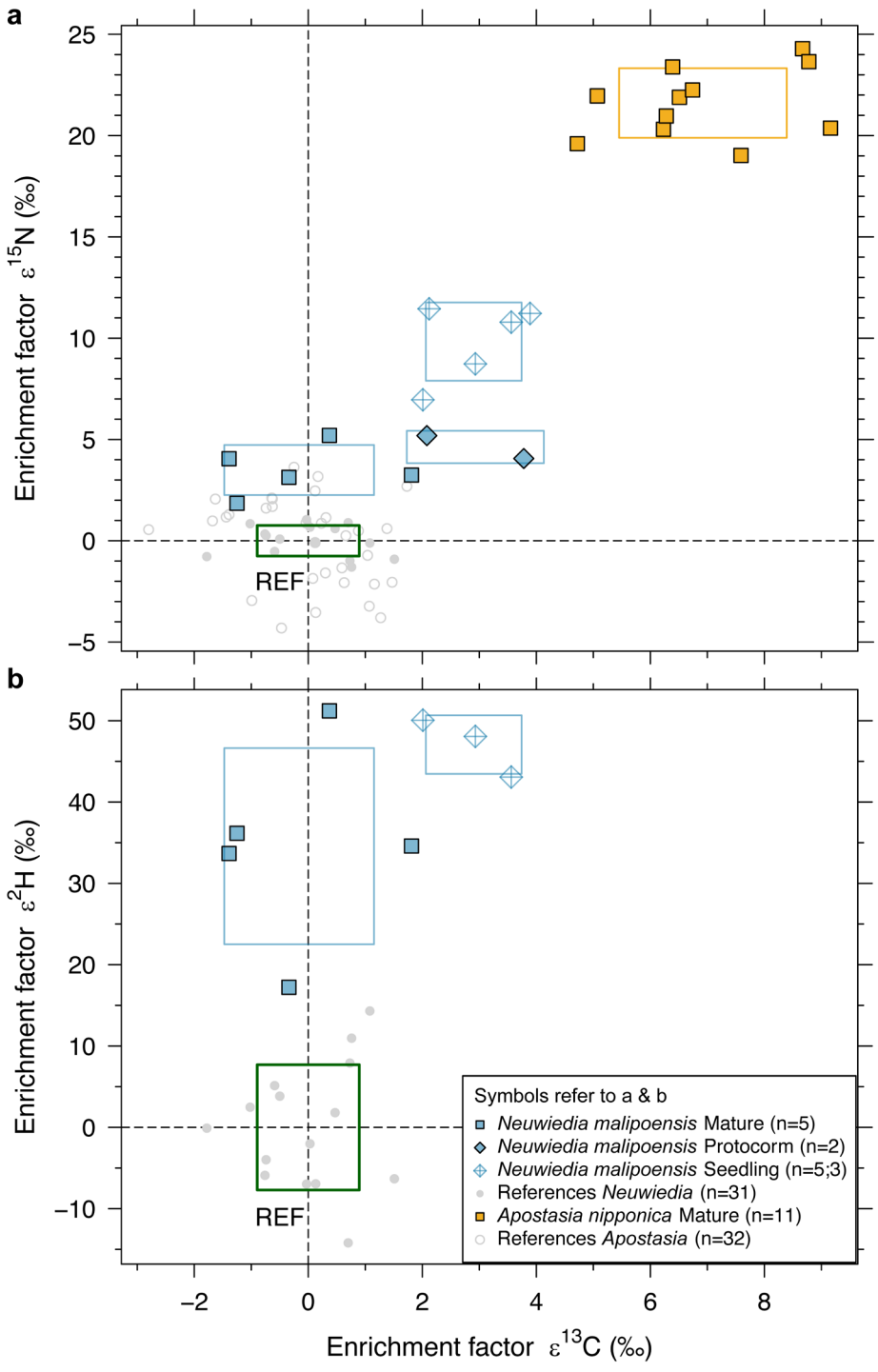

Fig. 4 Carbon and nitrogen (a) and carbon and hydrogen (b) stable isotope enrichment factors ε of *Neuwiedia malipoensis* protocorms, seedlings and adults, *Apostasia nipponica* and of photosynthetic reference plants. Data on *Apostasia nipponica* extracted from Suetsugu and Matsubayashi (2021). Frames represent the standard deviation from the mean enrichment factors ε of each group. Symbols denote single plant individuals. The green frame represents the standard deviation of autotrophic reference plants around a mean enrichment factor of zero, by definition.

DISCUSSION

Shifts in fungal associates and nutritional modes during ontogenesis of *N. malipoensis*

Assessing three different ontogenetic stages of *N. malipoensis*, our high-throughput sequencing data indicated a progressive switch of fungal partners from Ceratobasidiaceae at the protocorm stage to Tulasnellaceae at the adult stage (Fig. 2). Sampling *N. veratrifolia* at three different locations in Sabah, Borneo, Kristiansen et al. (2004) found two and four OTUs from pelotons or fungal isolates belonging to Ceratobasidiaceae and Tulasnellaceae, respectively. Isolated pelotons or fungal cultures represent the natural, symbiotic association between the mycorrhizal fungus and the plant host, but this approach may not capture the full diversity and abundance of different fungal species present within a sample. Increasing evidence suggests that orchid mycorrhizal interactions exhibit significant variability, both spatially (Lin et al., 2020) and temporally (Bidartondo and Read, 2008; Ventre-Lespiaucq et al., 2021). Some orchids consistently form associations with the same fungal partner, while others display large variability of fungal partners either across different locations or over time.

In this study, the ontogenetic shift in mycorrhizal partners coincided with a decrease in C and N supply by fungi during ontogenesis of *N. malipoensis* indicated by decreasing ^{13}C, ^{2}H and ^{15}N enrichment and decreasing total N concentration in orchid tissues.

As the Ceratobasidiaceae mycobiont (OTU1) was dominant at the protocorm stage of *N. malipoensis*, it should have a crucial role in the initial mycoheterotrophic stage of seed germination and protocorm growth. Likewise, the Ceratobasidiaceae mycobiont could facilitate a partially mycoheterotrophic nutrition und nutrient uptake of seedlings required for further development. While the degree of carbon acquisition abilities of mature *N. malipoensis* through a mycorrhizal association with mainly Tulasnellaceae remain inconspicuous based on ^{2}H data, ^{15}N enrichment and total N concentrations of mature *N. malipoensis* relative to reference plants hints towards a partially mycoheterotrophic nutrition.

Shifts in fungal partner across ontogenetic stages have been reported in both chlorophyllous (McCormick et al., 2004; Zahn et al., 2022) and achlorophyllous orchids (Chen et al., 2019; Li et al., 2022; Xu and Guo, 2000). Although shifts in fungal partners

seem riskier than associating with the same partner during development, mycorrhizal shifts could be beneficial in terms of resource acquisition, such as the utilization of a more stable and/or higher amount of nutrients (Ogura-Tsujita et al., 2021; Taylor and Bruns, 1997). Partial replacement of mycorrhizal partners during ontogenesis may reduce the risk of failing to establish new fungal connections in the transition stage (McCormick et al., 2006), and this strategy is regarded as the most frequent scenario of temporal turnover in orchid-mycorrhizal communities (Ventre-Lespiaucq et al., 2021).

Diversity of mycobionts in earliest-diverging orchid lineage

The dominant fungi associating with *N. malipoensis* in our study were members of the families Ceratobasidiaceae and Tulasnellaceae, both classified under Basidiomycota. Our results strongly agree with the observation of Kristiansen et al. (2004), wherein they similarly identified Ceratobasidiaceae and Tulasnellaceae as the primary fungal associates of *N. veratrifolia*. This implies that the potential mycobionts associated with *Neuwiedia* are probably limited to particular lineages within the families Ceratobasidiaceae and Tulasnellaceae. However, since Kristiansen et al. (2004) based their identification on mitochondrial ribosomal large subunit DNA sequences, incorporating their data into our phylogenetic analysis using ITS sequences is not directly possible. Therefore, the exact relationship between the mycobionts of *N. malipoensis* and *N. veratrifolia* remains uncertain.

Recently, adult *A. nipponica* plants were identified to form associations with the ectomycorrhizal Ceratobasidiaceae species, confirming the evolution of partially mycoheterotrophy with the association of ECM-forming fungi in the early divergent lineage of orchid family (Suetsugu and Matsubayashi, 2021). The association of ECM-forming Ceratobasidiaceae fungi has been also reported in a few other orchids, e.g. adult plants of a photosynthetic orchid, *Platanthera minor* (Yagame et al., 2012), and two fully mycoheterotrophic orchids, *Rhizanthella gardneri* (protocorm samples) (Bougoure et al., 2010) and *Chamaegastrodia sikokiana* (Yagame et al., 2008). Notably, our phylogenetic analyses reveal that *N. malipoensis* differs from *A. nipponica* in its fungal association. The fungal sequence of OTU1 in *N. malipoensis* belongs to the saprotrophic clade of Ceratobasidiaceae, distinctly separate from the ECM-forming clade detected in the aforementioned orchids (Fig. 3a). It is noticeable that Apostasioid species recruit both saprotrophic and ECM-forming Ceratobasidiaceae clades as the fungal partners. Our

results imply that multiple Ceratobasidiaceae lineages evolved in the mycorrhizal association with the early divergent subfamily Apostasioideae. Furthermore, the ECM-forming ability of Tulasnellaceae has been documented in the mycorrhizal association with the achlorophyllous liverwort, *Cryptothallus mirabilis* (Bidartondo et al., 2003). The fungal sequence of OTU2 in *N. malipoensis,* mainly detected in mature plant roots, clustered in a clade of Tulasnellaceae sequences obtained from *Cypripedium* species, clearly distinguished from the ECM-forming clade identified in the mentioned achlorophyllous liverwort (Fig. 3b).

Accordingly, mycorrhizal partners isolated from all development stages of *N. malipoensis* belong to the polyphyletic Rhizoctonia group. This is further supported by the detected stable isotope patterns of all three developmental stages of *N. malipoensis*, which is typical of Rhizoctonia-associated green orchids (Gebauer and Schweiger, 2021, Fig. 4). In the present study, we identified not ECM-forming/ Rhizoctonia mycorrhizal fungi as potential mycobionts throughout three different stages of ontogenesis up to maturity of *Neuwiedia,* and therefore extend our knowledge on mycorrhizal associations in the Apostasioideae. Unlike *A. nipponica*, which has the ability to obtain most of its carbon demand during the adult stage by exploiting assured ectomycorrhizal networks (Suetsugu and Matsubayashi, 2021), *N. malipoensis* seems to have less specialized in annidation within ECM-network but rather in mycorrhiza with ubiquitous Rhizoctonia potentially allowing a broader habitat range. Association with the Rhizoctonia fungi, like found for *N. malipoensis*, enables the successful settlement of the Orchidaceae in an extremely wide range of habitats worldwide. In contrast, *A. nipponica* limits its distribution range to forests with ECM networks that certainly allow a higher carbon supply from fungi and even cover the carbon demand of achlorophyllous orchids (Bidartondo, 2005).

It is noted that species of *Apostasia* and *Neuwiedia* occupy different ecological niches (Kolanowska et al. 2016). *Apostasia* species grows in regions with low precipitation values recorded in the driest month, while *Neuwiedia* representatives prefer rainfall of about 100-130mm at this time of year. The diversity in mycorrhizal fungal partners within the Apostasioideae, implying ECM and non-ECM/ Rhizoctonia mycorrhizal partners, enables variation in resource acquisition and therefore opens up different adaptions to environmental conditions and habitat options already in this early diverging orchid

group. While found in other Orchidaceae linages (Ogura-Tsujita et al., 2021; Wang et al., 2021), according to present knowledge no mycorrhizal interaction with non-Rhizoctonia saprotrophic fungi (e.g. *Mycenaceae* and *Psathyrellaceae*) or Ascomycota (Schiebold et al., 2017) appears in the earliest-diverging Apostasioideae. Despite the absence of fully mycoheterotrophic, achlorophyllous species in the subfamily Apostasioideae, diverse mycorrhizal fungal partners with multiple lifestyles enable a partially mycoheterotrophic nutrition of orchid species within both genera of Apostasioideae.

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AUTHOR CONTRIBUTIONS

YIL and GG had the idea for the research project. FEZ analyzed the isotope abundance data. HJ carried out the sampling. YIL carried out the molecular and phylogenetic analyses. FEZ, YIL and GG wrote the manuscript. FEZ and HJ contributed equally to this study. All co-authors approved the final version.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest to declare.

DATA AVAILABILITY

Isotope data are available in the Supporting Information. For the metagenome data of this study, the accession on NCBI Sequence Read Archive is: PRJNA982444.

SUPPORTING INFORMATION

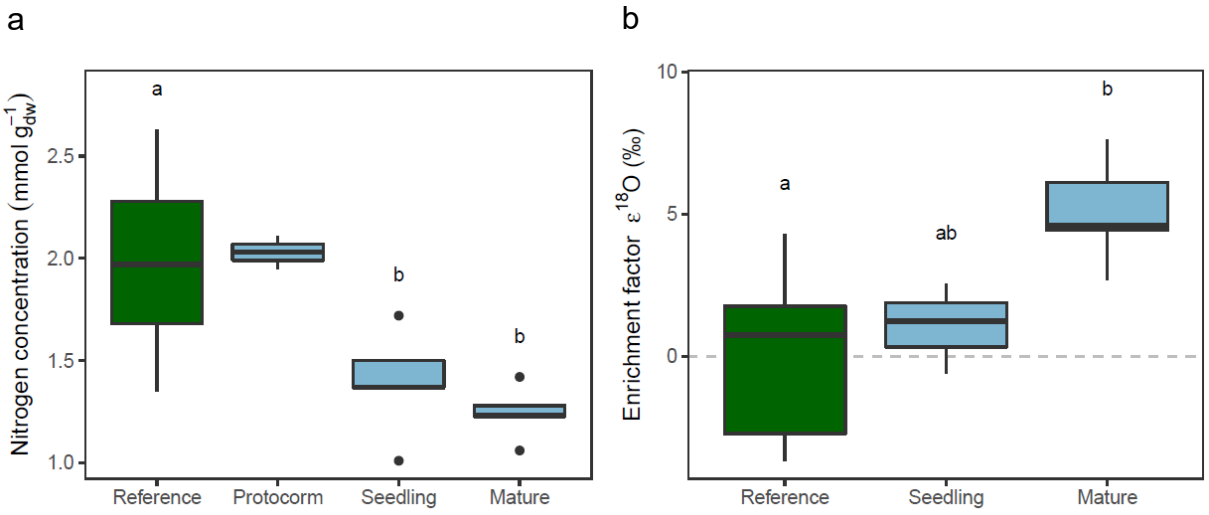

Fig. S1 Nitrogen concentration (a) and enrichment factor $\varepsilon^{18}O$ (b) for *Neuwiedia malipoensis* protocorms (n = 2; 0), seedlings (n = 5; 3), adults (n = 5) and reference plants (n = 15). The box spans the first and third quartile, while the horizontal line in the box represents the median; whiskers extend to 1.5 * interquartile range. Different letters indicate statistically significant differences (ANOVA with *post-hoc* Tukey HSD, cf. Table S3) between groups.

Table S1 Summary of fungal operational taxonomic units (OTUs) and their frequencies detected protocorms and roots using the Illumina Miseq platform.

OTU ID	Closest GenBank Match			BLAST Identity	Protocorm			Seedling			Adult			
	Accession	Length (bp)	Match (%)		P1	P2	P3	S1	S2	S3	A1	A2	A3	Total
Otu1	Ceratobasidiaceae	MN684576	199	99	10362	52397	43436	3980	16860	4689	2070	2952	3665	140411
Otu2	Tulasnellaceae	GU166424	197	99	45	100	4158	16721	48145	15493	20325	22310	28293	155590
Otu4	*Ilyonectria* sp.	MK139849	200	100	1410	4	1	0	0	2	0	0	0	1417
Otu5	Mortierella	GQ302682	199	99	1	0	2	0	0	546	0	0	0	549
Otu6	Uncultured Calcarisporiella	KP235796	102	93	493	0	0	0	0	0	0	0	0	493
Otu8	*Leotiomyceta* sp.	JQ272351	192	96	311	1	0	0	0	0	0	0	0	312
Otu9	*Pyrenochaetopsis* sp.	MT453283	200	100	323	1	0	0	0	0	0	0	0	324
Otu10	Sebacinales	HE814123	197	99	412	0	0	0	0	1	0	0	0	413
Otu12	*Beltrania* sp.	KR093912	200	100	440	2	1	0	0	0	0	0	0	443
Otu14	Uncultured Volutella	MG707568	200	100	262	0	0	0	0	0	0	0	0	262
Otu15	*Mucoromycotina* sp.	MK429879	186	92	219	0	0	0	0	0	0	0	0	219
Otu23	*Rhamphoriopsis* sp.	OP377812	189	92	154	0	0	0	0	0	0	0	0	154
Otu28	*Periconia* sp.	MT446212	200	100	130	0	0	0	0	0	0	0	0	130
Otu31	Uncultured Trechisporales	KF574243	200	100	102	0	0	0	0	0	0	0	0	102
					14664	52505	47598	20701	65005	20731	22395	25262	31958	300819

Table S2 Mean and single $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ and total nitrogen concentration data of Neuwiedia malipoensis adults, protocorms and seedlings sampled at Malipo County, Yunnan Province, China in 2016.

Sample	Plot	Total N [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\delta^{2}H$ [‰]	$\delta^{18}O$ [‰]
***Neuwiedia malipoensis* Z.J.Liu, L.J.Chen & K.Wei Liu (Orchidaceae)**						
Neuwiedia malipoensis - Mature 1	1	1.42	6.08	-33.97	-71.33	22.35
Neuwiedia malipoensis - Mature 2	2	1.28	4.31	-31.73	-71.10	19.79
Neuwiedia malipoensis - Mature 3	3	1.23	5.44	-33.77	-74.69	21.29
Neuwiedia malipoensis - Mature 4	4	1.23	3.03	-34.11	-62.10	21.14
Neuwiedia malipoensis - Mature 5	5	1.06	5.36	-32.92	-43.90	23.50
Neuwiedia malipoensis - Seedlings 1	1	1.01	8.99	-30.57	-54.94	17.48
Neuwiedia malipoensis - Seedlings 2	2	1.37	12.29	-29.65	NA	NA
Neuwiedia malipoensis - Seedlings 3	3	1.37	13.10	-29.87	-48.84	18.02
Neuwiedia malipoensis - Seedlings 4	4	1.50	12.63	-30.74	NA	NA
Neuwiedia malipoensis - Seedlings 5	5	1.72	8.89	-30.36	-47.04	18.42
Neuwiedia malipoensis- Protocorm 1	1	2.11	6.09	-28.80	NA	NA
Neuwiedia malipoensis- Protocorm 2	2	1.95	6.26	-31.46	NA	NA
***Elatostema multicanaliculatum* B.L.Shih & Yuen P.Yang (Urticaceae)**						
Elatostema multicanaliculatum 1	1	2.21	3.07	-32.61	-111.97	12.56
Elatostema multicanaliculatum 2	2	1.68	1.67	-33.07	-103.88	12.63
Elatostema multicanaliculatum 3	3	2.35	3.15	-34.45	-89.43	15.46
Elatostema multicanaliculatum 4	4	2.02	1.51	-33.62	-104.16	13.86
Elatostema multicanaliculatum 5	5	2.41	-0.62	-35.07	-95.21	12.37
***Dendrocalamus latiflorus* Munro (Poaceae)**						
Dendrocalamus latiflorus 1	1	2.63	2.28	-33.32	-108.98	19.17
Dendrocalamus latiflorus 2	2	1.35	1.00	-33.41	-112.63	16.82
Dendrocalamus latiflorus 3	3	1.68	2.39	-33.93	-88.08	22.91
Dendrocalamus latiflorus 4	4	1.87	1.85	-32.83	-100.28	18.77
Dendrocalamus latiflorus 5	5	1.63	1.05	-32.59	-109.31	17.49
***Selaginella* sp. (Selaginellaceae)**						
Selaginella sp. 1	1	1.86	0.73	-31.82	-94.04	16.98
Selaginella sp. 2	2	1.65	0.54	-34.13	-100.56	16.65
Selaginella sp. 3	3	1.97	1.40	-31.92	-98.23	17.48
Selaginella sp. 4	4	2.16	0.19	-32.13	-90.35	17.05
Selaginella sp. 5	5	2.37	0.05	-32.21	-80.80	17.80
Mean values						
Neuwiedia malipoensis - Mature		**1.25**	**4.84**	**-33.30**	**-64.62**	**21.61**
s.d.		*0.13*	*1.20*	*0.99*	*12.49*	*1.39*
Neuwiedia malipoensis - Seedlings		**1.39**	**11.18**	**-30.24**	**-50.27**	**17.97**
s.d.		*0.26*	*2.07*	*0.46*	*4.14*	*0.47*
Neuwiedia malipoensis- Proctocorm		**2.03**	**6.17**	**-30.13**	**NA**	**NA**
Reference plants		**1.99**	**1.35**	**-33.14**	**-99.19**	**16.53**
s.d.		*0.36*	*1.09*	*0.97*	*9.53*	*2.85*

Table S2 continued

Sample	Plot	Mean ref $\delta^{15}N$ per plot	$\varepsilon^{15}N$ [‰]	Mean $\varepsilon^{15}N$ [‰] *s.d*	Mean ref $\delta^{13}C$ per plot	$\varepsilon^{13}C$ [‰]	Mean $\varepsilon^{13}C$ [‰] *s.d*	Mean ref $\delta^{2}H$ per plot	$\varepsilon^{2}H$ [‰]	Mean $\varepsilon^{2}H$ [‰] *s.d*	Mean ref $\delta^{18}O$ per plot	$\varepsilon^{18}O$ [‰]	Mean $\varepsilon^{18}O$ [‰] *s.d*
***Neuwiedia malipoensis* Z.J.Liu, L.J.Chen & K.Wei Liu (Orchidaceae)**													
Neuwiedia malipoensis - Mature 1	1		4.05	**3.49**		-1.38	**-0.16**		33.67	**34.57**		6.11	**5.08**
Neuwiedia malipoensis - Mature 2	2		3.25	*1.24*		1.81	*1.31*		34.59	*12.05*		4.42	*1.87*
Neuwiedia malipoensis - Mature 3	3		3.12			-0.33			17.22			2.67	
Neuwiedia malipoensis - Mature 4	4		1.84			-1.25			36.16			4.58	
Neuwiedia malipoensis - Mature 5	5		5.20			0.37			51.21			7.61	
Neuwiedia malipoensis - Seedlings 1	1		6.97	**9.83**		2.01	**2.91**		50.06	**47.07**		1.24	**1.05**
Neuwiedia malipoensis - Seedlings 2	2		11.22	*1.93*		3.89	*0.84*		NA	*3.60*		NA	*1.57*
Neuwiedia malipoensis - Seedlings 3	3		10.79			3.57			43.07			-0.60	
Neuwiedia malipoensis - Seedlings 4	4		11.45			2.12			NA			NA	
Neuwiedia malipoensis - Seedlings 5	5		8.73			2.94			48.07			2.53	
Neuwiedia malipoensis- Protocorm 1	1		4.06	**4.63**		3.78	**2.93**		NA			NA	
Neuwiedia malipoensis- Protocorm 2	2		5.20	0.80		2.08	1.20		NA			NA	
***Elatostema multicanaliculatum* B.L.Shih & Yuen P.Yang (Urticaceae)**													
Elatostema multicanaliculatum 1	1		1.04	0.41		-0.03	-0.63		-6.98	-1.74		-3.68	-3.16
Elatostema multicanaliculatum 2	2		0.60	*0.71*		0.47	*0.87*		1.81	*4.41*		-2.74	*0.44*
Elatostema multicanaliculatum 3	3		0.84			-1.02			2.48			-3.16	
Elatostema multicanaliculatum 4	4		0.33			-0.76			-5.90			-2.70	
Elatostema multicanaliculatum 5	5		-0.78			-1.78			-0.10			-3.52	
***Dendrocalamus latiflorus* Munro (Poaceae)**													
Dendrocalamus latiflorus 1	1		0.25	0.36		-0.74	-0.08		-3.98	-4.66		2.94	2.50
Dendrocalamus latiflorus 2	2		-0.07	*0.40*		0.12	*0.56*		-6.94	*6.63*		1.45	*1.16*
Dendrocalamus latiflorus 3	3		0.08			-0.50			3.84			4.29	
Dendrocalamus latiflorus 4	4		0.67			0.03			-2.02			2.21	
Dendrocalamus latiflorus 5	5		0.89			0.70			-14.21			1.60	
***Selaginella* sp. (Selaginellaceae)**													
Selaginella sp. 1	1	2.03	-1.29	-0.77	-32.58	0.77	0.70	-105.00	10.96	6.40	16.24	0.74	0.66
Selaginella sp. 2	2	1.07	-0.53	*0.46*	-33.54	-0.59	*0.79*	-105.69	5.13	*7.89*	15.37	1.29	*1.14*
Selaginella sp. 3	3	2.31	-0.92		-33.43	1.52		-91.91	-6.31		18.62	-1.14	
Selaginella sp. 4	4	1.18	-1.00		-32.86	0.73		-98.26	7.91		16.56	0.49	
Selaginella sp. 5	5	0.16	-0.11		-33.29	1.09		-95.11	14.31		15.89	1.92	

Table S3 ANOVA with post-hoc Tukey HSD and Kruskal-Wallis H-tests with *post-hoc* Mann Whitney U-test of leaf isotope signatures and total N concentrations across sample types (references, mature, seedling), Note: protocorm samples not included in statistical tests due to n=2.

ANOVA			
	F	**df**	***p***
$\varepsilon^{13}C$	18.25	2	**<0.001**
$\varepsilon^{2}H$	57.94	2	**<0.001**
$\varepsilon^{18}O$	8.54	2	**0.002**
Total N conc.	14.09	2	**<0.001**

Kruskal-Wallis *H*-test			
	chi-squared	**df**	***p***
$\varepsilon^{15}N$	18.46	2	**<0.001**

TukeyHSD				
	diff	**lwr**	**upr**	***p* adj**
$\varepsilon^{13}C$				
seedling-reference	2.90	1.60	4.20	**<0.001**
target-reference	-0.16	-1.40	1.10	0.950
target-seedling	-3.06	-4.60	-1.50	**<0.001**
$\varepsilon^{2}H$				
seedling-reference	47.00	33.00	60.60	**<0.001**
target-reference	35.00	23.00	45.60	**<0.001**
target-seedling	-12.00	-28.00	3.20	0.130
$\varepsilon^{18}O$				
seedling-reference	1.10	-2.75	4.90	0.765
target-reference	5.10	1.97	8.20	**0.001**
target-seedling	4.00	-0.38	8.40	0.077
Total N conc.				
seedling-reference	-0.60	-1.00	-0.19	**0.004**
target-reference	-0.75	-1.15	-0.34	**<0.001**
target-seedling	-0.15	-0.65	0.35	0.732

Mann Whitney *U*-test (correction method = Holm)			
	U	***p* adj**	**effect size**
$\varepsilon^{15}N$			
seedling-reference	0	**<0.001**	0.7
target-reference	0	**<0.001**	0.7
target-seedling	25	0.008	0.8

MANUSCRIPT 3

Variation in mycorrhizal communities and the level of mycoheterotrophy in grassland and forest populations of *Neottia ovata* (Orchidaceae)

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ABSTRACT

1. Orchid mycorrhiza forms unique symbiotic associations between members of the Orchidaceae and multiple ecological guilds of fungi. Because orchids associate with a wide variety of fungi with different ecological functions, they represent an ideal study system to address fundamental questions about the evolution and ecophysiology of mycorrhizal symbiosis. Although it is well established that shifts in mycorrhizal associations are linked to transitions in plant trophic mode, it remains unclear what ecological drivers promote these evolutionary changes.
2. Here, we investigated mycorrhizal communities and isotope signatures across six populations of the terrestrial orchid *Neottia ovata* growing under contrasting light conditions in temperate Europe. We hypothesized that plants growing in forests would associate with different mycorrhizal fungi than plants occurring in grasslands and that the limited light availability in forests leads to a higher contribution of fungi to the carbon budget of orchids.
3. Our results showed that *N. ovata* predominantly associated with rhizoctonia fungi of the family Serendipitaceae in both habitats, but plants in forests also recruited ectomycorrhizal fungi. Root communities highly resembled soil communities and variation in root communities was significantly related to habitat type and edaphic factors. In contrast, isotope signatures (^{13}C, ^{15}N, ^{2}H and ^{18}O) and N concentration showed no significant relationship with habitat type. In addition, both ^{13}C and ^{2}H were not significantly correlated to habitat's light availability.
4. Although it has been suggested that the presence of a wide variety of ectomycorrhizal fungi in root communities of orchids can serve as a precursor for evolutionary shifts to partial mycoheterotrophy (mixotrophy), the presence or absence of ectomycorrhizal fungi did not substantially influence the isotope signatures of *N. ovata*. These results indicate that rhizoctonia fungi played the major functional role in C and nutrient supply and that ectomycorrhizal fungi did not substantially contribute to the carbon budget of the plants.

INTRODUCTION

Orchid mycorrhiza is exclusively formed in the highly diversified plant family Orchidaceae and represents a unique study system to address fundamental questions about the evolutionary trajectory of plant trophic mode and mycorrhizal associations (van der Heijden *et al.* 2015; Rasmussen *et al.* 2015; Jacquemyn & Merckx 2019). All orchid species are mycoheterotrophic at the early stages of development when their minute seeds and achlorophyllous protocorms have an obligate demand for fungal-derived C (Rasmussen 1995; Merckx 2013; Dearnaley *et al.* 2016). Although most adult orchids are assumed to become autotrophic (being capable of performing photosynthesis), fully or partially mycoheterotrophic orchids continue to depend on fungal-derived C throughout their entire life cycle or in combination with photosynthesis, respectively (Merckx 2013; Selosse & Roy 2009; Těšitel *et al.* 2018). Partially mycoheterotrophic orchids usually show intermediate enrichment in stable isotopes (^{13}C, ^{15}N, and ^{2}H) between autotrophic and fully mycoheterotrophic species (Gebauer & Meyer 2003; Hynson *et al.* 2013; Gebauer *et al.* 2016; Jacquemyn *et al.* 2021). Recent measurements of ^{2}H in combination with the frequently used ^{13}C and ^{15}N stable isotope abundance have suggested that partial mycoheterotrophy may be much more widespread among orchids than previously acknowledged (Gebauer *et al.* 2016; Schiebold *et al.* 2018; Gebauer & Schweiger 2021). Mycoheterotrophy has been hypothesized to be an adaptation to survival in low-light habitats (Selosse & Roy 2009; Merckx 2013; Gomes *et al.* 2019b) and the degree of mycoheterotrophy can be variable and depend on the light availability of different habitats (Bidartondo *et al.* 2004; Preiss *et al.* 2010; Schweiger *et al.* 2019). Mycoheterotrophic orchids often rely on different mycorrhizal fungi compared to their autotrophic relatives, suggesting that a shift from autotrophy to mycoheterotrophy is accompanied by a shift in fungal symbionts (Jacquemyn & Merckx 2019; Wang *et al.* 2021; Selosse *et al.* 2022). However, we still don't know the ecological drivers behind these different fungal associations.

The evolutionary transition in trophic mode of orchids from autotrophy, via partial to full mycoheterotrophy, is accompanied by shifts in mycorrhizal partners, from associations with typical rhizoctonia fungi (members of three families Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae; Dearnaley *et al.* 2012) to non-rhizoctonia ectomycorrhizal fungi or wood/litter saprobes in the phyla Basidiomycota and

Ascomycota (Bidartondo *et al.* 2004; Selosse & Roy 2009; Motomura *et al.* 2010; Ogura-Tsujita *et al.* 2012, 2021; Yagame *et al.* 2016; Wang *et al.* 2021; Selosse *et al.* 2022). For instance, in the tribe Neottieae, one of the scarce lineages comprising a broad range of putatively autotrophic, partially and fully mycoheterotrophic species (Feng *et al.* 2016; Zhou & Jin 2018; Lallemand *et al.* 2019), mycorrhizal associations switch from rhizoctonia fungi in putatively autotrophic species to ectomycorrhizal fungi in fully mycoheterotrophic species (e.g. Sebacinaceae, Russulaceae, Tuberaceae, Pyronemataceae) (Selosse *et al.* 2002; Bidartondo *et al.* 2004; Girlanda *et al.* 2005; Roy *et al.* 2009; Těšitelová *et al.* 2012; Suetsugu *et al.* 2017). Yet, the environmental conditions under which this increase in mycorrhizal partner breadth occurs, remain unclear.

Neottia ovata is a chlorophyllous terrestrial orchid species that is sister to partially and fully mycoheterotrophic species in the genus *Neottia* (Feng *et al.* 2016; Zhou & Jin 2018; Lallemand *et al.* 2019). It therefore represents a good study species to investigate variation in symbiotic associations and the level of heterotrophy and how they are linked to environmental conditions. First, within the genus *Neottia*, symbiotic switches most likely occurred from rhizoctonia Serendipitaceae fungi to ectomycorrhizal Sebacinaceae fungi towards the evolutionary end point of full mycoheterotrophy (characterized by the fully mycoheterotrophic species *N. nidus-avis*) (Bidartondo *et al.* 2004; Jacquemyn *et al.* 2015, 2021; McKendrick *et al.* 2002; Oja *et al.* 2015; Selosse *et al.* 2002; Těšitelová *et al.* 2015; Yagame *et al.* 2016), showing that mycorrhizal shifts can occur within the fungal order Sebacinales (Weiß *et al.* 2016). Second, despite its inconsistent enrichment in ^{13}C across different habitats (Těšitelová *et al.* 2015; Gebauer *et al.* 2016; Schweiger *et al.* 2019), *N. ovata* has been reported to be significantly enriched in ^{2}H (Gebauer *et al.* 2016; Schweiger *et al.* 2019), possibly indicating a C gain from mycorrhizal fungi. Third, several studies using molecular identification techniques have shown that *N. ovata* mainly associated with rhizoctonia fungi of the family Serendipitaceae, while occasional associations with ectomycorrhizal fungi were observed as well in forest habitats (Oja *et al.* 2015; Těšitelová *et al.* 2015; Jacquemyn *et al.* 2015; Vogt-Schilb *et al.* 2020). These results suggest that *N. ovata* may have high plasticity in fungal associations and show increased levels of mycoheterotrophy in particular habitats (Wang *et al.* 2021; Selosse *et al.* 2022). This plasticity of associating with multiple fungal species of different functional groups potentially places *N. ovata* at the early stages of the evolutionary shift from autotrophy to mycoheterotrophy which repeatedly occurred within *Neottia* (Fig. 1).

A more detailed and simultaneous investigation of mycorrhizal communities and ecophysiology over contrasting habitats may therefore shed light on the ecological factors that influence these variables.

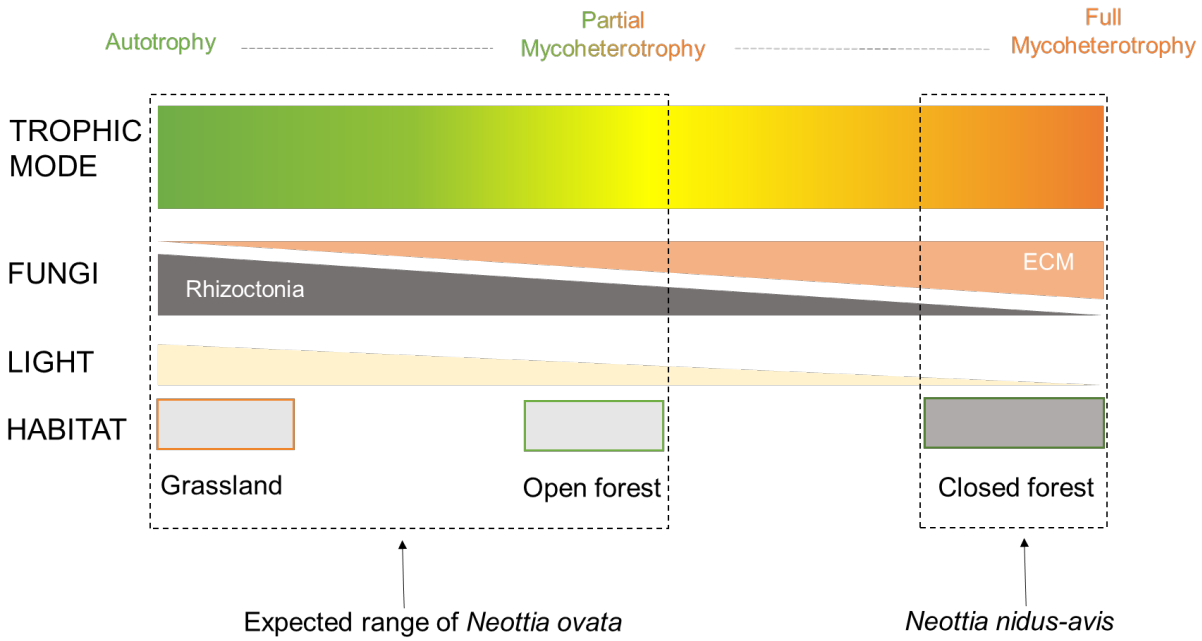

Fig. 1 Expected spectra of trophic mode, fungal associations and light availability for *Neottia ovata*. Compared with the fully mycoheterotrohic *N. nidus-avis* (Jersáková *et al.* 2022) that exclusively associates with ectomycorrhizal fungi (ECM), *N. ovata* (Kotilínek *et al.* 2015) is mainly associates with rhizoctonia fungi and hypothesized to be at an early stage of partial mycoheterotrophy (also broadly termed 'mixotrophy', Selosse & Roy 2009). The level of partial mycoheterotrophy is a continuum between autotrophy and full mycoheterotrophy and hypothesized to relate to habitat's light availability.

In this study, we examined whether mycorrhizal communities of *N. ovata* in specific microhabitats and whether variation in fungal community composition relates to changes in the degree of mycoheterotrophy. Mycorrhizal communities of *N. ovata* were investigated using high-throughput sequencing across six sites ranging from open grassland and shady forests in Europe. The full set of ^{13}C, ^{15}N, ^{2}H and ^{18}O stable isotope signatures was measured, as well as microhabitat conditions (light intensity and edaphic factors). Specifically, we hypothesized that orchid root mycorrhizal fungal communities (hereafter 'root communities') differ between forest and grassland populations (#H1). We further expected that the composition of root communities is determined by fungal availability in the soil and influenced by edaphic factors (#H2). Lastly, we expected that the level of mycoheterotrophy (represented by ^{13}C and ^{2}H signatures) is significantly related to light availability (#H3).

MATERIALS AND METHODS

Study species and sampling procedures

A total of 30 individuals of *N. ovata* were sampled at six sites (forest sites: BE, VP and VS; grassland sites: KA, ZI and KL) in North-East Bavaria (Germany) and the Netherlands in June 2021 (Table 1).

Table 1 Overview of all sampling sites of *Neottia ovata* used in this study.

Country	Site	Site ID	Habitat description	Collection date
Germany	Betzenstein (49.40°N/11.23°E)	BE	Mixed beech forest (*Fagus sylvatica*)	June 10, 2021
Germany	Velden (plain site) (49.37°N/11.34°E)	VP	Mixed pine forest (*Pinus sylvestris*) with spruce (*Picea abies*) and beech (*Fagus sylvatica*)	June 9, 2021
Germany	Velden (slope site) (49.40°N/11.23°E)	VS	Monodominant pine forest (*Pinus sylvestris*)	June 9, 2021
Germany	Kalkberg (50.04°N/11.14°E)	KA	Dry calcareous grassland	June 7, 2021
Germany	Zips (49.37°N/11.34°E	ZI	Wet grassland	June 10, 2021
The Netherlands	Klip (52.15°N/4.38°E)	KL	Humid dune slack	June 8, 2021

Within each site, five 1 m^2 plots containing *N. ovata* were randomly selected as soon as the vegetative parts of *N. ovata* could be identified. Within each plot, a light sensor (silicon photodiode BPW 21, Infineon, Germany) was installed beside the targeted orchid individual at a height of approximately 15 cm to record the irradiance level till sample collection (2 weeks to 1 month later) using a data logger (HOBO H8, ONSET, USA). Following the calibration procedure (Preiss *et al.* 2010), the light intensity received by each orchid individual during the measuring period was calculated (Appendix S1). We collected leaf, root and soil samples within each plot when the targeted orchid individual was at a budding or early flowering stage. One or two pieces of leaf of *N. ovata* ($n = 30$) and autotrophic reference plants ($n = 90$) were collected for stable isotope measurements. Reference plants were always growing in close spatial proximity and therefore under identical light conditions as the respective orchid individuals. For each

orchid individual, at least five root pieces (3 ~ 5 cm) were randomly chosen for DNA extraction and microscopic observation of fungal pelotons. Within each plot, five topsoil cores were randomly taken with a 2.5-cm-diameter soil auger to a depth of 5 cm below the litter layer and mixed as one homogenized bulk-soil sample for soil nutrient analysis. All soil samples were preserved in a cooling box immediately after collection and stored in a fridge (4 °C) in the laboratory until further processing.

Fungal barcoding

Root pieces were surface sterilized using 1% sodium hypochlorite and rinsed in sterile distilled water. Total DNA from five root sections (1 mm thickness) was extracted using the CTAB method (Doyle 1987). DNA from bulk soil (0.25 g) was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN). The forward primer fITS7 (5'-GTGARTCATCGAATCTTTG-3'; Ihrmark *et al.* 2012) and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.* 1990) were used to amplify the fungal nuclear ITS2 region for both root and soil samples. According to our pilot study (Appendix S1; Table S1), the primer pair fITS7 and ITS4 amplified a wider range of orchid mycorrhizal fungi than the primer pair ITS3 and ITS4OF specifically targeting orchid mycorrhizal fungi (Taylor & McCormick 2008). Fungi from the family Tulasnellaceae forming typical orchid mycorrhizal fungi were not captured by both sets of primers in our pilot study (Table S1). Since this is potentially due to the high variability of the ITS2 region of Tulasnellaceae (Waud *et al.* 2014; Oja *et al.* 2015; Li *et al.* 2021), we designed new forward primers to detect Tulasnellaceae fungi: Tul1F (5'-CGTYGGATCCCTYGGC-3') and Tul2F (5'-TGGATCCCTTGGCACGTC-3') positioning at the 5.8S region to match with reverse primer ITS4Tul2 (5'-TTCTTTTCCTCCGCTGAWTA-3'; Oja *et al.* 2015) based on sequence alignment (Appendix S1; Table S2). Thus, each sample was amplified using the fungal general primer pair and the newly designed primer pair. PCR reactions were duplicated for each DNA template to reduce PCR stochasticity and to increase the diversity of detected fungi (Alberdi *et al.* 2018). After checking the amplification products by gel electrophoresis, duplicates of amplicons were pooled after the first PCR step and attached with identical Nextera indexes (Illumina) in the second PCR according to the manufacturer's guidelines. The end pool was sequenced to generate 300-bp paired-end reads using the Illumina MiSeq platform (BaseClear, Leiden, the Netherlands).

After demultiplexing, paired reads of each sample were merged and low-quality sequences (error rate > 0.5) were filtered out by VSEARCH (Rognes *et al.* 2016). Merged sequences were separated by primer pair and subsequently trimmed from primers using CUTADAPT 1.0 (Martin 2011). Chimeric sequences were trimmed by the UCHIME chimera detection program (de novo algorithm) (Edgar *et al.* 2011). After quality filtering and chimera removal, fungal OTUs were clustered based on a 97% similarity threshold using VSEARCH. Global singletons were removed because they may reduce the accuracy of diversity estimates (Ihrmark *et al.* 2012; Waud *et al.* 2014). The remaining OTUs were assigned with taxonomic identities to the best match by USEARCH (Edgar 2010) using the Unite reference dataset (utax_reference_dataset_10.05.2021.fasta, https://unite.ut.ee/repository.php) as annotation resources. Finally, OTUs were manually screened for possible orchid mycorrhizal fungal families summarized in the literature (Dearnaley *et al.* 2012; Wang *et al.* 2021) and reported in previous studies on *N. ovata* (Oja *et al.* 2015; Těšitelová *et al.* 2015; Jacquemyn *et al.* 2015). The quality-filtered sequencing data generated a total of 3,342 OTUs (1,514,336 sequences) for 30 root and 60 soil samples, of which 543 OTUs (693,154 sequences – 45.8% of all filtered sequences) were assigned to putative orchid mycorrhizal fungi belonging to fifteen fungal families (Table S3; Appendix 1). Because the specific Tulasnellaceae primer pair did not yield considerably more Tulasnellaceae OTUs than the general primer pair, only orchid mycorrhizal fungi captured by the general primer were retained for further analyses.

Stable isotope signatures

The leaf material of all sampled plants was oven-dried at 105°C, ground to fine powder in a ball mill (Retsch Schwingmühle MM2, Haan, Germany), and stored in a desiccator before measuring stable isotope abundances of C, N, H, O and total N concentrations. All measurements of stable isotope signatures were conducted in the BayCEER Keylab of Isotope Biogeochemistry. Relative natural abundance analysis of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes as well as N concentrations were determined simultaneously using an EA-IRMS coupling combining an elemental analyser (EA IsoLink CN, Thermo Fisher Scientific, Bremen, Germany) with a continuous flow isotope ratio mass spectrometer (delta V advantage, Thermo Fisher Scientific). Relative natural abundances of hydrogen ($^{2}H/^{1}H$) and oxygen isotopes ($^{18}O/^{16}O$) were measured separately using a TC-IRMS coupling, which links a thermal conversion through pyrolysis unit (HTO,

HEKAtech, Wegberg, Germany) to a continuous flow isotope ratio mass spectrometer (delta V advantage, Thermo Fisher Scientific). Device coupling was always via a ConFlo IV open-split interface (Thermo Fisher Scientific). For H isotope abundance, each sample was measured four times in a row with the first three measures being neglected and we analysed target orchid samples and respective reference plant samples together in identical sample batches (Gebauer *et al.* 2016). The oxygen isotope abundances were measured to assess a potential transpiration effect on the relative enrichment in ^{13}C and ^{2}H caused by differences in stomata regulation and transpiration between orchids and the non-orchid reference plants. Transpiration affects C, H and O isotope abundance in plant tissues simultaneously (da Silveira Lobo Sternberg L 1989; Ziegler 1989; Cernusak *et al.* 2004). A transpiration effect can only be excluded if the ^{18}O isotope abundance of the target species is similarly distributed as the ^{18}O isotope signature of the non-orchid reference plants (Gebauer *et al.* 2016).

Measured relative isotope abundances (δ values) were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1{,}000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. To enable comparisons of stable isotope abundances across sampling sites, we used isotope enrichment factors (ε) to normalize δ values using the equation $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ or $\delta^{2}H$ value of an orchid individual and δ_{REF} is the mean value of all autotrophic reference plants within the same plot (Preiss & Gebauer 2008). The δ and ε values and total N concentrations of all orchid samples and their autotrophic references are available in Table S4.

Data analyses

Fungal diversity

Because of the variation in sequencing reads among samples, the abundance of OTUs of all samples was rarefied to the sample with the fewest reads. Unless stated otherwise, the "picante" R package (Kembel *et al.* 2010) was used for all community analyses. To investigate whether alpha diversity (α-diversity) of orchid mycorrhizal fungi differed between sites and habitats, we calculated for each sample OTU richness (SR) and phylogenetic α-diversity, Faith's (1992) PD. The phylogeny of fungal OTUs was reconstructed using the Maximum Likelihood method (see details about phylogenetic

analyses in Appendix S1) and used for the calculation of PD. Analysis of variance (ANOVA) or Kruskal-Wallis rank-sum tests was used to evaluate differences in mean SR and PD of root and soil samples among sites, respectively. If the null hypothesis was rejected, post hoc Tukey HSD or Dunn's-test was performed to analyze pair-wise differences. To test the hypothesis (#H1) that fungal α-diversity of root or soil samples differ between habitats, we compared measures of SR and PD between habitats by fitting linear mixed models with 'Habitat' as fixed effect and 'Site' as random effect using the R package 'lme4' (Bates *et al.* 2015).

Fungal community structure

To assess differences in mycorrhizal community structure between sampling sites and habitat types (β-diversity), we calculated Bray-Curtis (Bray & Curtis 1957) and UniFrac dissimilarity metrics (Lozupone & Knight 2005) based on the presence-absence information of OTUs. The Bray-Curtis index is widely used for assessing community dissimilarity in community ecology (Anderson *et al.* 2006), while the UniFrac distance considers the phylogenetic relatedness of fungal OTUs by measuring the unique fraction of the phylogenetic distances not shared between two samples. Variation in mycorrhizal community structure of root or soil samples among sites was visualized using non-metric multidimensional scaling (NMDS) using the "vegan" R package (Oksanen *et al.* 2019). After checking for homogeneity of variances with the function 'betadisper' from the R package "vegan" (Oksanen *et al.* 2013), we performed a PERMANOVA (Anderson 2001) using 'adonis2' to test whether mycorrhizal communities differed between sites. To test the hypothesis that mycorrhizal communities differed between habitats, a nested PERMANOVA was performed and 'strata = Site' was specified to constrain random permutations between sites. In addition, we ran indicator species analyses on root samples to identify fungal OTUs that were characteristic for a given site using the 'indval' function from the "labdsv" R package (Roberts 2019). Indicator species are species that preferentially occur in a community, and the indicator value of a species reflects species importance in the community (Dufrêne *et al.* 1997).

Effect of soil properties and soil fungi on root communities

To test the hypothesis (#H2) that root fungal communities are determined by the availability of fungal OTUs in the soil, we first related fungal alpha diversity (SR and PD) of soil samples to that of root samples using linear regressions. Second, Mantel tests with

10,000 permutations were used to examine the similarity between the Bray-Curtis metrics of root communities and bulk-soil samples. Furthermore, we calculated Bray-Curtis metrics for both root samples and the corresponding bulk-soil samples and examined the dissimilarity in fungal composition among sample types by PERMANOVA. In addition, to investigate whether particular fungal families are preferentially recruited in orchid roots from soil, the relative sequence abundance (MiSeq reads) of each fungal family was compared between root and soil sample within each site.

To test the effect of edaphic factors on root community composition, we conducted a distance-based redundancy analysis (db-RDA) in "vegan". Explanatory variables tested in the db-RDA model were soil organic content (SOC), extractable N (including nitrate concentration – NO_3^- and ammonium – NH_4^+), extractable P, pH and soil moisture content (see detailed measurements of soil properties in Appendix S1). To test the proportion of variation in root fungal communities explained by edaphic factors after controlling for differences by habitat type (forest vs. grassland), we performed a partial db-RDA model that used habitat type as a covariate. Edaphic factors that best fit the db-RDA model were selected using the 'ordiR2step' function from "vegan". The significance of the selected variables was assessed by the function 'anova.test' in "vegan".

Phylogenetic analyses of Sebacinaceae fungi associated with *Neottia* species

To explore the phylogenetic relationship of Sebacinaceae fungi between *N. ovata* and its fully mycoheterotrophic relatives (*N. nidus-avis*, *N. camtschatea*, and *N. accuminata*), we download the fungal ITS sequences reported in the literature (McKendrick *et al.* 2002; Selosse *et al.* 2002; Tesitelova *et al.* 2015; Chen *et al.* 2019) from NCBI GenBank database. Those downloaded sequences were aligned with Sebacinaceae OTUs of *N. ovata* in this study and used to reconstruct maximum likelihood phylogenetic trees using RAxML in Geneious Prime v. 2019. The three Sebacinaceae sequences (KJ188478; KJ188509; KJ188545) of *N. ovata* reported by Tesitelová *et al.* (2015) were also included for phylogenetic reconstruction, and the Serendipitaceace OTUs detected by this study were used as outgroup sequences.

Orchid stable isotope signatures and correlation with light intensity

For each site, isotope compositions ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{18}O$, $\varepsilon^{2}H$ and total N) of orchid and reference plant leaves were compared by fitting linear mixed models. All models included

'plot' as random effect due to our plot-wise sampling scheme. Differences in isotope compositions of orchid samples between sites were assessed using ANOVA or Kruskal-Wallis rank-sum tests. Linear mixed models were performed to test the effect of habitat on isotope composition using 'Site' as random effect. In addition, linear mixed models using 'Site' as random effect were used to assess the relationship between light intensity and $\varepsilon^{13}C$ and $\varepsilon^{2}H$, individually (#H3).

RESULTS

Mycorrhizal fungal community of *N. ovata* encompasses rhizoctonia symbionts and site-dependent ectomycorrhizal fungi

Within root communities, rhizoctonia fungi of the Serendipitaceae were present in all samples regardless of site (Fig. S1A and S1B), while the presence of ectomycorrhizal fungi was highly site-dependent. Apart from associations with members of the Serendipitaceae, forest root communities comprised several ectomycorrhizal fungi of Sebacinaceae (sites BE, VP and VS), Inocybaceae (sites BE, VP and VS) and Thelephoraceae (sites VP and VS), whereas grassland root communities comprised rhizoctonia fungi of Tulasnellaceae (sites KA and ZI) and Ceratobasidiaceae (sites KA, KL and ZI) (Fig. S1A and S1B). Besides these, other fungal families occasionally occurred in root communities of *N. ovata* with $< 1\%$ sequence abundance, including Hymenogastraceae, Tuberaceae, Cortinariaceae, Pyronemataceae, Pezizaceae, Psathyrellaceae and Tricholomataceae (Fig. S1A and S1B). The preference of forest and grassland individuals for ectomycorrhizal and rhizoctonia fungi was also shown by phylogenetic mapping of fungal OTUs and indicator species analyses (Fig. 2; Table S5). Each site (except site KL) included different Serendipitaceae OTUs as indicator species. In addition, forest sites had indicator species belonging to ectomycorrhizal families (BE – Sebacinaceae; VP – Inocybaceae, VS – Sebacinaceae, Thelephoraceae, Inocybaceae, Atheliaceae and Russulaceae), whereas grassland sites had rhizoctonia indicator species (KA – Tulasnellaceae and Ceratobasidiaceae; KL – Ceratobasidiaceae).

Root communities of *N. ovata* differed significantly in α-diversity (SR and PD) between sites (Table S6), with forest sites VP and VS showing significantly higher α-diversity than grassland sites KA and KL (Fig. 3A and 5.3B; Table S6). Significant variation in α-diversity was also found between habitat types (SR: $R^2 = 0.424$, $P < 0.001$; PD: $R^2 = 0.324$,

$P < 0.001$; Table S7). Root community structure differed significantly between sites (Bray-Curtis metric: $R^2 = 0.305$, pseudo-$F = 3.164$, $P < 0.001$; UniFrac metric: $R^2 = 0.217$, pseudo-$F = 2.065$, $P < 0.001$) (Fig. 3C and 5.3D) and between habitat types (Bray-Curtis metric: $R^2 = 0.142$, pseudo-$F = 4.463$, $P < 0.001$; UniFrac metric: $R^2 = 0.179$, pseudo-$F = 5.884$, $P < 0.001$).

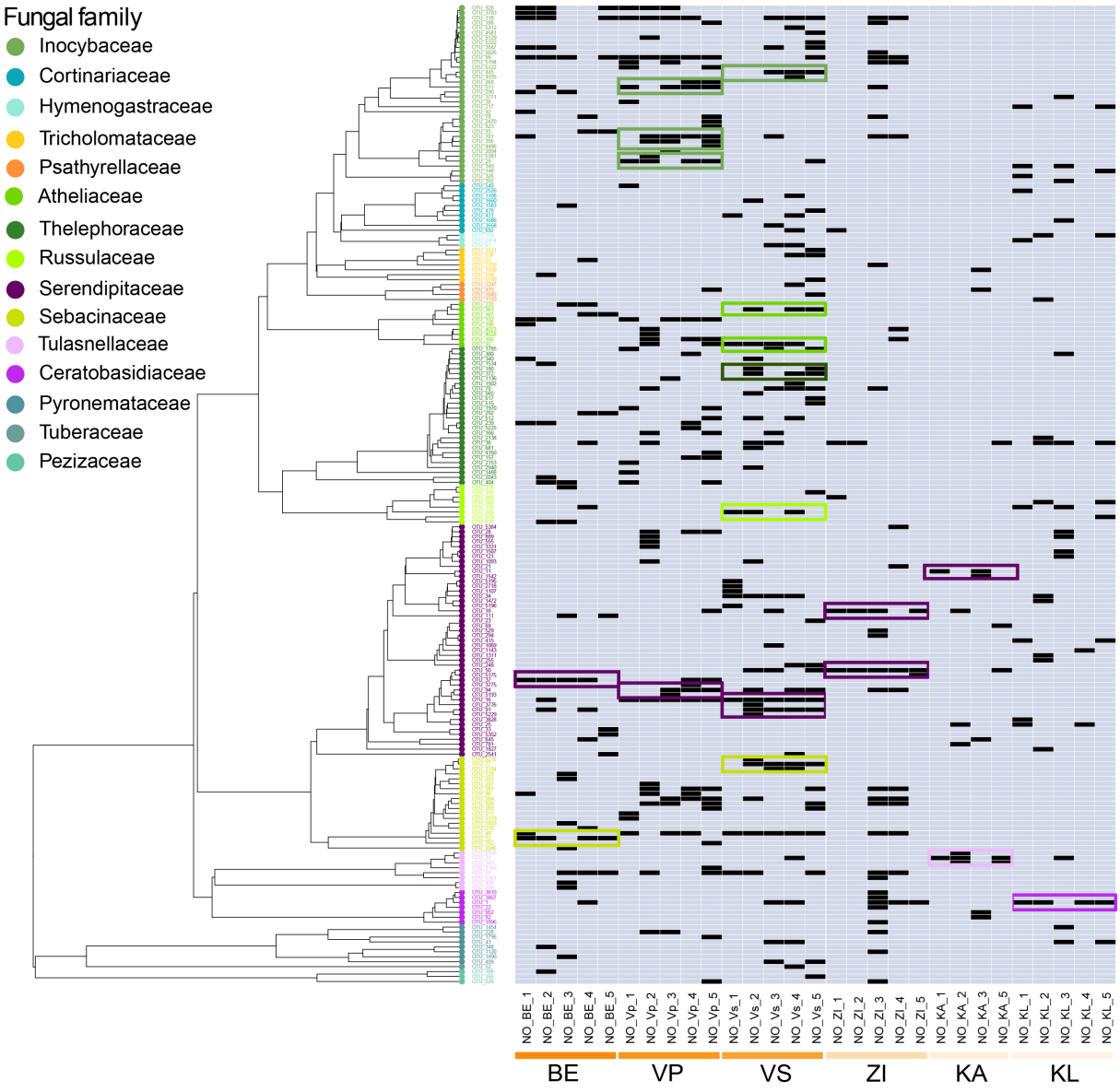

Fig. 2. Phylogenetic mapping of orchid mycorrhizal OTUs with indication of indicator species for each site. Tips of the tree are colored by fungal family. Rhizoctonia, ectomycorrhizal and saprotrophic families (in tips of the tree) are in shades of purple, green and yellow, respectively. Indicator species for each site are marked with rectangular boxes and show identical colors with tree tips. The light availability of orchid individuals among sites is displayed by the opacity of orange color bar at the bottom of the plot. The forest site BE, VP and VS exhibited lower light intensity: 69, 84 and 99 µmol photons m^{-2} s^{-1} on average, while grassland site KA, KL and ZI exhibited higher light intensity: 474, 593, 650 µmol photons m^{-2} s^{-1} on average.

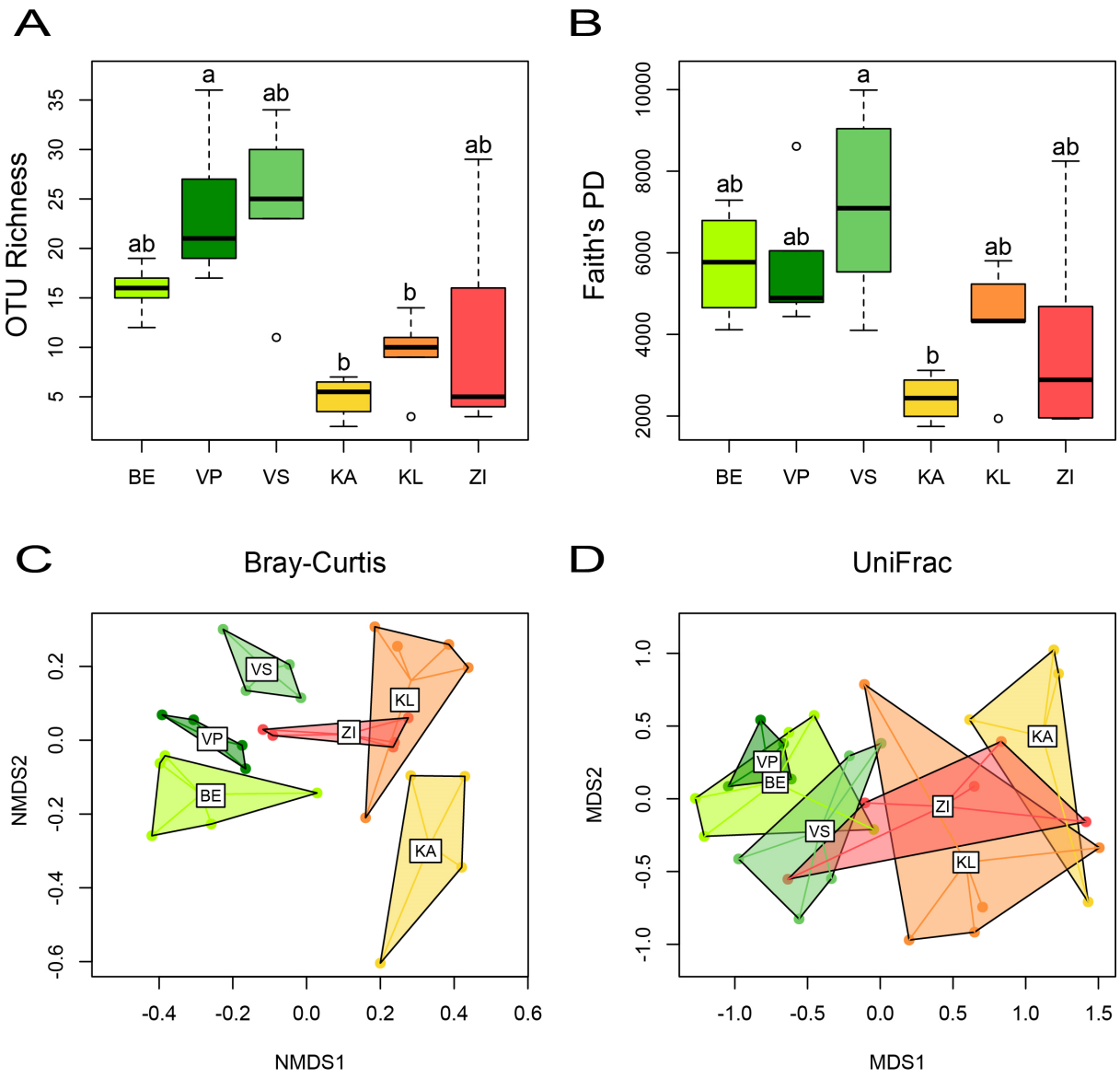

Fig. 3 Diversity and community structure of fungal communities in *Neottia ovata* roots sampled at different grassland and forest sites. A) OTU richness (SR) and (B) Faith's PD of root communities. Letters above the bars indicate the outcomes of post hoc analysis conducted to compare the difference between sites. Root community structure is visualized by NMDS plots based on (C) Bray-Curtis and (D) UniFrac distance metrics. Forest sites (BE, VP and VS) and grassland sites (KA, KL and ZI) are distinguished by color.

Roots recruit available fungi from the soil

Bulk soil samples (416 OTUs) had higher diversity of orchid mycorrhizal fungi than root samples (253 OTUs) (Fig. S2). Similar to the variation in root communities, soil fungal communities varied substantially between sites and habitats in composition (Fig. S1C and 5.1D) and α-diversity (Fig. S3; Table S6 and S7). Soil fungal community structure was significantly different between sites (Bray-Curis metric: $R^2 = 0.444$, pseudo-$F = 3.669$, $P < 0.001$; UniFrac metric: $R^2 = 0.473$, pseudo-$F = 4.131$, $P < 0.001$) and between habitat types (Bray-Curtis metric: $R^2 = 0.110$, pseudo-$F = 3.321$, $P < 0.001$; UniFrac metric: $R^2 = 0.208$, pseudo-$F = 7.100$, $P < 0.001$). There was a significant positive correlation between α-diversity indices of soil and root samples (Fig. S4). Mantel tests also revealed a high similarity in fungal community composition between root and soil samples ($R = 0.504$, $P < 0.001$). Furthermore, strong similarity in fungal community structure was found between root and soil samples (Fig. 4A) (Bray-Curtis metric: $R^2 = 0.086$, pseudo-$F = 0.703$, $P = 0.866$).

Root fungal community composition was significantly related to edaphic variables (pseudo-$F = 1.721$, $P = 0.001$, $R^2_{adj} = 0.134$) (Fig. 4B; Table S8). NO_3^-, SOC, P and pH significantly explained differences in root fungal communities between sites (RDA1: $F = 3.767$, $P = 0.001$; Table S9). Edaphic variables remained significant after controlling for the effect of habitat type (partial db-RDA, $R^2_{adj} = 0.064$, pseudo-$F = 1.6$, $P = 0.002$).

Despite the high similarity in fungal community composition between root and soil samples, the relative abundance of one fungal family in the roots did not always respond to its relative abundance in the soil (Fig. 4C). Rhizoctonia fungi, especially those belonging to the family Serendipitaceae, exhibited relatively higher abundances in the roots than in soil, indicating that *N. ovata* preferably recruits these fungi from the soil even though they were not dominant in the soil community.

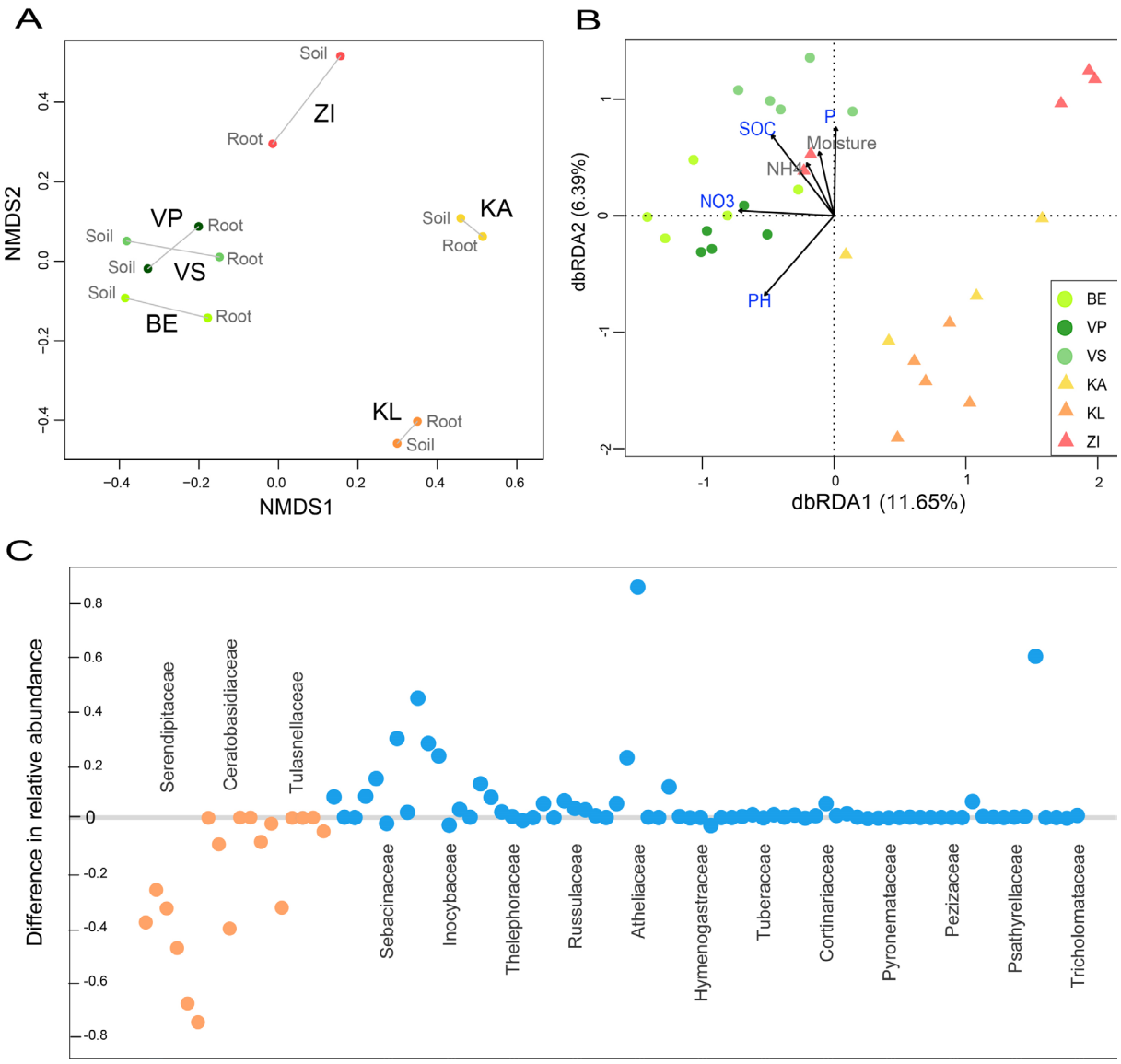

Fig. 4 Relationship between soil fungal community and soil properties and root communities. (A) NMDS graph displaying the similarity in fungal community structure among root and soil samples across sites. (B) dbRDA model shows the effect of soil properties on root communities. Significant soil variables are colored in blue. SOC: soil organic content; NO_3^-: nitrate concentration; NH_4^+: ammonium concentration; Moisture: soil moisture content. (C) Differences in the relative abundance of fungal families found in soil and root samples. For each site, the relative abundance (MiSeq reads) of each fungal family is calculated for soil and root samples separately and subsequently the difference in relative abundance is calculated as the relative abundance in soil - relative abundance in roots. Points below or above the zero line are interpreted as being more represented in root or soil sample, respectively. Rhizoctonia and non-rhizoctonia fungal families are shown in orange and blue, respectively.

The Sebacinaceae fungi detected in *N. ovata* are phylogenetically clustered with those in mycoheterotrophic relatives

Our phylogenetic analysis showed that the Sebacinaceae OTUs of *N. ovata* was highly clustered with the Sebacinaceae sequences of fully mycoheterotrophic relatives (*N. nidus-avis*, *N. accuminata*, and *N. camtschatea*) (Fig. S5). Conversely, the three Sebacinaceae sequences detected by Tesitelova *et al.* (2015) and one sequence of *N. accuminata* was placed at the basal and close to the Serendipitaceae OTUs (Fig. S5).

The level of mycoheterotrophy does not vary substantially between habitats of *N. ovata*

At each site, leaf samples of *N. ovata* were significantly enriched in ^{15}N (except for the individuals from the forest site BE) and ^{2}H, but not in ^{13}C, compared with autotrophic references (Table 2; Table S10). Notably, negative enrichment in ^{18}O was found for *N. ovata* at the three forest sites (BE, VP and VS) and grassland site ZI (Table S10), suggesting that a higher transpiration rate of orchids at these sites might have underestimated the heterotrophic C gain indicated by the ^{2}H enrichment.

Significant differences among sites were found for leaf enrichment factors $\varepsilon^{2}H$ and $\varepsilon^{15}N$, but not for $\varepsilon^{13}C$ and total nitrogen concentration (Fig. 5; Table S11). However, no consistent, significant difference in $\varepsilon^{2}H$ and $\varepsilon^{15}N$ of *N. ovata* was found between sites according to post hoc pairwise comparisons (Fig. 5; Table S11). Specifically, a significantly higher $\varepsilon^{15}N$ was found for *N. ovata* collected from grassland sites (KA and KL) compared with individuals collected from forest sites (BE and VP). A significant difference in $\varepsilon^{2}H$ was only found between the grassland site KL and the forest site VS. Our linear mixed models showed that $\varepsilon^{15}N$ ($R^2 = 0.436$, $P < 0.001$) and total nitrogen concentration ($R^2 = 0.149$, $P < 0.05$) differed significantly between forest and grassland sites, but no such differences were found for $\varepsilon^{13}C$ ($R^2 = 0.001$, $P = 0.838$) and $\varepsilon^{2}H$ ($R^2 = 0.137$, $P = 0.177$) (Table S12). While light intensity differed greatly between forest and grassland sites (Appendix S1), no significant relationship between light intensity and $\varepsilon^{13}C$ ($R^2 = 0.002$, $P = 0.823$) or $\varepsilon^{2}H$ ($R^2 = 0.014$, $P = 0.633$) was observed (Table S13).

Table 2 Mean enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ and total nitrogen concentration of *Neottia ovata* sampled at three forest and three grassland sites. Mean values in bold represent significant differences ($P < 0.05$) in leaf isotope composition between *N. ovata* and autotrophic references within each site using linear mixed models. All models include 'plot' as random effect and *P* values were computed using a Wald t-distribution approximation (see detailed results in Table S10).

Site	Habitat	$\varepsilon^{13}C$		$\varepsilon^{15}N$		$\varepsilon^{2}H$		$\varepsilon^{18}O$		Total N	
		mean	SD	Mean	SD	mean	SD	mean	SD	mean	SD
BE	Forest	0.66	1.53	-0.09	1.03	15.70	5.73	-3.05	0.91	2.56	0.59
VP	Forest	-0.72	1.33	0.81	0.23	19.30	0.91	-1.99	0.63	2.61	0.38
VS	Forest	0.01	1.67	1.90	1.13	9.56	4.11	-3.65	0.99	2.29	0.35
KA	Grassland	0.24	0.74	4.55	1.25	16.40	7.85	-0.98	0.86	2.02	0.31
KL	Grassland	-0.39	1.51	3.99	2.91	22.50	3.07	-0.10	1.10	2.21	0.40
ZI	Grassland	0.39	0.67	2.97	1.87	19.60	3.92	-1.62	0.80	2.27	0.23

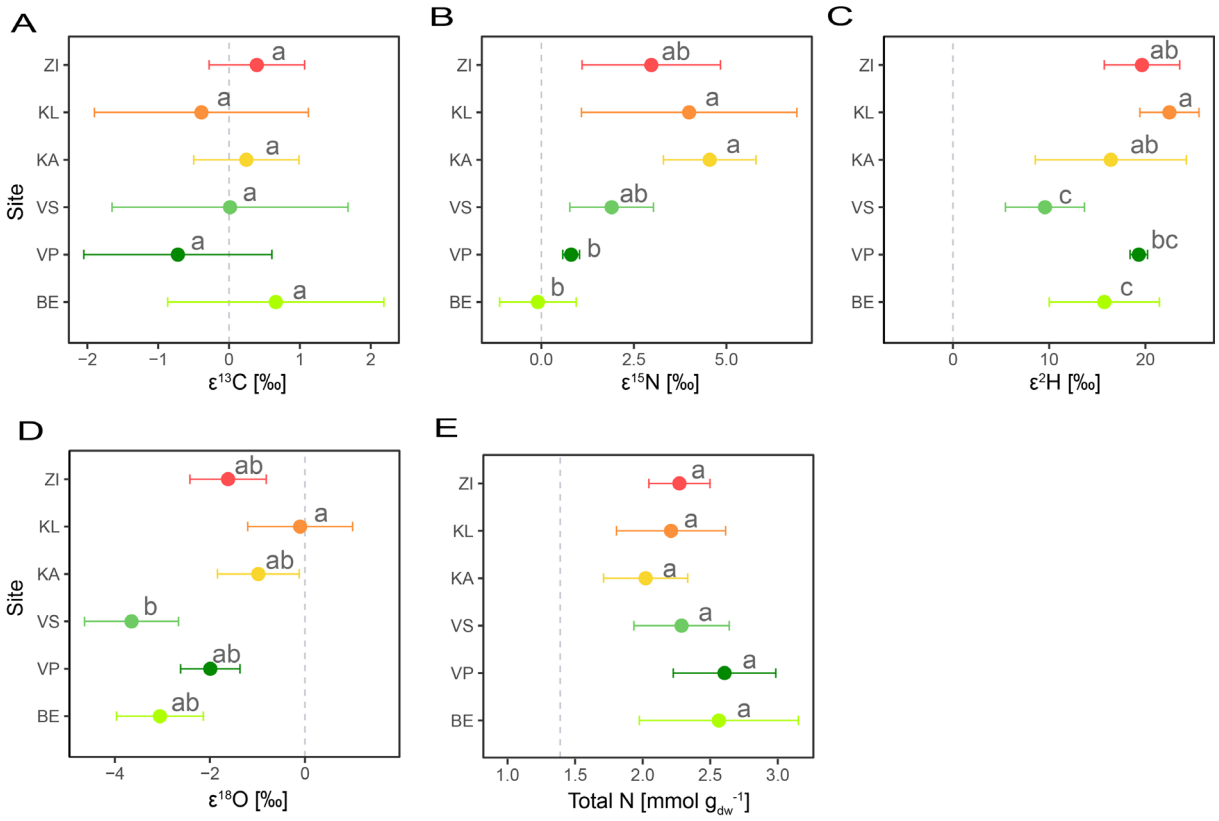

Fig. 5 Stable isotope signatures ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, and total N) of *Neottia ovata* sampled at six different sites in Europe. Forest sites include BE, VP and VS whereas grassland sites include KA, KL and ZI. Sites with different letters are significantly ($P < 0.05$) different from each other.

DISCUSSION

In line with previous studies (Oja *et al.* 2015; Těšitelová *et al.* 2015; Jacquemyn *et al.* 2015; Vogt-Schilb *et al.* 2020), we found that *N. ovata* predominantly associated with rhizoctonia fungi from the Serendipitaceae, while recruiting a wide range of site-dependent ectomycorrhizal fungi (Fig. 2) – this is common if not general in rhizoctonia-associated orchids (Selosse *et al.* 2022). Our phylogenetic analyses showed that *N. ovata* has already recruited the set of ectomycorrhizal Sebacinaceae fungi that tightly associated with its fully mycoheterotrophic relatives (Fig. S5). In support of our first hypothesis (#H1), the diversity and composition of root communities varied greatly between sites, especially between forests and grasslands (Fig. 3; Fig. S1). Moreover, variation in root communities was highly correlated with variation in soil communities (Fig. 4A; Fig. S3-S4) and significantly related to edaphic factors (Fig. 4B), supporting our second hypothesis (#H2). In general, we found significant enrichment in ^{15}N and ^{2}H, but not in ^{13}C (Table 2; Fig. 5), confirming the partially mycoheterotrophic mode of *N. ovata* and the usual trend for rhizoctonia-associated orchids (Gebauer *et al.* 2016; Schiebold *et al.* 2018; Schweiger *et al.* 2018). However, stable isotope signatures did not significantly differ between sites and habitat types (Fig. 5; Table S11-S12) and no correlation was found between ^{13}C or ^{2}H and light availability (Table S13), rejecting our third hypothesis (#H3).

Recruitment of ectomycorrhizal fungi precedes the evolutionary transition to heterotrophy in *Neottia*

The trend of symbiotic shifts from rhizoctonia Serendipitaceae to ectomycorrhizal Sebacinaceae fungi in parallel with an increased gain of fungal C is conceivable in the *Neottia* genus (Yamage *et al.* 2016). Such evolutionary changes have been supported by both stable isotope analyses and molecular identification of mycorrhizal associates of leafy and leafless *Neottia* species displaying different degrees of mycoheterotrophy. It has been well reported that *Neottia nidus-avis*, positioning at the evolutionary endpoint of full mycoheterotrophy, is significantly enriched in ^{13}C, ^{15}N and ^{2}H (Bidartondo *et al.* 2004; Gebauer & Meyer 2003; Preiss *et al.* 2010; Gebauer *et al.* 2016; Schiebold *et al.* 2018; Jacquemyn *et al.* 2021) and specializes on ectomycorrhizal Sebacinaceae fungi (McKendrick *et al.* 2002; Selosse *et al.* 2002; Yamage *et al.* 2016; Jacquemyn *et al.* 2021). Previous studies have showed that ectomycorrhizal Sebacinaceae fungi occurred in root communities of *N. ovata* (Oja *et al.* 2015; Těšitelová *et al.* 2015; Jacquemyn *et al.* 2015)

and several other leafy *Neottia* species (Těšitelová *et al.* 2015; Yamage *et al.* 2016). The presence of Sebacinaceae in root communities of *N. ovata* in forest sites is confirmed by this current study (Fig. 2). In disagreement with previous findings of phylogenetic separation of Sebacinaceae symbionts between *N. ovata* and the fully mycoheterotrophic *N. nidus-avis* (Těšitelová *et al.* 2015), our phylogenetic analyses further showed that Sebacinaceae fungi detected in *N. ovata* are closely related to mycorrhizal symbionts of its fully mycoheterotrophic relatives (*N. nidus-avis*, *N. accuminata*, and *N. camtschatae*) (Fig. S5). Our findings indicate that the predisposition of Sebacinaceae fungi in *Neottia* may have opened the door for symbiotic shifts at the fully mycoheterotrophic stage, which is in line with the results of broad-scale evolutionary inferences in Orchidaceae (Wang *et al.* 2021) and is predicted under the 'waiting room hypothesis' (Selosse *et al.* 2022).

Functional switches to ectomycorrhizal fungi were not detected for *N. ovata* in the studied habitats

Ectomycorrhizal fungi tend to have different types of access to nutrient resources compared with soil-dwelling rhizoctonia fungi and may therefore explain the distinct stable isotope signatures observed in orchids predominantly associating with ectomycorrhizal fungi (Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Schiebold *et al.* 2018; Gebauer & Schweiger 2021). It is well supported that ectomycorrhizal-dominant mycoheterotrophic orchid species are more enriched in ^{13}C, ^{15}N and ^{2}H than rhizoctonia-associated orchid species. Here, we expected that populations associating with a higher proportion of ectomycorrhizal associations would have a higher ^{13}C enrichment of orchid leaves. However, we found no significant variations in stable isotope signatures between forest and grassland individuals of *N. ovata* (Fig. 5). Considering the predominant association with rhizoctonia fungi of the Serendipitaceae (Fig. 2; Fig. 3) and the non-negligible host preference towards those fungi (Fig. 4C), we speculate that ectomycorrhizal fungi contribute little to the nutritional budget of *N. ovata* in the studied habitats when rhizoctonia fungi still take the major functional role in external C and nutrient supply from fungal resources. Thus, our results hint towards an early stage of partial mycoheterotrophy for *N. ovata* in line with the waiting room hypothesis (Selosse *et al.* 2022), a stage when mycorrhizal partners for a later stage have already been recruited (as endophytes) but the functional symbiotic switch to new partners probably has not yet been achieved.

Does the heterotrophic level of *N. ovata* vary with the irradiance level?

Light availability was hypothesized to be negatively correlated with the level mycoheterotrophy. Orchid individuals in low-light habitats were therefore hypothesized to gain a higher amount of fungal C to compensate for their reduced photosynthetic C than individuals exposed to high irradiance levels (Gonneau *et al.* 2014; Preiss *et al.* 2010; Schweiger *et al.* 2019). However, we found no correlation between enrichment factor ^{13}C of *N. ovata* and light intensity (Table S13). Our results are not in line with previous findings for partially mycoheterotrophic *Cephalanthera* species that associate with ectomycorrhizal fungi (Preiss *et al.* 2010) and *Ophrys insectifera* that associates with rhizoctonia fungi (Schweiger *et al.* 2019), but in agreement with previous reports on *N. ovata* (Schweiger *et al.* 2019; Onipchenko *et al.* 2023) and other rhizoctonia-associated orchids, such as *Cypripedium calceolus* (Preiss *et al.* 2010), *Goodyera repens* (Liebel *et al.* 2015) and *Epipactis palustris* (Lallemand *et al.* 2018). Particularly, *E. palustris* and *N. ovata* reside in different genera consisting of mycoheterotrophic species within in the same tribe Neottieae. ^{2}H enrichment was considered as suitable nutrition indicator for rhizoctonia-associated orchids because of their usually inconspicuous ^{13}C enrichment (Gebauer *et al.* 2016). Because *N. ovata* was not enriched in ^{13}C (Fig. 5), we further expected to find a correlation between ^{2}H and light intensity. However, this expectation was also not met in the current study (Table S13).

Several factors may influence the stable isotope signatures of *N. ovata* and hamper our understanding of its heterotrophic nutrition. Those factors include the transpiration effect (Ziegler 1989; Cernusak *et al.* 2004; Onipchenko *et al.* 2023), the sampling range of light availability (Schweiger *et al.* 2019), the plants' alternative strategies of adaption (Liebel *et al.* 2015) and soil nutrient availability or stoichiometry (Gomes *et al.* 2019a). Detailed elaborations on those factors can be found in Appendix S1. Nonetheless, understanding the exact factors influencing the mycoheterotrophic nutrition of orchid species awaits future *in situ* and *ex situ* studies in which environmental factors can be manipulated.

CONCLUSIONS

Our comprehensive investigations showed that *N. ovata* is capable of dual associations with rhizoctonia symbionts and supplementary ectomycorrhizal fungi in relatively low-light forest habitats. However, the presence of ectomycorrhizal fungi did not substantially influence the isotope signatures of *N. ovata* in forests. Although we cannot rule out the possibility that the C and H enrichments were masked by higher transpiration rates of forest individuals, our findings indicate that the contribution of ectomycorrhizal fungi to the C and nutrition budget of *N. ovata* is probably minimal in the studied habitats, and that rhizoctonia fungi play the major functional role in its nutritional supply from fungal resources.

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AUTHOR CONTRIBUTIONS

VSFTM, DW, GG and HJ designed the experiment. GG, FEZ, HJ and HVDH contributed to the selection of the sampling site. DW, VSFTM, HJ, FEZ and JL collected and processed the samples. DW analysed data with input from VSFTM, GG, HJ, FEZ and SIFG. DW wrote the first manuscript draft. All authors commented and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

Additional Supporting Information will be found online in the Supporting Information section at the end of the article. Illumina sequencing data have been deposited with links to BioProject accession number PRJNA952620 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

SUPPORTING INFORMATION

The following additional information is available in the online version of this article (at https://besjournals.onlinelibrary.wiley.com/doi/10.1111/1365-2435.14354):

Appendix S1 Methodological details and supplementary results.

Fig. S1 Fungal community composition of root and soil samples across site.

Fig. S2 Orchid mycorrhizal fungal OTU abundance of root and soil samples.

Fig. S3 Alpha diversity of mycorrhizal community in bulk soil.

Fig. S4 Linear regression of SR and PD values between root and soil samples.

Fig. S5 Phylogenetic relationship of the Serendipitaceae fungi detected in *Neottia ovata* and its fully mycoheterotrophic relatives.

Table S1 A pilot study to test the efficiency of two commonly used primer pairs for capturing orchid mycorrhizal fungi.

Table S2 Sequence list and alignment of Tulasnellaceae for primer design.

Table S3 Taxonomic assignments of fungal OTUs of root and soil samples.

Table S4 Stable isotope signatures of orchid individuals and reference plants, habitat type, light intensity and soil properties.

Table S5 Indicator species with indicator value larger than 0.5 and significant *P*-value.

Table S6 ANOVA, Kruskal-Wallis tests and post hoc pairwise comparisons of alpha diversity index (SR and PD) of root and soil samples across sites.

Table S7 Habitat effect on SR and PD of root and soil samples using linear mixed models. 'Site' was used as a random effect.

Table S8 Soil properties of sampling sites.

Table S9 Results of the dbRDA model.

Table S10 Model difference between leaves of orchid and reference plants within each site, plot as random effect, e.g. lmer ($\varepsilon^{13}C \sim$ Category + (1|Plot), data=BE).

Table S11 ANOVA, Kruskal-Wallis tests and post hoc pairwise comparisons of leaf isotope signatures and total N concentration across sites.

Table S12 Habitat effect on stable isotope signatures and total N concentration of *N. ovata* leaves. 'Site' was used as random effect, e.g. lmer ($\varepsilon^{13}C \sim$ Habitat + (1|Site).

Table S13 Test the effect of light on ^{13}C and ^{2}H enrichment of *N. ovata* leaves. 'Site' was used as random effect, e.g. lmer ($\varepsilon^{13}C \sim$ Light + (1|Site).

MANUSCRIPT 4

Novel insights into orchid mycorrhiza functioning from stable isotope signatures of fungal pelotons

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Keywords: *Anoectochilus, Epipactis*, mycoheterotrophy, mycorrhiza, Orchidaceae, peloton, rhizoctonia, stable isotopes

SUMMARY

- Stable isotope signatures of fungal sporocarps have been instrumental in identifying carbon gains of chlorophyllous orchids from a fungal source. Yet, not all mycorrhizal fungi produce macroscopic sporocarps and frequently fungi of different taxa occur in parallel in orchid roots.

- To overcome this obstacle, we investigated stable isotope signatures of fungal pelotons extracted from orchid roots and compared these data to the respective orchid and reference plant tissues. *Anoectochilus sandvicensis* and *Epipactis palustris* represented specialized or unspecialized rhizoctonia-associated orchids. *Epipactis atrorubens* and *Epipactis leptochila* are orchids considered ectomycorrhiza-associated with different preferences for Basidio- and Ascomycota.

- ^{13}C enrichment of rhizoctonia pelotons was minor compared to plant tissues and significantly lower than enrichments of pelotons from ectomycorrhizal *Epipactis* species. ^{15}N values of pelotons from *E. leptochila* and *E. atrorubens* showed similar patterns as known for respective sporocarps of ectomycorrhizal Ascomycota and Basidiomycota, however, with an offset towards lower ^{15}N enrichments and nitrogen concentrations.

- Our results suggest an explicit fungal nutrition source of orchids associated with ectomycorrhizal fungi, whereas the low ^{13}C enrichment in rhizoctonia-associated orchids and fungal pelotons hamper the detection of carbon gains from fungal partners. ^{15}N isotopic pattern of orchids further suggests a selective transfer of ^{15}N-enriched protein-nitrogen into orchids.

INTRODUCTION

The roots of around 90% of higher plants form a symbiotic association with mycorrhizal fungi (Brundrett & Tedersoo, 2018). The mycorrhizal symbiosis is generally considered as a nutrient-for-carbon exchange between fungi and plants (Smith & Read, 2008). In contrast to this classical view on the functioning of mycorrhizae, Orchidaceae access essential nutrients, and can simultaneously complement or even replace the carbon (C) acquisition via photosynthesis by mycoheterotrophic nutrition (Jacquemyn & Merckx, 2019). This fungus-to-plant C transfer is particularly important during germination and seedling development of orchids and for achlorophyllous orchids during their entire life span (Burgeff, 1936; Leake, 1994; Rasmussen, 1995; Rasmussen & Whigham, 1998; Arditti & Ghani, 2000). Likewise, chlorophyllous mature orchids (cf. Figure 1A) rely on C supply from mycorrhizal fungi to varying extents (e.g. Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Hynson *et al.*, 2013; Schiebold *et al.*, 2018). The degree of partial mycoheterotrophy of green orchid species can be investigated via stable C and nitrogen (N) isotope analysis of target orchids, autotrophic reference plants, and respective fungal hosts (e.g. Gebauer and Meyer 2003; Bidartondo *et al.* 2004). Previously, it has been frequent practice to examine stable isotope natural abundance of mycorrhizal fungi's fruit bodies in order to elucidate the C and N isotope signatures of the fungal source (Trudell *et al.*, 2003; Julou *et al.*, 2005; Ogura-Tsujita *et al.*, 2009; Lee *et al.*, 2015; Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). Yet, this proves difficult for rhizoctonia, the fungal partners of most orchids, particularly non-forest orchid species, as many of these ubiquitous fungi do not produce fruit bodies. Moreover, fungi of various taxa frequently co-occur in orchid roots and our knowledge about their functional role in orchid nutrition is still largely unexplored. Extracting intracellular hyphae, so-called pelotons, directly from orchid roots could solve these issues and would provide a more straightforward approach even if fruit body material is obtainable.

So far, analogous research has examined isotope signatures of intra-radical hyphae of arbuscular mycorrhizal fungi (Klink *et al.*, 2020). Together with Gomes *et al.* 2023, this study provides the first ^{15}N and ^{13}C natural abundance stable isotope composition data of fungal pelotons, the typical morphological feature of mycorrhizal fungi in orchid roots (Fig. 1B, C). Gomes *et al.* 2023 focused on arbuscular mycorrhizal fungi and orchid mycorrhizal rhizoctonia fungi and thus on gaining stable isotope data of mycorrhizal fungi

which do not produce fruit bodies and can only be extracted from plant roots. In this study, we centred specifically upon orchid mycorrhiza. In addition to orchids associated with rhizoctonia fungi, we investigated orchid species that are part of tripartite ectomycorrhizal (ECM) networks with Ascomycota and/or Basidiomycota. In the case of ectomycorrhizal fungi, stable isotope data of respective fungi fruit bodies exist and can be used for comparison with fungal pelotons when originating from the same sampling site (Taylor *et al.*, 2003; Gebauer *et al.*, 2016; Schiebold *et al.*, 2017).

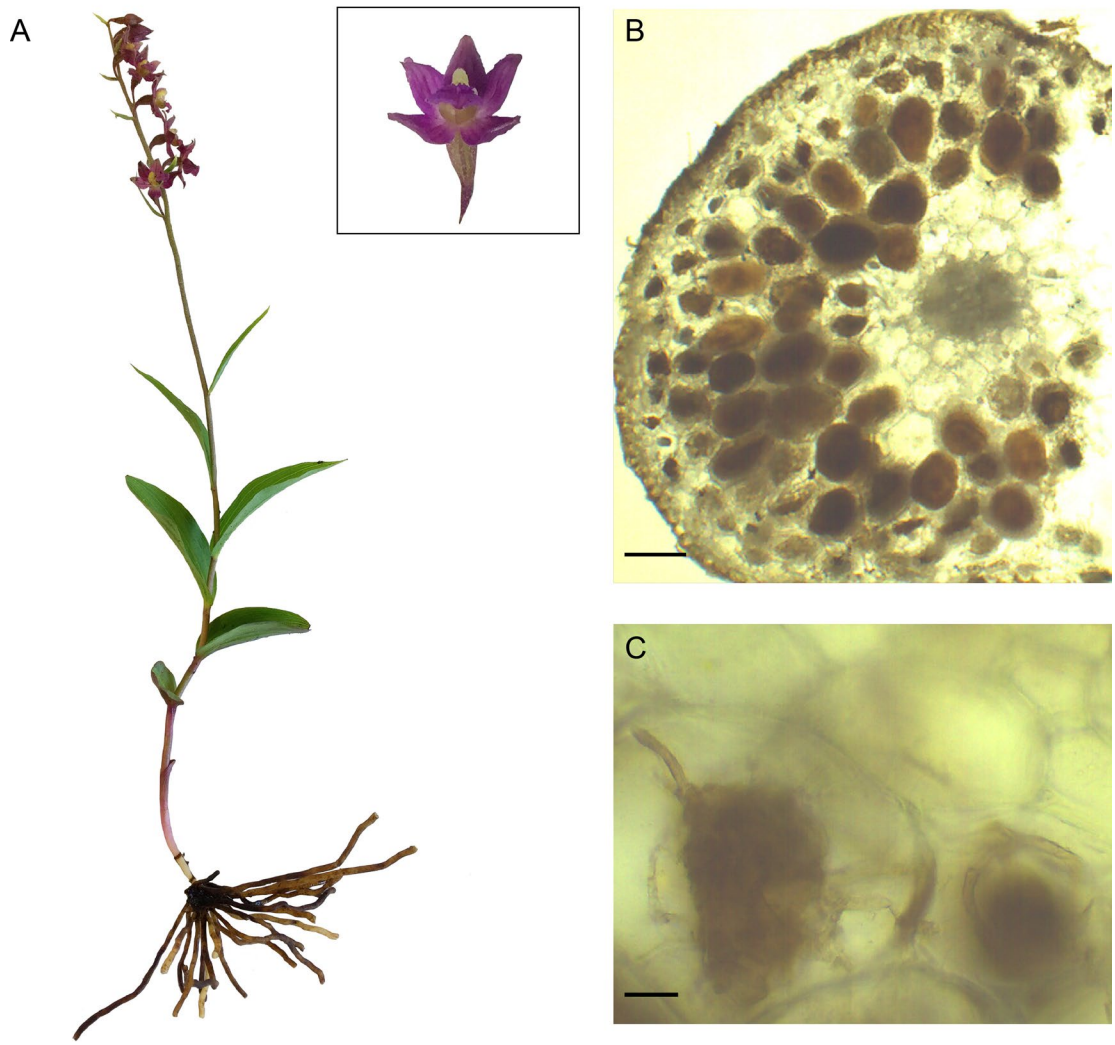

Fig. 1 (A) *Epipactis atrorubens* with magnified flower. (B) Transverse section of an *E. atrorubens* root colonized with intracellular hyphal coils (pelotons). Scale bar = 100 µm. (C) Fungal pelotons in cortex cells. Scale bar = 30 µm.

As a rhizoctonia-associated orchid species, we selected the Hawaiian endemic orchid *Anoectochilus sandvicensis* Lindl. because it shows, unlike many other rhizoctonia-associated species, extreme fungal specificity, associating with a single operational taxonomic unit (OTU) of *Ceratobasidium* per orchid individual, and only two closely related OTUs among individuals at our sampling site on Mount Ka'ala, O'ahu, Hawai'i USA (Swift *et al.*, 2019), and has shown some indication of partial mycoheterotrophy based on leaf $\delta^{15}N$ enrichment (Hynson, 2016). Further we chose to analyse pelotons of three *Epipactis* spp. as this orchid genus can be characterised by various habitats ranging from open meadows to dense forests. Also, *Epipactis* is well studied in respect of isotopy and fungal partners mostly based on Sanger sequencing (Bidartondo *et al.*, 2004; Bidartondo & Read, 2008; Jacquemyn *et al.*, 2016; Schiebold *et al.*, 2017). While grassland orchid *Epipactis palustris* (L.) Crantz was classified as rhizoctonia-associated (White *et al.*, 1990; Bidartondo *et al.*, 2004; Illyés *et al.*, 2009; Jacquemyn *et al.*, 2016, 2017; Lallemand *et al.*, 2018; Schweiger *et al.*, 2018), *Epipactis leptochila* (Godfery) Godfery and *Epipactis atrorubens* (Hoffm.) Besser are forest-dwelling and entangled in ECM networks in temperate Europe (Schiebold *et al.*, 2017). *Epipactis leptochila* was previously shown to associate with *Tuber excavatum* Vittad., an ECM Ascomycota (Schiebold *et al.*, 2017), whereas for *E. atrorubens* both, ECM Ascomycota and ECM Basidiomycota, were identified as orchid mycorrhizal fungi (Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Bidartondo & Read, 2008). To verify existing knowledge about root fungi of our investigated orchid species we used a fungal sequencing approach here.

By providing the first natural abundance ^{15}N and ^{13}C data of fungal pelotons with our approach, we further elucidate the complex relationship between orchids and their fungal symbionts and aim to address the following two aspects:

i) *Partial mycoheterotrophy of green orchids.* ^{13}C enrichment of green orchid leaves relative to reference plant leaves is an indicator for partial mycoheterotrophy, at least for orchids associating with ECM-type fungi. However, this remains challenging for rhizoctonia-associated orchids, because leaves of green orchids with rhizoctonia fungal partners are usually not enriched in ^{13}C relative to autotrophic reference plants (Hynson *et al.*, 2013). Yet, leaves of rhizoctonia-associated orchids tend to be slightly enriched in ^{15}N, have higher total N concentrations (Bidartondo *et al.*, 2004; Hynson *et al.*, 2013; Stöckel *et al.*, 2014), and a hydrogen isotope (^{2}H) approach provides evidence for a hidden

C transfer from fungi to orchids (Gebauer *et al.*, 2016; Schiebold *et al.*, 2018). In this approach, a significant ^{2}H enrichment of orchid leaves serves as an indicator for a fungus-to-plant organic matter (i.e. carbon) transfer based on the finding that secondary heterotrophic organic compounds, i.e. originating from fungi, are enriched in ^{2}H compared to primary photosynthetic compounds (Yakir, 1992). Interestingly, seedlings of rhizoctonia-associated orchids (so called protocorms) were found to be enriched in ^{13}C and ^{15}N, but their low ^{13}C enrichment made them hardly distinguishable from their respective adult stages and neighbouring autotrophic plants (Stöckel *et al.*, 2014). Altogether, there is reason to presume that ^{13}C enrichment of rhizoctonia fungi is relatively small resulting in no suspicious enrichment of their orchid associates' leaves (Selosse & Martos, 2014). We, thus, expect rhizoctonia pelotons isolated from *A. sandvicensis* and *E. palustris* to be less ^{13}C enriched than pelotons from orchids in tripartite ECM networks with forest trees (*E. leptochila* and *E. atrorubens)* or even not enriched in ^{13}C at all.

ii) *Fungal associates and their function*. When utilizing organic matter as N source, fungi become enriched with the heavy isotope ^{15}N in relation to their bulk substrate (Gebauer & Taylor, 1999). Certainly, ^{15}N values of fruit bodies show distinct patterns depending on their fungal guild, e.g. *Tuber* species (ECM Ascomycota) display higher $\delta^{15}N$ values than ECM Basidiomycota (Schiebold *et al.*, 2017). Commonly different fungi species have characteristic isotopic signature depending on their ecology and habitat (cf. Selosse & Martos, 2014). Based on the fungal guild a plant associates with, studies have shown predictable patterns in plant isotope enrichment (Hobbie & Colpaert, 2003; Hobbie & Högberg, 2012; Hynson *et al.*, 2013; Schiebold *et al.*, 2017). We thus expect isotope compositions of orchid leaves to reflect the isotopic signature of respective pelotons, and thus to provide predictable patterns of their functional, nutrition-specific fungal associates. For orchids having fungal hosts additional to or other than rhizoctonia (presumably *Epipactis leptochila* and *Epipactis atrorubens*) we predict pelotons to mirror the ^{15}N enrichment of fruit bodies of the respective fungal guilds they associate with.

MATERIALS AND METHODS

Study species and sampling locations

Three *Epipactis* species were investigated during orchid flowering season in June and July 2021 in north-eastern Bavaria (Germany): *E. atrorubens* (Hoffm.) Besser (n=5) growing in a mixed forest dominated by *Fagus sylvatica* L. and *Picea abies* (L.) H.Karst., *E. leptochila* (Godfery) Godfery (n=5) in a *Fagus sylvatica* forest, and *E. palustris* (L.) Crantz (n=5) from a wet grassland. Sampling of orchids and accompanying autotrophic reference plants (see Table S1) followed the plot-wise sampling scheme of Gebauer and Meyer (2003). Additionally, the Hawaiian endemic orchid *Anoectochilus sandvicensis* Lindl. was studied in a tropical montane cloud forest at Mount Ka'ala bog (O'ahu, Hawai'i USA) with a mixed community of *Metrosideros polymorpha* Gaudich. and endemic shrubs. Due to the stoloniferous nature of *A. sandvicensis*, it is difficult to differentiate individuals, thus orchid root samples within 0.5 m were considered to be from a single plant. Samples were taken in December 2020 and February 2021 from three orchid individuals that were separated by at least 100 m, along with replicates of three to five autotrophic reference plant species (see Table S1), each with a maximum distance of 0.5 m from the orchid.

For orchids, the entire plant was dug out (Fig. 1A), and soil was washed off from roots. For *A. sandvicensis* only leaves and roots were studied, while each *Epipactis* individual was divided into the compartments 'leaves', 'stem and blossom', and 'roots' (Table S2). Root systems were stored at 4 °C one week maximum until further processing. All other orchid compartments and leaves of reference plants were immediately oven dried at 105 °C and stored in a desiccator until further processing.

Isolation of pelotons and sample preparation

Pelotons were extracted from roots using mechanical approaches. For *Epipactis* spp., 1 cm root segments with and without pelotons were separated based on optical differentiation, which was validated by microscopic observation, and cleaned by sonication (5 min, 35 kHz) in deionised water. Roots without pelotons and a subsample of the colonized root samples with pelotons were kept for isotopic analysis, thus dried at 105° C and stored in a desiccator. The isolation method for colonised root segments of *Epipactis* is based on Klink *et al.* (2020) with adjustments for orchid roots: Colonised root cells were opened by longitudinal sections using a razor blade, followed by another sonication (15 min) in

deionised water to release the pelotons from the sliced roots. The solution containing the extracted pelotons was transferred to another centrifuge tube using a pipette. This step was repeated several times by consistently adding deionised water to the root fragments until the solution appeared no longer turbid. Purification steps comprised a combination of stacked 250 µm, 200 µm, 125 µm, 90 µm and 63 µm sieves (Retsch test sieve, stainless steel, DIN/ISO 3310-1, Germany) to separate pelotons from remaining plant residues. The sieve surfaces were rinsed with deionised water several times, thus remaining contaminates were sifted out and maximal purity of pelotons was achieved depending on mesh size. We observed the largest pelotons and the highest colonisation in the roots of *E. leptochila*, followed by *E. atrorubens* and *E. palustris*. Most pelotons were retained by the 90 µm sieve for *E. atrorubens*, and by the 63 µm sieve for *E. palustris* and *E. leptochila* which were then pipetted into Eppendorf tubes and centrifuged (10 s at 1306×g, Eppendorf Centrifuge 5415 C, Eppendorf AG, Hamburg, Germany). The supernatant liquid was removed because it did not comprise any pelotons and the peloton pellet was dried at 105°C for 72h and placed in tin (sample weight range of 50 to 2000 µg) or annealed silver (sample weight range of 1000 to 2000 µg) capsules. Note that all cleaning steps were monitored by microscopic observation. Pelotons within *Anoechtochilus sandvicensis* roots were extracted from roots (approximately 2 cm in length) by maceration in sterile deionized water and pipetting, to generate a concentrated peloton solution, which was directly pipetted into tin capsules and lyophilized (Labconco FreeZone 230V, Labconco, Kansas City, Missouri, USA) to generate 10 samples of varying weights within a range of 100 to 500 µg. For both peloton isolation methods applied here, we assume a similar level of purification and thus no large effect of different extraction methods on isotope signatures. All peloton samples were stored in a desiccator until isotope analysis (Table S3).

Leaf, stem and blossom, and root samples of the orchids and reference plant samples were ground to a homogenous, fine powder using a ball mill (Retsch Schwingmühle MM2, Haan, Germany) before transferring them into tin or annealed silver capsules. Sample quantities were determined using micro balances (Sartorius CPA2P & MSE3.6P-000-DM, Göttingen, Germany & Mettler AT21, Gießen, Germany).

Stable isotope analyses

Relative natural abundance analysis of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes as well as N concentrations were determined simultaneously using an EA-IRMS coupling combining an elemental analyser with a continuous flow isotope ratio mass spectrometer (see Table S4). Relative natural abundances of hydrogen ($^{2}H/^{1}H$) and oxygen isotopes ($^{18}O/^{16}O$) was measured separately using a TC-IRMS coupling which links a thermal conversion through a pyrolysis unit to a continuous flow isotope ratio mass spectrometer (see Table S4). For H isotope abundance, each sample was measured four times in a row with the first three measures being neglected to avoid a memory bias of the previous sample owing to the extraordinary high frequency difference of the isotopes ^{1}H and ^{2}H. Furthermore, we analysed H isotope abundance of target orchid samples and respective reference plant samples together in identical sample batches and calculated differences (ε values, see further down) to account for a bias of post-sampling H atom exchange between organically bound hydroxyl groups in our samples and H_2O in ambient air (Gebauer *et al.*, 2016). Note that H and O isotope signatures are only available for *E. leptochila* due to the limited amount of fungal peloton sample material. Measured relative isotope abundances are denoted as δ values (Table S2, Table S3): $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000$ ‰, where R_{sample} and $R_{standard}$ are the ratios of heavy to the light isotope of the samples and the respective standard (Table S4). Stable isotope natural abundances of plant tissues are influenced by local environmental conditions, so we normalized data for interspecies comparisons using the isotope enrichment factor (ε) approach (Preiss & Gebauer, 2008): $\varepsilon = \delta S - \delta REF$, where δS is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O$ value of an orchid individual tissue or an autotrophic reference plant and δREF is the mean value of all autotrophic reference plants by plot (Preiss & Gebauer, 2008).

Isotope compositions ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$) of orchid and reference plant leaves were compared fitting linear mixed models 'lmer' (package lme4, version 1.1.29; Bates *et al.*, (2015)). Due to our plot-wise sampling scheme and the data normalization per plot, the model included the 'plot' as a random effect. 95% Confidence Intervals (CIs) and *P*-values were computed using a Wald *t*-distribution approximation. Analogously, we fitted linear mixed models to compare isotopic compositions of sample types, particularly orchid leaves and pelotons, across orchid species. For *post hoc* pairwise comparison between

species, we used the 'emmeans' and 'contrast' functions (package emmeans, version 1.7.5; Russell *et al.*, 2022) with Bonferroni-Holm *P*-value adjustment. To test for differences in total N concentration of orchid compartments, pelotons and reference plants, we conducted non-parametric Kruskal-Wallis *H*-tests and *post hoc* Dunn's test with Bonferroni-Holm correction for multiple comparisons (Dinno, 2017). We used a significance level of $\alpha = 0.05$ for the statistical tests. Statistical analyses were conducted in R version 4.2.0 (R Core Team, 2022).

Fungal sequencing

Root fungi of *A. sandvicensis* at our sampling site have already been identified by Swift *et al.*, (2019), and confirmed by fungal ITS Sanger sequencing a subset of individual root fragments to be *Ceratobasidium* sp. 1 (sensu Swift *et al.*, 2019). For the three *Epipactis* species (*E. leptochila*, *E. atrorubens* and *E. palustris*), five individuals of each species were used for DNA extraction and fungal metabarcording, which was done as follows. Root pieces were surface sterilized using 1% sodium hypochlorite, and rinsed in sterile distilled water. For each sample, five root sections with 1 mm thickness were randomly cut and chosen for DNA extraction using a CTAB method (Doyle & Doyle, 1987). The fungal forward primer fITS7 (GTGARTCATCGAATCTTTG, (Ihrmark *et al.*, 2012)) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC, (White *et al.*, 1990)) were used to amplify the nuclear ITS2 region. This primer pair has been shown to amplify the substantial diversity of orchid mycorrhizal fungi and other soil fungal communities (Waud *et al.*, 2014; Li *et al.*, 2021). It is important to note that the primer pair used in this study may not capture all fungal taxa reside in orchid root communities because of the potential primer bias. Particularly for the family Tulasnellaceae, its abundance might be underestimated due to the high variability of the ITS2 region of Tulasnellaceae (Waud *et al.*, 2014; Oja *et al.*, 2015; Vogt-Schilb *et al.*, 2020; Li *et al.*, 2021). PCR reactions were duplicated for each DNA template as PCR replicates were found to reduce PCR stochasticity and increase the detected fungal diversity (Alberdi *et al.*, 2018). PCR replicates were pooled before being tagged. After checking the amplification products by electrophoresis gels, amplicons of the PCR replicates in the first PCR were pooled together, cleaned with magnetic beads and attached with identical Nextera indexes in the second PCR. After quality assessment using Fragment Analyzer 5100, amplicons of 15 *Epipactis* samples, together with another 385 samples were pooled in equimolarity

per sample. The end pool was processed to generate pair-end reads of 300 bp using the MiSeq platform within one run (BaseClear, Leiden Bio Science Park, The Netherlands).

Raw sequences were processed using the DADA2 pipeline (Callahan *et al.*, 2016) in R. Taxonomic assignment was performed by querying the resulting 258 ASVs (amplicon sequence variants) against the UNITE database (Table S5) (Kõljalg *et al.*, 2013). To analyse the fungi associated with the orchid roots, we rarefied the dataset to 2031 reads per sample based on the sample with the fewest reads (Table S6), and only considered ASVs present in a sample if represented by at least six reads (15 samples with 137 ASVs). For further analysis, we selected fungi belonging to the typical rhizoctonia families (Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae; (Dearnaley *et al.*, 2012)), ectomycorrhizal fungi classified according to the FungalTraits database (Põlme *et al.*, 2020), and others previously detected in the roots of the selected orchids (Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Bidartondo & Read, 2008; Schiebold *et al.*, 2017). We retained 14 samples and 80 ASVs assigned to the taxonomic groups of interest. To distinguish between taxa within the order Sebacinales which can be ectomycorrhizal (Sebacinaceae) or rhizoctonia fungi (Serendipitaceae), we used phylogenetic analysis for their identification. We used available sequences from GenBank that belong to both families (and including one outgroup) to place the OTUs from our study within the order Sebacinales. The phylogenetic tree (Fig. S1) was made using MAFFT (Katoh & Standley, 2013) to obtain the highest likelihood tree, using RaxML HPC-SSE3 (Stamatakis, 2014) using the GTR+CAT model of substitution.

RESULTS

^{13}C stable isotope signatures

The mean enrichment factor $\varepsilon^{13}C$ of leaves of the four orchid species ranked as follows: *E. leptochila* (4.38 $\pm$ 1.04 [SD] ‰) > *E. atrorubens* (1.70 $\pm$ 0.63 ‰) > *E. palustris* (0.51 $\pm$ 0.51 ‰) > *A. sandvicensis* (-0.75 $\pm$ 1.86 ‰). The linear mixed models comparing leaf $\varepsilon^{13}C$ estimates depending on orchid species showed significant effects for most pairwise species comparisons (Table 1). However, $\varepsilon^{13}C$ values of *E. palustris* leaves were neither statistically distinct from *E. atrorubens* nor from *A. sandvicensis* leaves.

Within-site comparison showed that leaf ^{13}C signature differed between sample types (reference vs. orchid) only for *E. leptochila* and *E. atrorubens*. Leaf ^{13}C compositions of

E. palustris and *A. sandvicensis* were not statistically distinct from respective reference plants (Table 2). Compared to other plant compartments (stem & blossom, roots) the orchid leaves tended to be least enriched or even depleted in ^{13}C (*A. sandvicensis*) relative to reference plants (Fig. 2).

For all orchid species, $\varepsilon^{13}C$ of extracted pelotons was higher than $\varepsilon^{13}C$ of the respective orchids' leaves (Fig. 2). The mean $\varepsilon^{13}C$ of pelotons and respective orchid leaves differed by approx. 3 ‰ for all investigated orchids. Regarding *E. leptochila* and *E. atrorubens,* fungal pelotons showed the highest $\varepsilon^{13}C$ relative to all plant samples (Fig. 2). ^{13}C enrichments of pelotons extracted from *A. sandvicensis* and *E. palustris* were not significantly different, while all other pairwise comparisons of pelotons' $\varepsilon^{13}C$ values across orchid species were significantly different (Table 1).

Table 1 Pairwise comparisons of orchid species effect (*Epipactis leptochila, Epipactis atrorubens, Epipactis palustris* and *Anoectochilus sandvicensis)* on $\varepsilon^{13}C$ and e $\varepsilon^{15}N$ of leaves and pelotons, respectively, obtained from linear mixed models' post hoc tests comparing difference*s* in means. *P*-values were adjusted using Bonferroni-Holm correction. Underlying models were: Lmer ($\varepsilon^{13}C_{Leaves}$ ~ orchid species + (~1|plot)): $R^2 = 0.78$, Lmer ($\varepsilon^{15}N_{Leaves}$ ~ orchid species + (~1|plot)): $R^2 = 0.97$, Lmer ($\varepsilon^{13}C_{Pelotons}$ ~ orchid species + (~1|plot)): $R^2 = 0.80$, Lmer ($\varepsilon^{15}N_{Pelotons}$ ~ orchid species + (~1|plot)): $R^2 = 0.90$).

Leaves			**$\varepsilon^{13}C$**					**$\varepsilon^{15}N$**				
contrast			estimate	SE	df	*t*	*P*	estimate	SE	df	*t*	*P*
A. sandvicensis	*vs.*	*E. atrorubens*	-2.50	0.76	11	-3.20	**0.011**	-7.90	0.96	11	-8.30	**<0.001**
A. sandvicensis	*vs.*	*E. leptochila*	-5.10	0.76	11	-6.80	**<0.001**	-19.70	0.96	11	-20.40	**<0.001**
A. sandvicensis	*vs.*	*E. palustris*	-1.30	0.76	11	-1.70	0.122	-2.00	0.96	11	-2.10	0.063
E. atrorubens	*vs.*	*E. leptochila*	-2.70	0.63	10	-4.30	**0.003**	-11.70	0.80	10	-14.70	**<0.001**
E. atrorubens	*vs.*	*E. palustris*	1.20	0.63	10	1.90	0.107	5.90	0.80	10	7.40	**<0.001**
E. leptochila	*vs.*	*E. palustris*	3.90	0.63	10	6.10	**<0.001**	17.70	0.80	10	22.10	**<0.001**
Pelotons			**$\varepsilon^{13}C$**					**$\varepsilon^{15}N$**				
contrast			estimate	SE	df	*t*	*P*	estimate	SE	df	*t*	*P*
A. sandvicensis	*vs.*	*E. atrorubens*	-2.51	0.62	13	-4.08	**0.002**	-5.00	1.06	15	-4.70	**<0.001**
A. sandvicensis	*vs.*	*E. leptochila*	-4.91	0.55	12	-8.85	**<0.001**	-10.00	0.97	13	-10.00	**<0.001**
A. sandvicensis	*vs.*	*E. palustris*	-0.49	0.55	12	-0.88	0.390	0.00	0.97	13	0.00	1.000
E. atrorubens	*vs.*	*E. leptochila*	-2.39	0.56	11	-4.29	**0.002**	-5.00	0.99	12	-5.10	**<0.001**
E. atrorubens	*vs.*	*E. palustris*	2.02	0.56	11	3.62	**0.005**	5.00	0.99	12	5.10	**<0.001**
E. leptochila	*vs.*	*E. palustris*	4.42	0.52	11	8.54	**<0.001**	10.00	0.91	11	11.00	**<0.001**

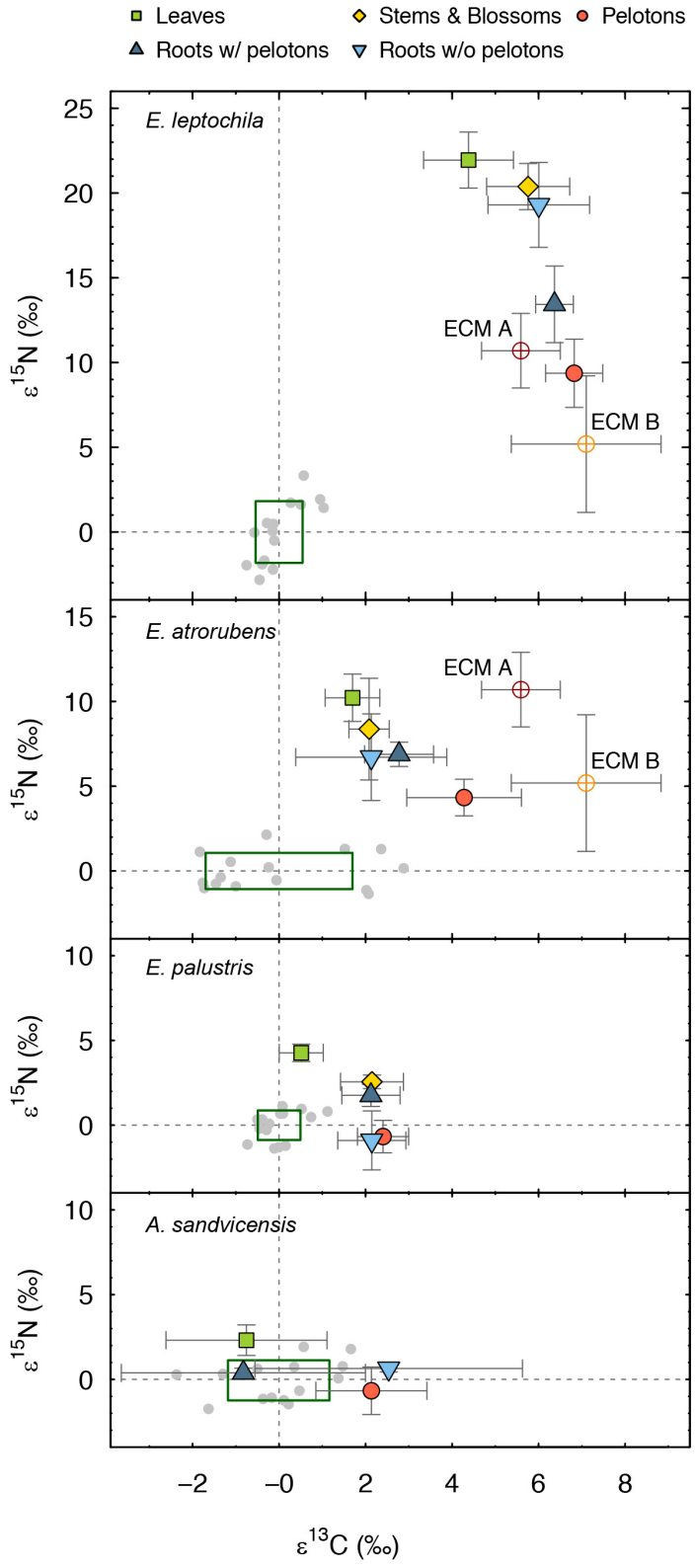

Fig. 2 Carbon and nitrogen stable isotope enrichment factors ε (mean ± SD) of orchid parts ('leaves', 'stems & blossoms', 'roots with pelotons', 'roots without pelotons') and isolated pelotons for *Epipactis leptochila, Epipactis atrorubens, Epipactis palustris and Anoectochilus sandvicensis.* Enrichment factors ε of autotrophic reference plants are indicated by the green frame (standard deviation around a mean enrichment factor of zero, by definition) and grey dots (single values). For n please refer to Table S1-S3. For comparison, carbon and nitrogen stable isotope enrichment factors ε (mean ± SD) of sporocarps of four ECM ascomycete *Tuber* species (ECM A) from Schiebold *et al.* (2017), and 11 ECM basidiomycete species (ECM B) from (Gebauer *et al.*, 2016).

Table 2 Summaries of linear mixed models (estimated using REML and nloptwrap optimizer, R function: lmer) predicting $\varepsilon^{13}C$ and $\varepsilon^{15}N$, respectively with Type (orchid vs. reference) for the orchid species *E. leptochila, E. atrorubens, E. palustris and A. sandvicensis*. For fixed effects Type [Orchid] is the baseline. All models include 'plot' as random effect. Model diagnostic was applied. 95% Confidence Intervals (CIs) and p-values were computed using a Wald t-distribution approximation.

E. leptochila

	$\varepsilon^{13}C$					$\varepsilon^{15}N$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(16)*	*P*	*Estimate*	*SE*	*CI*	*t(16)*	*P*
(Intercept)	4.38	0.31	[3.73 – 5.03]	14.31	**<0.001**	21.95	0.80	[20.26 – 23.64]	27.50	**<0.001**
Type [Ref]	-4.38	0.35	[-5.13 – -3.63]	-12.39	**<0.001**	-21.95	0.92	[-23.90 – -19.99]	-23.81	**<0.001**
Random effects	σ	n		*Marginal R^2*	*0.890*	σ	n		*Marginal R^2*	0.968
Plot	0.69	5		*AICc*	*52.422*	1.78	5		*AICc*	*86.921*

E. atrorubens

	$\varepsilon^{13}C$					$\varepsilon^{15}N$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(16)*	*P*	*Estimate*	*SE*	*CI*	*t(16)*	*P*
(Intercept)	1.70	0.68	[0.25 – 3.14]	2.49	**0.024**	10.22	0.51	[9.13 – 11.31]	19.87	**<0.001**
Type [Ref]	-1.70	0.79	[-3.37 – -0.03]	-2.15	**0.047**	-10.22	0.59	[-11.48 – -8.96]	-17.21	**<0.001**
Random effects	σ	n		*Marginal R^2*	*0.196*	σ	n		*Marginal R^2*	*0.940*
Plot	1.53	5		*AICc*	*81.287*	1.15	5		*AICc*	*71.102*

E. palustris

	$\varepsilon^{13}C$					$\varepsilon^{15}N$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(16)*	*P*	*Estimate*	*SE*	*CI*	*t(16)*	*P*
(Intercept)	0.51	0.22	[0.04 – 0.98]	2.31	**0.035**	4.27	0.39	[3.45 – 5.09]	11.01	**<0.001**
Type [Ref]	-0.51	0.26	[-1.05 – 0.03]	-1.99	0.064	-4.27	0.45	[-5.22 – -3.329]	-9.53	**<0.001**
Random effects	σ	n		*Marginal R^2*	*0.173*	σ	n		*Marginal R^2*	*0.827*
Plot	0.50	5		*AICc*	*40.863*	0.87	5		*AICc*	*60.934*

A. sandvicensis

	$\varepsilon^{13}C$					$\varepsilon^{15}N$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(13)*	*P*	*Estimate*	*SE*	*CI*	*t(13)*	*P*
(Intercept)	-0.75	0.74	[-2.36 – 0.85]	-1.01	0.329	2.31	0.66	[0.88 – 3.75]	3.49	**0.004**
Type [Ref]	0.74	0.82	[-1.02 – 2.51]	0.91	0.379	-2.37	0.73	[-3.95 – -0.79]	-3.24	**0.006**
Random effects	σ	n		*Marginal R^2*	*0.049*	σ	n		*Marginal R^2*	*0.397*
Plot	1.29	3		*AICc*	*65.197*	1.15	3		*AICc*	*61.805*

^{15}N stable isotope signatures

ε^{15}N values of leaves of the four orchid species arranged as follows: *E. leptochila* (21.95 ± 1.65 ‰) > *E. atrorubens* (10.22 ± 1.40 ‰) > *E. palustris* (4.27 ± 0.84 ‰) > *A. sandvicensis* (2.31 ± 0.9 ‰), and thus displaying the same order as ε^{13}C (Fig. 2). However, enrichment in ^{15}N relative to reference plants was always highest in orchid leaves compared to other orchid compartments of the same orchid species.

We found that leaves of all investigated orchids were significantly enriched in ^{15}N relative to their respective reference plants (Table 2). Enrichment of orchid leaves in ^{15}N was distinct from each other when comparing all four species pairwise (Table 1), with one exception (*A. sandvicensis* vs. *E. palustris*).

Fungal pelotons extracted from all four orchid species mostly showed the lowest enrichment in ^{15}N when compared with orchid compartments (Fig. 2). Enrichment factor ε^{15}N of pelotons extracted from *A. sandvicensis* (-0.67 ± 1.40 ‰) and *E. palustris* (0.07 ± 2.32 ‰) was close to 0 and thus similar to reference plants. Yet, ε^{15}N of pelotons gained from *E. atrorubens (*4.33 ± 1.09 ‰) and *E. leptochila* (9.37 ± 2.01 ‰) were distinct from respective autotrophic plants. Comparison of ε^{15}N in pelotons across orchid species, displayed significant differences for all pairwise contrasts, except for *A. sandvicensis* vs. *E. palustris* – a similar pattern that has been observed for ε^{13}C (Table 1).

Total N concentrations

Mean total N concentrations of reference plant leaves were similar across the *Epipactis* sampling sites: 1.35 ± 0.25 mmol g_{dw}^{-1} at *E. leptochila* site, 1.24 ± 0.16 mmol g_{dw}^{-1} at *E. atrorubens* site, 1.20 ± 0.23 mmol g_{dw}^{-1} at *E. palustris* site. For reference plants of *A. sandvicensis* mean leaf total N concentration was slightly lower and showed more variation (0.93 ± 0.41 mmol g_{dw}^{-1}). Leaves of all investigated orchid species showed higher total N concentrations than respective reference plant leaves (Fig. 3); this difference was statistically significant for *E. leptochila* (2.52 ± 0.41 mmol g_{dw}^{-1}), *E. atrorubens* (2.03 ± 0.19 mmol g_{dw}^{-1}) and *A. sandvicensis* (1.82 ± 0.04 mmol g_{dw}^{-1}), but not for *E. palustris (*1.77 ± 0.09 mmol g_{dw}^{-1}). Other orchid compartments, such as stems and blossoms and roots, had lower N concentrations than orchids' leaves.

Mean total N concentrations of pelotons extracted from *E. leptochila* (2.44 ± 0.12 mmol g_{dw}^{-1}) *and E. atrorubens* (2.36 ± 0.53 mmol g_{dw}^{-1}) were similar to orchid leaf

N concentrations (2.51 ± 0.41 mmol g_{dw}^{-1} and 2.03 ± 0.19 mmol g_{dw}^{-1}, respectively). Peloton N concentrations extract from *E. palustris* (1.25 ± 0.35 mmol g_{dw}^{-1}) and *A. sandvicensis* (1.19 ± 0.26 mmol g_{dw}^{-1}) were in the range of those of reference plant leaves (Table S2, Table S3). For *A. sandvicensis*, extracted pelotons had significantly smaller total N concentrations than orchid leaves (Fig. 3, Table S3).

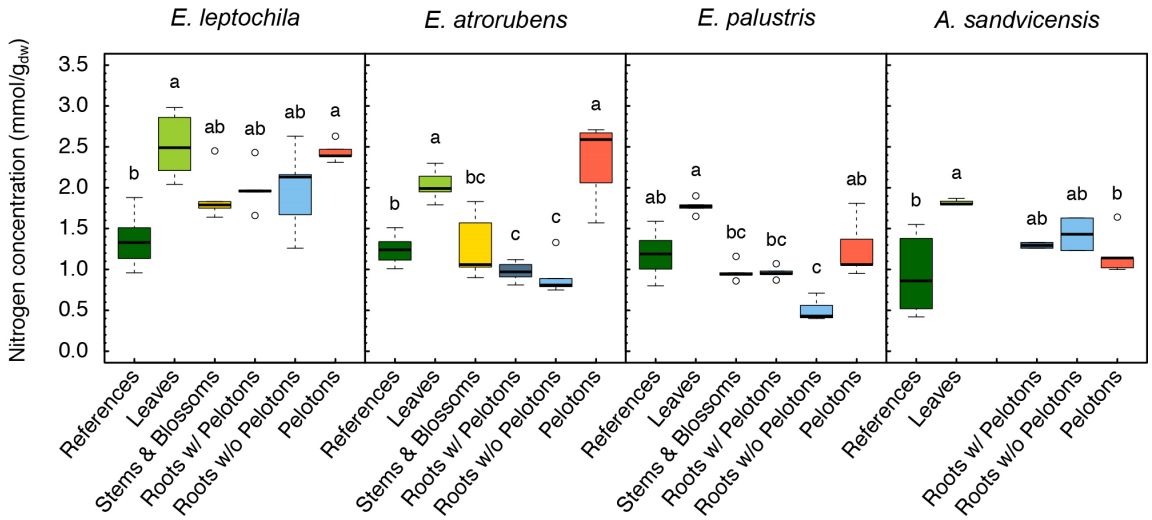

Fig. 3 Total nitrogen concentration of orchid parts ('leaves', 'stems & blossoms', 'roots with pelotons', 'roots without pelotons') for *Epipactis leptochila* (n = 5), *Epipactis atrorubens* (n = 5), *Epipactis palustris* (n = 5), *Anoectochilus sandvicensis* (n = 6) and extracted pelotons and reference plants. The box spans the first and third quartile, while the horizontal line in the box represents the median; whiskers extend to the 1.5*interquartile range. Different letters indicate statistically significant differences (Dunn's test with Bonferroni-Holm *P*-value adjustment) between groups. Means sharing a letter are not significantly different. For n please refer to Table S2-S3.

^{2}H and ^{18}O stable isotope signatures

$\varepsilon^{2}H$ and $\varepsilon^{18}O$ data are available for *E. leptochila* (Figure 4). Overall, fungal pelotons and orchid compartments were enriched in ^{2}H, while they were depleted in ^{18}O relative to the reference plants.

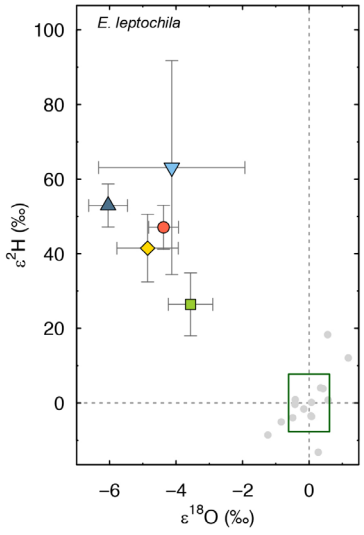

Fig. 4 Hydrogen and oxygen stable isotope enrichment factors ε (mean ± SD) of orchid parts 'leaves' (square, n = 5), 'stems & blossoms' (diamond, n = 5), 'roots with pelotons' (triangle upwards, n = 5), 'roots without pelotons' (triangle downwards, n = 5) and isolated pelotons (circle, n = 5) for *Epipactis leptochila.* Enrichment factors ε of autotrophic reference plants are indicated by the green frame (standard deviation around a mean enrichment factor of zero, by definition) and grey dots (single values).

Root fungi isolated from *Epipactis* species

Fungal sequencing detected rhizoctonia fungi belonging to the families Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae in the root samples of *E. palustris*. Fungi known to form ectomycorrhizas were predominant in the roots of both species, *E. atrorubens* and *E. leptochila* (Fig. 5).

We identified a variety of fungal families belonging to the Ascomycota (Gloniaceae, Pyronemataceae, Helvellaceae, Tuberaceae) and Basidiomycota (Sebacinaceae, Melanogastraceae, Inocybaceae, Thelephoraceae) in the roots of *E. atrorubens*, with the majority belonging to Gloniaceae (Ascomycota). For one sample of *E. atrorubens,* a high relative abundance of Tuberaceae was detected.

Tuberaceae were the most abundant fungi in all root samples of *E. leptochila*, with only one exception. For one *E. leptochila* individual we found a high relative abundance of Thelephoracea and Ceratobasidiaceae, the latter being assigned to the rhizoctonia guild (Figure 5). In lower relative abundances, Sebacinaceae and Gloniaceae were present in up to two *E. leptochila* root samples. In all root samples of investigated *Epipactis* spp. we detected ASVs that were assigned to the fungal family Helotiaceae with a high share in *E. palustris* samples.

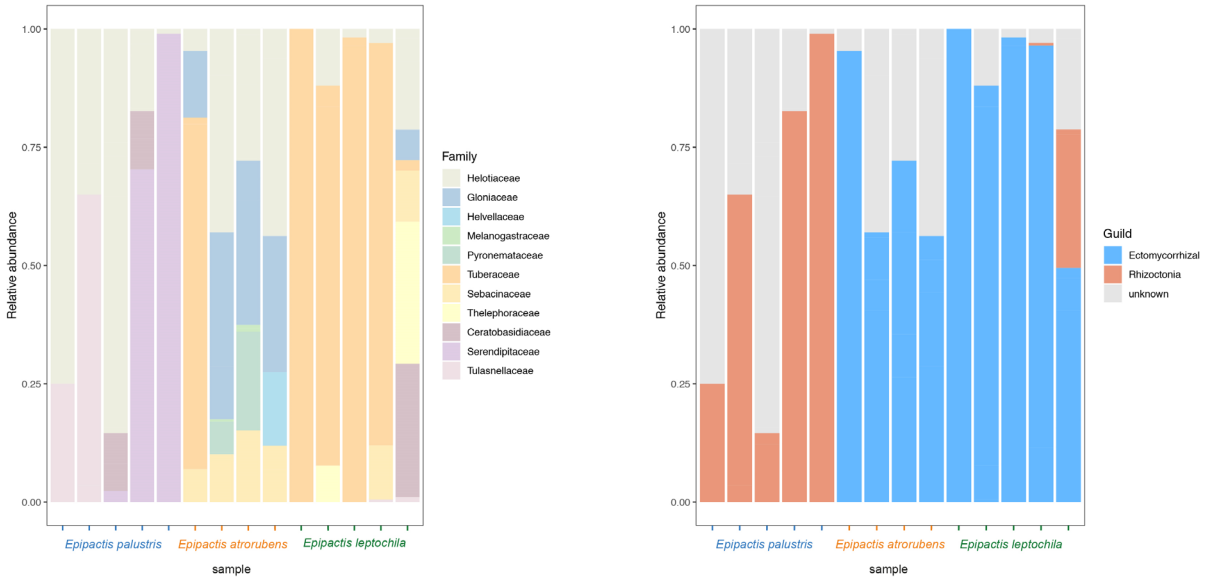

Fig. 5 Fungal diversity detected in the roots of individual plants of (A) the orchid species: *E. leptochila* (n = 5), *E. atrorubens* (n = 4), *E. palustris* (n = 5) represented per fungal family (left panel) and fungal guild (right panel). Note: The ecological role of Helotiaceae in orchid roots is unknown as yet (Tedersoo *et al.*, 2009, 2010; Jacquemyn *et al.*, 2016).

DISCUSSION

^{13}C enrichment of rhizoctonia pelotons smaller than of ECM-type pelotons

Obtaining stable isotope data of orchid mycorrhiza pelotons, we intended to investigate both, orchids associated with rhizoctonia and orchid species that are involved in tripartite ECM networks with Ascomycota and/or Basidiomycota. Fungal sequencing overall substantiates earlier findings and our expectations, that *E. palustris* is mycorrhizal with fungi assigned to the rhizoctonia guild (Bidartondo *et al.*, 2004; Illyés *et al.*, 2009; Jacquemyn *et al.*, 2016, 2017; Schweiger, 2018). Apart from Ceratobasidiaceae and Serendipitaceae, we also detected Tulasnellaceae as major symbionts of *E. palustris* which was rather not reported by previous studies. While *A. sandvicensis* is also rhizoctonia-associated, it displays extreme fungal specificity to closely related OTUs of *Ceratobasidium* at our study site (Swift *et al.*, 2019). In contrast, roots of *E. atrorubens* and *E. leptochila* were predominantly colonised by fungi that are ectomycorrhizal with trees (cf. Schiebold *et al.*, 2017). The ecological role of endophytic Helotiaceae in orchid roots is largely unknown as yet (Tedersoo *et al.*, 2009, 2010; Jacquemyn *et al.*, 2016), nonetheless, we detected Helotiaceae in all *Epipactis* spp. samples. Notably, Helotiaceae can serve beneficial dark septate endophytes (DSEs) for plants (Newsham, 2011), and the important ecological function of DSEs in the nutrition of orchids particularly in stressful environments is only just at the beginning of being addressed (Liu *et al.*, 2022).

We expected the smallest enrichment in ^{13}C for pelotons extracted from *A. sandvicensis* and *E. palustris* because leaf material of rhizoctonia-associated orchids is usually not enriched in ^{13}C (Hynson *et al.*, 2013). Indeed, as hypothesised, enrichment in ^{13}C of pelotons extracted from *A. sandvicensis* and *E. palustris* was similar irrespective of fungal specificity and considerably smaller than $\varepsilon^{13}C$ of pelotons isolated from the forest-dwelling, ECM-associated *E. leptochila* and *E. atrorubens.* Although pelotons of all investigated orchid species (incl. *Ophrys insectifera* and *Orchis militaris,* Gomes *et al.* 2023) displayed by approx. 3 ‰ higher mean $\varepsilon^{13}C$ values than leaves of respective reference plants, the relatively small ^{13}C enrichment of rhizoctonia pelotons extracted from *A. sandvicensis* and *E. palustris* may explain the inconspicuous ^{13}C enrichment of rhizoctonia-associated orchids. Yet, the small $\varepsilon^{13}C$ of rhizoctonia pelotons (< 3 ‰) limits the suitability as a nutrition indicator for mycoheterotrophic C gain of rhizoctonia-associated orchids. Nonetheless, enrichment in ^{15}N and higher N concentrations relative

to reference plant leaves hint at the capability of *A. sandvicensis* and *E. palustris* for a cryptic manifestation of partial mycoheterotrophy as previously suggested (Schiebold *et al.*, 2017; Lallemand *et al.*, 2018; Swift *et al.*, 2019). Yet, autotrophy remains as a possible nutrition mode of *A. sandvicensis* and *E. palustris*. Pursuing the ^{2}H approach according to Gebauer *et al.*, (2016), future ^{2}H and ^{18}O analysis of extracted pelotons and leaves could contribute substantially to resolving the degree of mycoheterotrophic nutrition for rhizoctonia-associated orchids, like from *A. sandvicensis* and *E. palustris.* So far, we were able to get the first ^{2}H and ^{18}O data merely of pelotons extracted from *E. leptochila* because isolating sufficient material remains challenging.

At the same time, our data confirm a distinct fungus-to-plant C transfer for investigated orchids associated with ECM-type fungi (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Hynson *et al.*, 2016; Schiebold *et al.*, 2017). In this respect, leaves of *E. leptochila* and *E. atrorubens* were enriched in ^{13}C relative to reference plants and they mirror the relatively high ^{13}C values of extracted pelotons. Additionally, the highest ^{15}N enrichment and significantly higher total N concentrations of *E. leptochila* and *E. atrorubens* leaves compared to autotrophic plants hint at partially mycoheterotrophic nutrition. For *E. leptochila,* ^{2}H data adduce an additional proof (Gebauer *et al.*, 2016), and ^{13}C enrichment factors indicated the highest C gain from fungal partners among all investigated orchids, with the latter being concordant with earlier findings (Hynson *et al.*, 2016). Yet, a comparison between *E. leptochila* and *E. atrorubens* in terms of ^{13}C natural abundance of orchid compartments and pelotons, respectively, demonstrated similar isotopic arrays for both orchid species.

Isotopic signature reveals nutrient exchange function of root fungi

The impact of the fungal phyla an orchid associates with on the orchid's isotopic signature is controversial. Besides differences in mycorrhizal communities (Schiebold *et al.*, 2017), physiological variations between species (Jacquemyn *et al.*, 2021) were adduced to influence isotopic signatures of orchid leaves. We aimed to tackle this issue by comparing the first stable isotope data of orchid mycorrhizal pelotons to data from fungal fruit bodies and orchid leaves (Fig. 2) in combination with molecular barcoding of mycobionts.

As suspected, ^{15}N values of pelotons extracted from *E. leptochila* and *E. atrorubens* roots showed the same array as fruit bodies of ECM Ascomycota and ECM Basidiomycota, their dominant mycorrhizal root fungi (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). However,

pelotons of rhizoctonia-associated orchids (*A. sandvicensis* and *E. palustris)* were least enriched in ^{15}N, a pattern being reflected in the ^{15}N isotopic signature of the orchid leaves and also reported for rhizoctonia-associated *Ophrys insectifera* L. and *Orchis militaris* L. (Gomes *et al.* 2023).

Focusing on the ECM-associated orchids, we identified a difference in isotopic signatures coming along with variation in fungal partners. The higher $\varepsilon^{15}N$ values found for extracted pelotons and orchid tissues of *E. leptochila* (9.37 ± 2.01 ‰) relative to *E. atrorubens* (4.33 ± 1.09 ‰) can be explained by the dominance of *Tuber* species in *E. leptochila* roots as *Tuber* species (Ascomycota) have been shown to have higher ^{15}N enrichment than ECM Basidiomycota (10.70 ± 2.20 ‰ vs. 5.19 ± 4.03 ‰) collected at identical locations (Schiebold *et al.*, 2017). Also, *E. atrorubens* roots were mainly colonized by ECM Ascomycota fungi, with a relatively high share of the *Cenococcum* species (Gloniaceae, Ascomycota), a widespread ectomycorrhizal fungi clade in temperate forest ecosystems (Spatafora *et al.*, 2012). One *E. atrorubens* roots sample even revealed *Tuber* as the dominant root fungi. However, besides ECM Ascomycota we isolated ECM Basidiomycota from *E. atrorubens* roots most likely causing lower ^{15}N natural values of orchid tissues and respective pelotons compared to *E. leptochila.* Remarkably, $\varepsilon^{15}N$ values of pelotons from *E. leptochila* (9.37 ± 2.01‰) and *E. atrorubens* (4.33 ± 1.09 ‰) showed the same range as fruit bodies of ECM Ascomycota (10.74 ± 2.18 ‰) and ECM Basidiomycota (5.19 ± 4.04 ‰), respectively (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017) and therefore mirror the ^{15}N enrichment of sporocarps of the respective fungal phyla they associate with (Fig. 2). While results of fungi DNA analysis within one orchid species can vary substantially across root samples (e.g. *E. leptochila* and *E. atrorubens*, Fig. 5), it is essential to consider that only a small cut-out of the roots is being analysed. Stable isotope analyses of bulk samples, however, enable interferences of plants' physiological processes integrated over space and time (Dawson *et al.*, 2002). We provide evidence that pelotons display distinct ^{15}N patterns depending on the present fungal guild like previously proposed for fruit bodies (Schiebold *et al.*, 2017). Considering that pelotons act as the orchids' direct fungal source and that the difference in isotopy of pelotons is reflected in ^{15}N stable isotope patterns of orchid leaves, we further argue that the isotopic signature of orchid leaves reveals the functional purpose of the present fungi regarding the fungus-to-plant nutrient transfer. Other than Jacquemyn *et al.* (2021), our findings support a linkage between 'functional' mycorrhizal partners and isotope signatures as suggested by

Schiebold *et al.* (2017). Further, we claim that a high prevalence of fungal taxa in orchid roots may not be directly indicative of playing a major role in their nutrition. As ^{15}N of mycorrhizal fungi is related to the N sources used (organic N, NH_4^+, NO_3^-), the depth of soil at which the mycelium occurs, and metabolic fractionations (Taylor *et al.*, 1997) the variety of fungi present in orchid roots can be narrowed to a selection of fungi with relevance for the orchids nutrition. Stable isotope analysis thus considerably complements the information about the presence of root fungi with evidence about their functional role in the plants' nutrition. Nonetheless, more data are needed to substantiate if there is a distinct difference in orchid leaf ^{15}N signature depending on Asco- vs. Basidiomycota mycobionts or on a smaller taxonomic level (e.g. fungal family). To this end, the future extraction of larger quantities of fungal pelotons from orchid roots in order to utilize peloton samples for both, stable isotope analysis and fungal sequencing, would allow the isotope data from the pelotons to be attributed directly to the fungal taxa that form them.

Peloton lysis and selective utilisation of ^{15}N-enriched protein-N by orchid

Although the ^{15}N isotopic signature of pelotons from *E. leptochila* and *E. atrorubens* showed the same arrangement as sporocarps of ECM Ascomycota and ECM Basidiomycota, respectively, mean $\varepsilon^{15}N$ values tended to be smaller (9.37 ± 2.01 & 4.33 ± 1.09 ‰ vs. 10.70 ± 2.20 & 5.19 ± 4.03 ‰) and N concentrations lower (2.44 ± 0.12 & 2.36 ± 0.53 mmol g_{dw}^{-1} vs. 2.90 ± 0.38 & 2.81 ± 0.95 mmol g_{dw}^{-1}) (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). Certainly, the cap of a mushroom displays higher $\delta^{15}N$ values and N concentration than its stipe (Taylor *et al.*, 1997; Hobbie *et al.*, 2012; Vaario *et al.*, 2019), while fruit bodies overall are more enriched in ^{15}N than extraradical hyphae (Kohzu *et al.*, 1999; Hobbie & Colpaert, 2003; Wallander *et al.*, 2004).

The isotopic pattern of pelotons could be induced by a selective transport of certain compounds, as previous studies have indicated a nutrient transfer from fungus to orchid across the mycorrhizal interface or after a degeneration and lysis of pelotons in the orchid roots cells. (e.g. Kristiansen *et al.*, 2001; Selosse *et al.*, 2004; Cameron *et al.*, 2006; Chang & Chou, 2007; Hobbie & Högberg, 2012; Bougoure *et al.*, 2014; Kuga *et al.*, 2014; Suetsugu *et al.*, 2017; Favre-Godal *et al.*, 2020). Aside from soluble carbohydrates (Cameron *et al.*, 2006; Ponert *et al.*, 2021) amino acids, playing a significant role in N transfer, have been recognised to participate in C transfer in orchid mycorrhiza (Cameron *et al.*, 2006, 2008;

Dearnaley & Cameron, 2017; Fochi *et al.*, 2017; Favre-Godal *et al.*, 2020; Valadares *et al.*, 2021). Notably, fungal proteins and amino acids are enriched in ^{15}N by approx. 10 ‰ relative to fungal chitin, irrespective of the part of the fungus examined (Taylor *et al.*, 1997; Hobbie & Colpaert, 2003). Remarkably, pelotons of *E. leptochila* and *E. atrorubens* were by approx. 10 ‰ less enriched in ^{15}N compared to respective orchid leaves. Smaller mean ^{15}N enrichments of pelotons relative to fruit bodies and orchid symbionts could reflect preferential incorporation of ^{15}N-enriched, protein-derived N, i.e. hyphal cell content, in the plant, while ^{15}N-depleted fungal cell wall chitin-N remains as the extracted pelotons. This is further supported by similar or smaller total N concentrations of pelotons relative to fungal fruit bodies and orchid leaves. Similar reports exist for mycoheterotrophic plants (Trudell *et al.*, 2003; Hobbie & Högberg, 2012) and Ericaceae (Tedersoo *et al.*, 2007). Yet, the possibility of bidirectional transport (Cameron *et al.*, 2008; Suetsugu *et al.*, 2017) and the degree of peloton lysis (Kuga *et al.*, 2014) can cause variations in isotopic patterns of pelotons. Anyhow, a fungal nitrogen source other than mycorrhizal pelotons remains a possible option to be involved in the nutrition of orchids (e.g. other endophytic fungi, bacteria and non-mycorrhizal pelotons). For instance, Helotiaceae were abundant in the investigated orchid roots and DSEs being non-mycorrhizal fungi can also form peloton-like structures in orchids (Liu *et al.*, 2022) and have been shown to affect isotope N signatures of plants (Giesemann *et al.*, 2020). Though unlikely, we cannot entirely exclude extraction artefacts, i.e. selective loss of parts of the fungal cell content, to have additionally influenced isotopic signatures found for the pelotons.

Stable isotope signature of different orchid compartments

Comparing stable isotope signature and total N concentrations of different orchid compartments across all here investigated orchid species ^{13}C and ^{15}N patterns resembled and can be attributed to their tissue composition – a pattern similarly found for partially mycoheterotrophic *Pyrola japonica* (Matsuda *et al.*, 2020). Namely, compartments with no or lower photosynthetic capacity, such as ‘stems & blossoms’ and ‘roots’ mainly consist of cellulose and hemicellulose, which usually have higher $\delta^{13}C$ values (Gebauer & Schulze, 1991; Gleixner *et al.*, 1993). Photosynthetic leaf tissue, however, has a higher share of secondary metabolites like lipids and proteins with lower $\delta^{13}C$ values (Winkler *et al.*, 1978; Tieszen & Boutton, 1989) and higher total N concentration compared to other

orchid compartments (Field & Mooney, 1986; Gebauer *et al.*, 1988; Evans, 1989). Varying differences in N concentration between orchid and respective reference plant leaves among the studied orchid species are likely the result of variation in N surplus and deposition in orchid leaf tissue linked to their degree of nutrient gain from fungi. That we did not detect a clear pattern for ^{15}N and ^{13}C comparing roots with and without pelotons may be due to our separation method based on a rough optical assessment. Both types of root samples may exhibit different levels of purity. Particularly for highly colonized orchid roots, e.g. for *E. leptochila* and *E. atrorubens*, the presence of pelotons in 'roots without' samples cannot be fully ruled out. Roots without pelotons could also be colonized to a significant extent by endophytic hyphae, which do not form pelotons, but which contribute in some way to the nutrition of the orchid. ^{2}H enrichment of all *E. leptochila* orchid compartments and pelotons matched its mycoheterotrophic nutrition. Differences in transpiration between *E. leptochila* and reference plants, indicated by ^{18}O depletion of orchid leaves, could have even minimized the effect of mycoheterotrophy on ^{2}H enrichment (Ziegler, 1989; Cernusak *et al.*, 2004).

Conclusions and future directions

With this investigation, we present the first natural abundance of ^{15}N and ^{13}C data of fungal pelotons isolated from rhizoctonia-associated orchids (contemporaneous with Gomes *et al.* 2023) and of pelotons extracted from orchids entangled in ectomycorrhizal networks with Ascomycota and/or Basidiomycota. Because small ^{13}C enrichment of rhizoctonia pelotons is obviously not suited as a nutrition indicator of rhizoctonia-associated orchids, we suggest refining the ^{2}H approach with regards to pelotons. This could contribute to elucidate the relevance of partial mycoheterotrophy among rhizoctonia associated orchid species. Yet, isolating sufficient material proves to be difficult but we were able to make a start by providing novel ^{2}H and ^{18}O data of *E. leptochila* pelotons. Though currently limited to C isotopes, within-cell spatial microanalysis of natural stable isotope abundance using ablation-isotope ratio mass spectrometry following Rodionov *et al.* (2019) could be a further approach to solve this issue in the future.

Extracting intracellular hyphal material from plant roots contributes to understanding the complex relationship between plants and their fungal symbionts because it helps to recognise predictable stable isotope patterns of orchid leaves depending on their

functional, nutrition-specific fungal symbionts. Extracted pelotons have an advantage over fungal fruit bodies in terms of availability and the possibility to directly obtain the isotopic signature of the abundant multiple root endophytic fungi and thus provide straightforward insights into the fungi's functional role. Lysis of pelotons in the orchid roots followed by a selective transfer of the fungal cell content, i.e. rather ^{15}N-enriched protein-N, into orchids is the most likely explanation for our results.

Our investigations demonstrate that the combination of both stable isotope data and mycorrhizal fungal diversity information is highly beneficial but reasonable care needs to be given to interpretation as each of both methods has its explicit limitations and power. To further explore the functional role of the multiple fungi abundant in orchid roots, future investigations should aim to provide both isotopic data but also fungal sequencing data directly from extracted pelotons.

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AUTHORS' CONTRIBUTIONS

ES and FEZ collected and processed the samples of the *Epipactis* species. TKC and NAH contributed the samples of *A. sandvicensis.* FEZ analysed the isotope abundance data and wrote the first manuscript draft. DW conducted the molecular analyses, and together with SIFG and FEZ processed the data. GG developed the idea for the project and supervised the isotope abundance analyses. NAH, TKC, ES and FEZ contributed to the research design. JP provided equipment essential for the peloton extraction and contributed to the manuscript. All authors commented and approved the final version of the manuscript.

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COMPETING INTERESTS

None declared.

DATA AVAILABILITY

Isotope data are available in the Supporting Information. Raw sequencing data are available at GenBank with SRA accession numbers SRR24401816- SRR24401822 within the BioProject PRJNA966147.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article (at https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.18991):

Fig. S1 Phylogenetic placement of detected taxa within the order Sebacinales to distinguish between Sebacinaceae (ectomycorrhizal) and Serendipitaceae (rhizoctonia fungi) based on available sequences from GenBank.

Table S1 Mean and single $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, i.e., isotope shifts of individual plants relative to the mean isotopic composition of autotrophic reference plants, and total nitrogen concentration data of autotrophic reference species per target orchid species.

Table S2 Mean and single $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, i.e., isotope shifts of individual plant tissue relative to the mean isotopic composition of autotrophic reference plants and total nitrogen concentration data of orchid compartments (leaf, stem & blossom, roots with pelotons, roots without pelotons) of *Anoechtochilus sandvicensis*, *Epipactis palustris*, *Epipactis leptochila*, *Epipactis atrorubens.*

Table S3 Mean and single $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, i.e., isotope shifts of individual plant tissue relative to the mean isotopic composition of autotrophic reference plants, and total nitrogen concentration data of pelotons extracted from *Anoechtochilus sandvicensis*, *Epipactis palustris*, *Epipactis leptochila*, *Epipactis atrorubens*.

Table S4 Equipment and conditions as used for stable isotope abundance analysis.

Table S5 Sequencing data and taxonomic assignments of fungal ASVs of orchid root samples (including non-mycorrhizal ASVs) before quality control.

Table S6 Sum of reads per sample before and after quality control.

MANUSCRIPT 5

Stable isotope analysis indicates partial mycoheterotrophy in arbuscular mycorrhizal woody seedlings in tropical forests

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ABSTRACT

1. Chlorophyllous plants exhibiting partial mycoheterotrophy obtain carbon through photosynthesis and mycorrhizal interactions. In arbuscular mycorrhizal (AM) plants, the *Paris*-morphotype (i.e. hyphal coils) is considered essential for mycoheterotrophic carbon gains. Numerous tree species in tropical lowland forests form this morphotype, and under light- and nutrient-limitation, additional carbon gain would be beneficial. However, if seedlings of woody species in the understory of tropical lowland forests exhibit partial mycoheterotrophy remains unexplored.

2. Here we (a) examined the AM morphotype (*Paris*- or *Arum*-type) in seedlings of 42 tropical woody species, and (b) to determine if any of the target *Paris*-type species are partially mycoheterotrophic, we compared their multi-element stable isotope natural abundance (^{13}C, ^{2}H, ^{18}O, ^{15}N) to neighbouring autotrophic non *Paris*-type reference seedlings.

3. 50 % of the investigated species (and 80 % of the genera) exhibited the *Paris*-type, expanding the number of tropical plant genera with *Paris*-type AM. Enrichment in ^{13}C, but not in ^{18}O in target compared to neighbouring reference plants indicated partial mycoheterotrophy in seedlings of 6 of the 21 investigated *Paris*-type AM species.

4. Our results provide the first evidence that carbon gain through mycoheterotrophy occurs in seedlings of AM tropical tree species. In tropical forests, partial mycoheterotrophy during seedling establishment may confer so far unrecognised ecological advantages influencing seedling recruitment and ecosystem dynamics.

INTRODUCTION

The vast majority of plants are associated with mycorrhizal fungi, which optimise their nutrient and water supply from soil. In return, mycorrhizal fungi receive organic carbon (C) compounds from the plant associates (Smith & Read, 2008). This mutualistic interaction, however, can shift to an exploitive disparity, where plants often referred to as 'cheaters' gain C from their fungal partners – a nutritional mode called mycoheterotrophy (Leake, 1994; Merckx, 2013). Some plants use fungal-derived C (either from photosynthesizing plants or from saprotrophic nutrition) to such an extent that they lost their photosynthetic ability and became achlorophyllous. This phenomenon, which is known as full mycoheterotrophy (FMH), occurs in several hundred endo-mycorrhizal plant species (Merckx, 2013). Mycoheterotrophy leads to an enrichment of plant tissues in the heavy isotopes ^{13}C, ^{2}H and frequently ^{15}N in comparison to neighbouring autotrophic plants' isotopic signatures, which can be used to identify and quantify mycoheterotrophic C gain (Gebauer & Meyer, 2003; Merckx *et al.*, 2009; Gomes *et al.*, 2020).

Some chlorophyllous mycorrhizal plant species gain C from their associated fungi in addition to their own photosynthetic C gain, i.e. they exhibit partial mycoheterotrophy (PMH). This nutritional mode has been recognised in herbaceous plants, mostly from the Orchidaceae, Ericaceae and some Gentianaceae. It is considered, *inter alias*, a strategy to gain additional C where photosynthesis is severely light-limited (Gebauer & Meyer, 2003; Zimmer *et al.*, 2007; Cameron & Bolin, 2010; Preiss *et al.*, 2010; Hynson *et al.*, 2013). Recent evidence from stable isotope analyses shows that in herbaceous species additional C gain from fungal partners (i.e. PMH) is considerably more widespread than previously assumed (Giesemann *et al.*, 2021).

Over 70 % of flowering plant species are associated with endo-symbiotic arbuscular mycorrhiza (AM, Brundrett, 2017). The AM fungal partners belong to the subphylum Glomeromycotina within the phylum Mucoromycota (Spatafora *et al.*, 2016). AM is typical for most herbaceous (i.e. non-woody) plant species, and has been especially well studied for agricultural plants (Genre *et al.*, 2020). In forest ecosystems, AM associations dominate in tropical lowland trees (e.g. Alexander, 1989; Mangan *et al.*, 2004), while ectomycorrhizal associations prevail in boreal and temperate biomes (Baldrian *et al.*, 2023). Studies of C transfer between woody plant species have mainly focussed on

ectomycorrhizal plants (e.g. Simard *et al.*, 1997; Avital *et al.*, 2022; Cahanovitc *et al.*, 2022; Klein *et al.*, 2023), but rarely AM plants (e.g. Lerat *et al.*, 2002; Avital *et al.*, 2022), and these studies did not focus on partial mycoheterotrophy.

With about 43000 tree species, tropical forests contain by far the highest tree diversity of any biome, yet, they belong to the most threatened terrestrial systems (Wright, 2010). Intact tropical forests provide important ecosystem services, holding tremendous C pools, and acted as net C sinks in the past (Pan *et al.*, 2011; Brockerhoff *et al.*, 2017; Harris *et al.*, 2021). Vegetation-climate feedbacks with tropical forests are crucial for global climate change projections, and their C relations have thus received considerable attention (Strengers *et al.*, 2010; Hubau *et al.*, 2020). Yet, if woody plants in tropical forests supplement C from fungi through PMH has not been addressed (Kuyper & Jansa, 2023).

Seedlings in the shaded understory of tropical forests are severely light limited (Pearcy, 1987; Holste *et al.*, 2011), with light conditions often below the light compensation point (Baltzer & Thomas, 2007). Yet, seedlings of many species can survive, i.e. maintain a positive C balance, for many years (Hubbell *et al.*, 1999; Rüger *et al.*, 2009). Additionally, soil phosphorus (P) availability to plants is low in many tropical forests (Vitousek, 1984; Turner *et al.*, 2007; Turner & Engelbrecht, 2011), with nutrient limitation restricting plant growth (Holste *et al.*, 2011; Santiago *et al.*, 2012). Due to drought during the dry season water availability to plants is reduced, also limiting C gains from photosynthesis (Engelbrecht *et al.*, 2002; Comita & Engelbrecht, 2009; Santiago *et al.*, 2017). Under these conditions, an ability for additional C gain through mycoheterotrophy might provide a significant ecological and evolutionary advantage. In fact, mycoheterotrophic AM plant lineages probably evolved in tropical forests (Merckx & Bidartondo, 2008), where light and P (but not N) are limiting factors. At the same time, AM allowed various achlorophyllous FMH herb taxa to diversify in the tropics (Merckx, 2013; Gomes *et al.*, 2019).

A specific AM morphotype, the *Paris*-morphotype (named after its occurrence in *Paris quadrifolia*), occurs in all achlorophyllous FMH plants and is thus widely considered indispensable for their fungal C gain (Imhof, 1999). This morphotype is characterised by intracellular hyphal coils that colonise the plant root cells (Gallaud, 1905). In contrast, a second AM morphotype, the *Arum*-type (named after its occurrence in *Arum maculatum)*, has intercellular hyphae with emerging branched fungal structures (i. e. arbuscules).

Chlorophyllous herbaceous PMH AM plant species in the understory of temperate forests also all exhibited the *Paris*-type AM, while plants with *Arum*-type AM exhibited autotrophy (Giesemann *et al.*, 2020b, 2021). *Paris*-type AM is thus considered to be a prerequisite for fungal C gain in both achlorophyllous (FMH) and chlorophyllous (PMH) AM plant species, yet not all plant species with *Paris*-type AM gain C from fungi (Giesemann *et al.*, 2021; Murata-Kato *et al.*, 2022).

The *Paris*-morphotype occurs in a significant proportion (≈ 40 %) of all AM plants (Dickson *et al.*, 2007). Thus, a large share of AM plant species globally may potentially exhibit partially mycoheterotrophic C gain (Giesemann *et al.*, 2021). So far, research on PHM has focused on herbaceous understory plants (Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022).

Quite a number of common tropical tree species are in genera that form *Paris*-type AM, indicated for example by a comparison of a comprehensive review of AM morphotypes (Dickson *et al.*, 2007) with tree species occurring in forests of Central Panama (Condit *et al.*, 2013). 31 plant genera included in the review contain woody species that occur in the Panama Canal area.(Condit *et al.*, 2013) Thereof, 18 genera (which include 64 woody species in Central Panama) had previously been classified as *Paris*-type (and 13 genera with 51 species as *Arum*-type). Combined with the potential ecological advantages that PHM might confer to plants in the understory of tropical forests, we expect that mycoheterotrophy might occur, or even be widespread among seedlings of tropical tree species. Since all AM fungi are obligate biotrophic, i.e. gain their C from living host plants (Trépanier *et al.*, 2005), and lack saprotrophic capability (Tisserant *et al.*, 2013), that would imply C sharing among trees *via* common mycorrhizal networks (CMN, Horton, 2009). This in turn may have pervasive, yet entirely unrecognized implications for the composition, productivity and C cycle of tropical forests. However, investigations on PHM in AM woody species are currently lacking in tropical forest ecosystems as well as in other ecosystems.

Mycoheterotrophic understory plants that utilize obligate biotrophic AM fungi as a C source can be identified by comparing their natural stable isotope signature to autotrophic plants co-occurring under the same environmental conditions. Tissues of mycoheterotrophic plants are enriched in ^{13}C, reflecting a relative ^{13}C enrichment of their fungal C-source, i.e. the AM fungal hyphae (Courty *et al.*, 2011, Gomes *et al.*, 2020, 2023;

Klink *et al.*, 2020, 2022), which in turn is due to the fact that the fungi take up ^{13}C-enriched carbohydrates from their photosynthesising host plants (Gleixner *et al.*, 1993). Significant ^{2}H enrichment of leaves can serve as an additional and independent indication for fungus-to-plant C transfer (Gebauer *et al.*, 2016; Cormier *et al.*, 2018), because secondary heterotrophic organic compounds originating from fungi, are often enriched in ^{2}H compared with primary photosynthetic compounds (Yakir, 1992). However, ^{13}C and ^{2}H abundances of leaves are also strongly influenced by stomatal regulation. Leaf ^{18}O isotope abundances, which are affected by transpiration but not by mycoheterotrophic C exchange, can be used to separate the effect of mycoheterotrophic C gains on ^{13}C or ^{2}H abundance from a transpiration effect (Farquhar et al., 1982; da Silveira Lobo Sternberg, 1988; Cernusak et al., 2004; Barbour, 2007): ^{13}C enrichment compared to neighbouring autotrophic plants without parallel ^{18}O enrichment is indicative of heterotrophic C gain rather than higher transpiration and lower water use efficiency. Additionally, mycoheterotrophic plants tend to be generally enriched in ^{15}N and display higher total N concentrations than autotrophic reference plants, although these parameters are highly variable (Giesemann *et al.*, 2020a; Gomes *et al.*, 2020), and thus not necessarily diagnostic of PMH in plants associated with AM.

In this study, we evaluated if seedlings of (at least some) common tropical woody species (i.e. trees or shrubs) in the understory of tropical forests in Central Panama exhibit partial mycoheterotrophy, i.e. gain organic C from fungal AM partners in addition to their own photosynthesis. To this end, we (a) assessed the AM morphological type (*Paris*- or *Arum*-type) for tropical woody species from genera that include species with known AM-morphotype (based on Dickson *et al.,* 2007), and (b) compared the multi-element stable isotope natural abundance (^{13}C, ^{2}H, ^{18}O, ^{15}N) of potentially mycoheterotrophic target woody seedlings with *Paris*-type AM with neighbouring autotrophic reference plants (i.e. without *Paris*-type AM) to determine if they exhibit partially mycoheterotrophic C gain. Specifically, we tested if any *Paris*-type AM target species showed enrichment in the heavy isotopes ^{13}C without simultaneous enrichment in ^{18}O relative to surrounding non *Paris*-type plants, i.e. the isotopic signature indicative for partial mycoheterotrophy.

MATERIALS AND METHODS

Study area

This study was conducted in semi-deciduous lowland forests in Central Panama, in the Soberania National Park and on Barro Colorado Island, i.e. within an area of about 20 km * 3.5 km along the Panama Canal (approx. 9.07° - 9.16°N, 79.65° - 79.85°W). The climate is moist tropical with mean annual precipitation of about 2100 to 2600 mm, a distinct dry season from January to April (Engelbrecht *et al.*, 2007), and a mean annual temperature of approx. 25 °C (Autoridad Nacional del Ambiente Panamá, 2010). Soils formed on volcanic rocks or marine sediments and include Oxisols and Alfisols (Baillie *et al.*, 2007; Turner & Engelbrecht, 2011). Sampling took place in the understory of old secondary forest along established trails.

Study species and sampling design

We selected focal woody study species (all C3-photosynthesis and non-succulent) from genera for which information on the AM type (*Paris*- or *Arum*-type) was available in the comprehensive review by Dickson *et al.*(2007). From these species, we *a priori* selected focal species from *Paris*- and *Arum*-type genera that are relatively frequent and abundant in Central Panama (Condit *et al.*, 2013), and that are shade tolerant (Rüger *et al.*, 2009). We included two of the most speciose and common shrub genera in the understory of Central Panama, *Piper* and *Psychotria* (Foster & Hubbell, 1990), since they promised to allow for numerous and easily identifiable *Arum*-type reference plants (based on Dickson *et al.*, 2007). During sampling (see below), we opportunistically included additional woody species from genera for which AM morphotype information was available in Dickson *et al.* (2007). Overall, we collected samples from seedlings of 41 woody species within 20 genera and 15 families (see Table S1, including 8 *Psychotria* and 14 *Piper* species) with a wide range of abundances and light requirements. For comparison, we also collected the FMH, achlorophyllous herbaceous species *Voyria tenella* (Gentianaceae), which has *Paris*-type AM (Imhof, 1997).

Sampling of tree and shrub seedlings took place during the dry season (in March and April, Table S1), while *Voyria tenella*, which only emerges in the wet season, was sampled in September (together with its respective reference plants).

For *a posteriori* microscopic determination of the species' AM morphotype in our study area, we collected roots of two to three individuals per plant species. Roots were mostly collected on the same individuals as for leaf sampling (below) or occasionally from separate plants along forest trails. Sampled roots were washed with tap water and fixed in ethanol (60 %) until further processing for microscopic observations (see below).

The sampling design for leaf stable isotope analysis followed the approach of Gebauer & Meyer (2003), with few modifications. We aimed to compare the isotopic signature of potential PMH plants (i.e. species with *Paris*-type AM) with autotrophic reference species (i.e. without *Paris*-type AM) growing under similar environmental conditions in their direct vicinity. This design accounts for variation of environmental factors (e.g. nutrient availability, microclimate, light conditions) and sampling time, which are known to affect stable isotope signatures (Dawson *et al.*, 2002). We focussed on seedlings 30 to 80 cm in height. Specifically, walking along established trails we first searched for *a priori* target plants (*Paris*-type genus according to Dickson *et al.* (2007). If at least three (up to 6) *a priori* reference plants ('*Arum*-type AM' or 'non-mycorrhizal') grew in the immediate surrounding (up to 2 m distance, mostly considerably closer), we sampled leaves for analyses (see below). For each *a priori* target species, we sampled a minimum of five such sampling plots (total 105) with the plots separated by at least 30-50 m.

For stable isotope analyses, from each plant individual at least one healthy, fully developed leaf was collected, carefully cleaned and kept in a paper bag. If necessary, the number of leaves per sample was increased to ensure sufficient material for analyses. For *Voyria tenella*, we sampled the entire aboveground parts.

AM morphotype determination in roots

For microscopic determination of the AM morphological type, an arbitrary subsample of the fine roots of each plant species was prepared and stained according to a modified staining protocol based on Phillips & Hayman (1970) and Vierheilig *et al.* (2005) following recommendations of T. Camenzind (pers. comm.). 1-2 cm root segments were cleared in 10 % KOH (w/v) at 60 °C (water bath: W760, Memmert GmbH, Schwabach, Germany) for 1.5 h to 1.5 d (depending on hardness), bleached in 3 % (v/v) H_2O_2 at RT for 30 min to 4 h (depending on pigmentation), acidified in 1 % (v/v) HCl and stained for 0.5-1h in 0.05 % (w/v) Trypan Blue lactoglycerol solution at 60 °C. Stained roots were

mounted onto slides with lactoglycerol for assessment. We examined the mounted root samples with a compound microscope at 100x magnification (Motic BA210, Motic Instruments Inc., Richmond, Canada).

Based on the presence of fungal structures in the roots and their morphological characteristics, we *a posteriori* classified the investigated plant species into (1) species with *Paris*-type AM hyphal coil structures in their roots. They either had exclusively *Paris*-type AM or exhibited both *Paris*-type and *Arum*-type AM structures. In the following, we refer to these species, which were potentially PMH, as '*Paris*-type' plants. (2) Species without any *Paris*-type AM structures. They had either only *Arum*-type AM or were non-mycorrhizal. In the following we refer to these species as 'non *Paris*-type' plants, and consider them as autotrophic reference plants. Microscopically unclear plant species ('NA') were excluded from further analyses (Table S1).

Multi-element stable isotope analyses of leaves

Leaf samples (or entire aboveground plant samples in the case of *Voyria tenella*) were oven-dried to constant weight at 105 °C, ground to a homogenous, fine powder using a ball mill (Retsch Schwingmühle MM2, Haan, Germany), and weighed into tin and annealed silver capsules (micro balances: Sartorius CPA2P & MSE3.6P-000-DM, Göttingen, Germany). Relative nitrogen and C isotope natural abundances ($\delta^{15}N$, $\delta^{13}C$) were determined simultaneously using an EA-IRMS coupling and relative natural abundances of hydrogen ($\delta^{2}H$) and oxygen isotopes ($\delta^{18}O$) were measured using a TC-IRMS coupling at the BayCEER– Laboratory of Isotope Biogeochemistry as specified in Zahn *et al.*(2023). Relative isotope abundances were denoted as δ values relative to their respective standards: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R\text{sample}/R\text{standard} - 1) \times 1000$ ‰, where *R*sample and *R*standard are the ratios of heavy to the light isotope of the samples and the respective standard (for utilised standards refer to Zahn *et al.*, 2023).

Replication Statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Species	Plots	Minimum of 5, Mean of 14
Arbuscular mycorrhiza type	Pooled species	21 '*Paris*-type' species, 12 'non *Paris*-type' reference species

Data analysis

To normalise for environmental and temporal variation of the isotopic signature (see above), we compared within each plot the δ of the potentially PMH '*Paris*-type' target species to the neighbouring autotrophic 'non *Paris*-type' reference plants (classification based on our own *a posteriori* microscopic observations, see Table S1).

We calculated for the neighbouring plants in each plot their comparative enrichment, i.e. their enrichment factors ε according to Preiss & Gebauer (2008): $\varepsilon = \delta S - \delta REF$, where δS is a single $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ or $\delta^{15}N$ value of an individual plant ('*Paris*-type' target or 'non *Paris*-type' reference), and δREF is the mean value across all 'non *Paris*-type' reference plants in the respective plot. An analogous standardisation was carried out for Total N concentrations.

After the *a posteriori* reclassification of the species' AM morphotype, 79 plots contained a minimum of one '*Paris*-type' target individual and one 'non *Paris*-type' reference individual and were thus suitable for the calculation of ε. Mean sample size (i.e. number of plots) for each of the 21 *a posteriori* '*Paris*-type' target species was 14 (minimum 5, see Table S2 for details).

We initially strived to include a diverse range of co-occurring reference taxa to account for potential physiological signals inherent in the isotopic signature. After reclassification, each target plant had on average two reference individuals from two reference species in the same plot (Fig. S2), with most reference plants belonging to the genus *Piper* genus (Piperaceae). We included 11 different species of this physiologically and functionally highly diverse genus (Fredeen et al. 1996; Kyllo et al. 2003, plus *Alcalypha*, Euphorbiaceae) so we can exclude a systematic bias of isotopic signatures between target and reference plants.

We first tested for overall differences among the three groups ('*Paris*-type' target, 'non *Paris*-type' reference, FMH *Voyria*) in their isotopic enrichment compated to their respective neighbouring 'non *Paris*-type' reference plants ($\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$) and N concentrations (εTotalN). We fitted separate linear mixed models ('lmer', package lme4, version 1.1.29; Bates *et al.,* 2015) for each variable, including 'Species' and 'Plot' as random effects. 95 % Confidence Intervals (CIs) and *P*-values were computed using a Wald *t*-distribution approximation. For *post hoc* pairwise comparison between groups,

we used the 'emmeans' and 'contrast' functions (package emmeans, version 1.7.5; Russell *et al.*, 2022) with Bonferroni-Holm *P*-value adjustment. Excluding *Voyria* as a group in these analyses did not qualitatively change the results.

Subsequently, in order to identify individual '*Paris*-type' target species consistent with PHM, we then ran separate linear mixed models for each parameter, which compared individual '*Paris*-type' target species to all pooled neighbouring 'non *Paris*-type' reference plant species, including 'Plot' as random effect.

Calculations and statistics were conducted in R version 4.2.3 (R Core Team, 2023).

RESULTS

AM morphotypes

Overall, about half of the investigated species (21 out of 41 species, in 16 out of 20 genera) exhibited *Paris*-type AM, i.e. we detected coil structures in the roots. Of those, most (81 %) had only *Paris*-type AM, while the others showed both *Paris*- and *Arum*-type structures (i.e. also had arbuscules). 12 of the species we examined (in 2 genera) did not show any *Paris*-type structures: they exhibited only arbuscules characteristic for the *Arum*-type AM (4 species), or had no visible AM structures (8 species). In additional 8 species the AM morphotype could not be conclusively determined.

Comparing our microscopic root assessments of tropical woody species in Central Panama with the previous literature-based classification (Dickson *et al.*, 2007, Table S1), all genera which had been known to contain *Paris*-type AM species consistently exhibited *Paris*-type AM in our study, except for 2 species, which remained unclear. In our study, six additional genera exhibited *Paris*-type structures, which had exhibited *Arum*-type structures in other species in the same genera in Dickson *et al.* (2007). For instance, in *Psychotria*, which we had *a priori* considered to be *Arum*-type, 6 of the species showed *Paris*-type structures (while 2 remained unclear). Several of the genera including *Arum*-type AM species according to Dickson et al. (2007), exhibited no visible AM structures in our study (e.g. most *Piper* species), or the morphotype could not be determined.

From the *a posterior* AM morphotype determination, 21 of the investigated 42 species were considered as potentially PMH ('*Paris*-type' target species), and 12 species were

regarded as autotrophic 'non *Paris*-type' reference plants ('*Arum*-type' or 'non-mycorrhizal') (Table S1, Table S2).

Comparison of isotopic signatures among plants with different AM morphotypes

Overall, '*Paris*-type' woody target plants did not show significantly higher isotope enrichment (ε) of ^{13}C or ^{2}H than 'non *Paris*-type' reference plants, but instead they were depleted in ^{2}H and ^{18}O (Table 1). Thus, not surprisingly, across species, no general trend to partial mycoheterotrophy in the investigated '*Paris*-type' plants was indicated by the stable isotopes. In contrast, as expected, the FMH *Voyria* showed highly significantly stronger enrichment in both ^{13}C and ^{2}H relative to reference plants and showed no significant difference in ^{18}O and ^{15}N enrichment (Table 1). Enrichment of ^{15}N and εTotalN did not differ across groups (Table 1). We will not further elaborate on the N data due to their limited diagnostic power for PMH in AM plants (full results are available in the Supplementary in Table S5 and Fig. S1).

Table 1 Comparison of stable isotope enrichment factors (ε) and difference in total nitrogen concentrations between tropical woody seedlings without '*Paris*-type' (references), with '*Paris*-type' (target), and the fully mycoheterotrophic herbaceous species *Voyria tenella*. Shown are mean$\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ (‰) and εTotalN (mmol gdw^{-1}) and standard deviations (SD). Different letters indicate significant differences between groups ($P < 0.05$) based on linear mixed effect models and *post hoc* tests for pairwise comparisons. All models included 'species' and 'plot' as random effect and *P*-values were adjusted using Bonferroni–Holm correction (see Table S3 and S4 for detailed results).

	Woody seedlings without ***Paris*-type AM**	**Woody seedlings** with ***Paris*-type AM**	***Voyria tenella***
$\varepsilon^{13}C$	0.00 ± *1.02* [a]	0.57 ± *2.36* [a]	6.11 ± *1.37* [b]
$\varepsilon^{2}H$	0.00 ± *4.97* [a]	-5.27 ± *9.52* [b]	22.57 ± *9.16* [c]
$\varepsilon^{18}O$	0.00 ± *0.98* [a]	-0.93 ± *2.07* [a*]	0.39 ± *0.69* [a]
$\varepsilon^{15}N$	0.00 ± 0.71 [a]	-0.12 ± 1.87 [a]	1.50 ± 1.84 [a]
εTotalN	0.00 ± *0.25* [a]	-0.06 ± *0.53* [a]	0.23 ± *0.26* [a]

* Significant difference target and reference plants based on the linear mixed model and based on the *post hoc* pairwise comparison before Bonferroni-Holm correction, but not after correction.

Isotopic signatures of individual '*Paris*-type' target species compared to 'non *Paris*-type' reference plants

At the level of individual species, seedlings of 6 '*Paris*-type' target species (28.6 %) showed significant enrichment in ^{13}C compared to the neighbouring autotrophic 'non *Paris*-type' reference plants, while at the same time not showing an ^{18}O enrichment (Fig. 1, Table S5). Their combined C and O isotopic signature thus supports PMH. Significance

of ^{13}C enrichment in each of these species was based on at least six intra-plot comparisons (see Figure S3). One of these species, *Anacardium excelsum,* was additionally significantly enriched in ^{2}H compared to the 'non *Paris*-type' reference plants and fulfilled a second criterion for PMH. In contrast, the remaining 5 species with ^{13}C enrichment were depleted in ^{2}H (Fig. 1b, Table S5).

While no '*Paris*-type' target species showed significant ^{18}O enrichment, 7 species were significantly depleted in ^{18}O relative to reference plants (Fig. 1c, Table S5). This complicates interpretation with regards to PMH (see below for discussion).

Only one target species was significantly enriched in ^{2}H, but 18 target species were significantly depleted in ^{2}H compared to the 'non *Paris*-type' reference plants (Fig. 1b, Table S5).

Seedlings of 9 of the target species were not enriched in ^{13}C and ^{2}H, and indistinct in $\varepsilon^{18}O$ from reference plants, i.e. these species showed isotopic signatures that clearly do not support PMH.

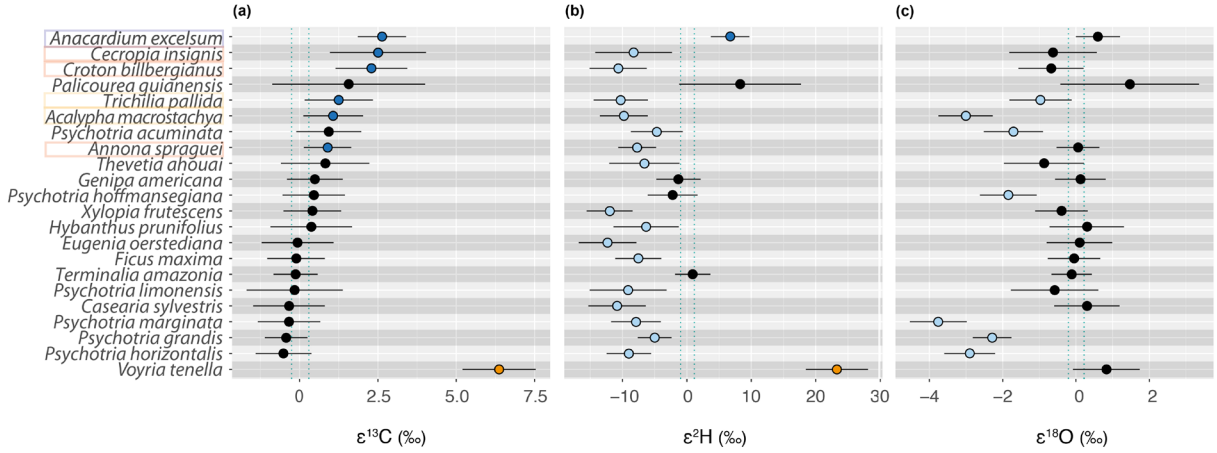

Fig. 1 Estimated enrichment factors $\varepsilon^{13}C$ (a), $\varepsilon^{2}H$ (b) and $\varepsilon^{18}O$ (c) and 95% CI of individual '*Paris*-type' species in comparison to pooled 'non *Paris*-type' reference plant species (95% CI displayed by dotted vertical lines) based on linear mixed models (Table S5). Dark and light blue symbols indicate significant enrichment or depletion of '*Paris*-type' species in comparison to 'non *Paris*-type' reference plants, respectively. Orange symbols indicate significant enrichment for FMH *Voyria.*

DISCUSSION

To our knowledge, this is the first study that investigated stable isotope natural abundances of tropical woody species with respect to PMH. Seedlings of six species exhibited isotopic signatures clearly supporting PMH, i.e. ^{13}C enrichment compared to neighboring autotrophic species without a parallel ^{18}O enrichment. For one of our study species, namely *Anacardium excelsum,* ^{2}H enrichment provides additional evidence for PMH.

AM morphotypes

The *Paris*-type AM is widely considered to be a pre-requisite for a fungus-to-plant C transfer in both achlorophyllous FMH and chlorophyllous PMH plants with AM (Imhof, 1999; Giesemann *et al.*, 2021, but see Murata-Kato *et al.*, 2022). We showed that *Paris*-type AM is indeed rather widespread in seedlings of tropical woody species, occurring in 50 % of the investigated species (21 of 42) and in 80 % of the genera (16 of 20) in lowland moist forests in Panama.

Several tropical woody plant genera can contain both *Paris*- and *Arum*-type species (based on comparison with Dickson *et al.*, 2007) and we occasionally observed two morphological structures in the same plant species, even within the same root system (e.g. *Anacardium excelsum, Cecropia insignis, Croton billbergianus, Acalypha macrostachya)*. In general, plant and AM fungal identity as well as the environment are considered to impact the formation of *Arum*- or *Paris*-type structures along a continuum (e.g. Ahulu *et al.*, 2006, 2007; Kubota *et al.*, 2005; Lovelock *et al.*, 2003). It remains poorly understood what exactly determines the formation of specific AM morphotype structures (Dickson *et al.*, 2007; Bennett & Groten, 2022), and consequently if a plant individual, species or genus may be capable of PMH. Thus, *in situ* AM-morphotype evaluation is pivotal. AM fungal communities in tropical forests are recognised as highly diverse (Muthukumar *et al.*, 2003; Alexander & Lee, 2005; Kottke *et al.*, 2006; Zhang *et al.*, 2021) with considerable spatial (Mangan *et al.*, 2004) and temporal (Husband *et al.*, 2002; Herre *et al.*, 2009) variation. Further investigations combing morphotype determination and fungal associate identification in different woody species along environmental gradients may help to better understand the formation of *Paris*-type AM structures.

Isotopic evidence for PMH in woody tropical seedlings

Seedlings of six species with *Paris*-type AM (*Anacardium excelsum, Annona spraguei, Cecropia insignis, Croton billbergianus, Acalypha macrostachya* and *Trichilia pallida*) displayed significant ^{13}C enrichment compared to the surrounding autotrophic 'non *Paris*-type' reference plants, and none of these species exhibited a parallel enrichment of ^{18}O. We can thus exclude that enrichment in ^{13}C in these species was caused by lower transpiration and a higher water use efficiency of target relative to reference plants (and all woody species in the system have C3 photosynthesis). The isotopic signature thus provides a clear indication for supplementary gain of enriched ^{13}C through fungus-to-plant C transfer, i.e. that these species exhibit PMH. The ^{13}C-enriched seedlings exhibited a similar isotope pattern to FMH *Voyria* in our study area (Fig. 1, Table S5) – of course with a smaller ^{13}C (and ^{2}H) enrichment – and to other FMH (Merckx *et al.*, 2010; Courty *et al.*, 2011; Gomes *et al.*, 2020) or PMH (Giesemann *et al.*, 2020b, 2021; Murata-Kato *et al.*, 2022) herbaceous AM plants. Leaf ^{13}C enrichment of mycoheterotrophic plants in the forest understory compared to autotrophic ones reflects a relative ^{13}C enrichment of their biogenic fungal C-source, i.e. the AM fungal hyphae (Courty *et al.*, 2011; Gomes *et al.*, 2020, 2023), which in turn is mainly due to the supply of ^{13}C enriched carbohydrates from canopy trees to the AM fungi (Gleixner *et al.*, 1993; Courty *et al.*, 2011). Leaf exposure to high irradiance leads to ^{13}C enrichment during photosynthesis in canopy trees (and thus AM fungi) compared to plants in the shaded understory (Courty *et al.*, 2011). Additionally, the C that plants in the forest understory assimilate through photosynthesis is ^{13}C-depleted due to CO_2 originating from soil respiration (Gebauer & Schulze, 1991). This enhances the contrast in the ^{13}C signature between photosynthetic and biogenic C sources in understory plants. Such effects are especially pronounced in tropical forests, due to higher rates of respiratory CO_2 released from the soil, leading to a higher vertical profile of air $\delta^{13}C$ in tropical forests, compared to temperate ones (Quay *et al.*, 1989; Hanba *et al.*, 1997; Courty *et al.*, 2011).

Recently some concerns have been raised that ^{13}C enrichment (without parallel ^{18}O enrichment) may not always be the result of mycoheterotrophy (Murata-Kato *et al.*, 2022). They argued that in herbaceous species in temperate forests, differences in plant phenology could also explain ^{13}C enrichment, with species that photosynthesise early before canopy closure being relatively ^{13}C enriched, because they grow under higher light

levels (limited ^{13}C discrimination) and lower soil respiration (less ^{13}C-depletion of ambient CO_2 at the forest floor) conditions than species that continue to photosynthesis throughout the growing season (Murata-Kato *et al.*, 2022). Similarly, more light is available and soil respiration is lower in the understory of tropical lowland forests in the dry season compared to the wet season (Newell *et al.*, 2002; Cusack *et al.*, 2019). However, photosynthetic CO_2 uptake is limited in the dry season (Restrepo-Coupe *et al.*, 2013; Comita & Engelbrecht, 2014), and seasonal light differences in the understory of semi-deciduous tropical forests are small compared to temperate forests (Gaviria *et al.*, 2017; Matsuo *et al.*, 2021). In addition, our investigated species all have long-lived leaves that most likely still developed in the wet season (Kursar & Coley, 1993; Kitajima *et al.*, 2013) and there is no evidence that our '*Paris*-type' species with ^{13}C enrichment would have different leaf phenology. PMH therefore is the most parsimonious explanation for their significant ^{13}C-enrichment (without ^{18}O enrichment) compared to neighbouring 'non *Paris*-type' species.

In our study, we considered 'non *Paris*-type' species (i.e. with *Arum*-type AM or non-mycorrhizal) as autotrophic (Giesemann *et al.*, 2021). If this is indeed a general pattern, or if fungus-to-plant C transfer can also occur in *Arum*-type species, has been questioned (Murata-Kato *et al.*, 2022). However, in our study none of the 'non *Paris*-type' species showed conspicuous ^{13}C enrichment ($\varepsilon^{13}C < 0.4$ ‰, except *Piper marginatum*, n=1, $\varepsilon^{13}C$ =3.38 ‰ and *Piper grande,* n=1, $\varepsilon^{13}C$ =1.6, Table S2). Even if *Arum*-type plants in our study would indeed exhibit some ^{13}C enrichment due to PMH, this would lead to under- rather than overestimating the occurrence of mycoheterotrophy in the investigated *Paris*-type AM plants. The finding of an apparent ^{13}C enrichment in some *Arum*-type species by Murata-Kato *et al.* (2022) may be traced back to a different sampling design. We always collected closely neighbouring leaves of our target and reference plants fulfilling the requirement of growth under identical light conditions, while Murata-Kato *et al.* (2022) collected their target and reference plants within an area of 45 m^2. On a forest ground with lots of small scale sun flecks 45 m^2 sampling area are probably already too large to represent identical light conditions.

In two of the emerging PMH species, *Acalypha macrostachya* and *Trichilia pallida,* the ^{13}C enrichment signature may indeed even underestimate fungus-to-plant C transfer. In these species, ^{18}O was depleted relative to 'non *Paris*-type' reference plants, indicating

higher transpiration and lower water use efficiency (Barbour, 2007). This counteracts any ^{13}C enrichment by heterotrophic nutrition. Higher transpiration and lower water use efficiency indicated by ^{18}O depletion may also explain their ^{2}H depletion (see below).

Five additional '*Paris*-type' species (all in the genus *Psychotria*) exhibited higher transpiration than their respective reference plants (indicated by ^{18}O depletion). In these species, which did not differ in $\varepsilon^{13}C$ from 'non *Paris*-type' reference plants, the transpiration effect leading to ^{13}C depletion may have completely masked possible ^{13}C enrichment through biogenic C gain. Thus, it is possible that these species also exhibit PMH, but currently we cannot conclusively resolve their nutritional mode based on the stable isotope signatures.

By identifying PMH in seedlings of nearly 30 % of the studied *Paris*-type AM tropical woody species, we showed for the first time that supplementary C gain through PMH exists in seedlings of tropical tree species with AM associations.

^{2}H depletion among PMH seedlings

A ^{2}H enrichment relative to 'non *Paris*-type' reference plants, which has been widely observed in FMH and PMH herbaceous AM species (Gomes *et al.*, 2020; Giesemann *et al.*, 2020, 2021) and is considered an additional independent indication for biogenic organic matter gain from fungi, occurred only in seedlings of one '*Paris*-type' tree species (*Anacardium excelsum*).

Instead, five out of the six ^{13}C-enriched '*Paris*-type' species exhibited even significant depletion of ^{2}H. This was unexpected and has not been described before, neither for FMH nor PMH AM plants (Gomes *et al.*, 2020; Giesemann *et al.*, 2020, 2021). In those species that also exhibit depletion of ^{18}O compared to the reference plants, lower transpiration may explain the observed ^{2}H depletion (i.e. for *Acalypha macrostachya* and *Trichilia pallida*). Yet, in the species where no ^{18}O depletion emerged, such a transpiration effect can be excluded (for *Annona spraguei, Cecropia insignis* and *Croton billbergianus*). A major fungus-to-seedling C supply in form of ^{2}H-depleted lipids (higher lipid-to-carbohydrate gain ratio) might potentially explain ^{2}H-depleted leaf tissues in these species: AM fungi receive substantial amounts of lipids (i.e. fatty acids) from their 'C donor plant' partner (Jiang *et al.*, 2017; Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017) and lipids represent the major C-storage compounds in AM fungi (Wipf *et al.*, 2019). Lipids have

been recognised to be ^{2}H-depleted compared to cellulose due to ^{2}H-fractionation during biosynthesis of plant organic compounds (Cormier *et al.*, 2018).

Possible ecological implications of PHM for tropical woody species

Our results showed for the first time that supplementary C gain through PMH exists in seedlings of tropical tree species with AM associations. Our results imply that plant-fungus-plant C transfer occurs in tropical tree species with AM *via* CMN: Since AM fungi are obligate biotrophic, i.e. they entirely depend on organic C supplied by a living plant partner (Trépanier *et al.*, 2005; Tisserant *et al.*, 2013), the PMH requires at least a tripartite association interconnecting a photosynthetic 'C donor plant' *via* an AM fungus to a MH 'C reviever plant'. Additional C gain and redistribution of C through PMH may have pervasive ecological implications considering the severe light, and frequently also P and water limitation to tree regeneration in the understory of tropical lowland forests (Holste *et al.*, 2011; Santiago *et al.*, 2017, Comita and Engelbrecht 2014).

PMH is considered a strategy to allow for seedling establishment, survival and growth under C limited conditions, especially light limitation (e.g. Preiss *et al.*, 2010). Seedlings of shade tolerant species can survive for decades in the deep shade of the tropical forest understory (Hubbell *et al.*, 1999; Rüger *et al.*, 2009). Biogenic C supply through PHM in addition to photosythetic C gain may lead to lower whole-plant light compensation points in some species, and help to maintain a positive C balance under these conditions. Additional C gain through PMH may also allow to alleviate the trait-based trade-off between survival in the shade and growth rates in those species that exhibit PMH (Ellers *et al.*, 2012). Yet, the species, for which we found PMH in seedlings, were not the most shade tolerant ones in the system (Rüger *et al.*, 2018) and even included pronounced pioneer species (*Cecropia insignis* and *Croton billbergianus*). These results imply that PMH may provide additional or alternative advantages to woody seedlings (particularly of pioneer species) beyond survival and growth under light-limited conditions.

PMH may additionally enable plants to cope with P limitation and/or drought during the dry season by providing organic C when photosynthesis is limited (Holste *et al.*, 2011; Comita & Engelbrecht, 2014; Santiago *et al.*, 2017). Fully mycoheterotrophic plants in tropical forest are associated with low-fertility sites (Sheldrake *et al.*, 2017; Gomes *et al.*, 2019) and the dependency of partially mycoheterotrophic plants on fungal derived carbon can increase under drought (McCormick *et al.*, 2022). The distribution of woody

species, for which we found PMH among seedlings, was associated with dry sites (with the exception of *Croton billbergianus*), while the association with soil P availability varied between species (Condit *et al.*, 2013). C gains from fungi can also be crucial for germination in plants with tiny seeds that provide only limited C resources (Eriksson & Kainulainen, 2011). In our study, PHM occurred among seedlings of AM tree species with a wide range of seed sizes. Seedlings of pioneer species with small seeds and thus low C reserves might benefit from additional biogenic C supply through PMH during initial survival and growth. Detecting PMH among seedlings of species with variable light, P and water requirements and different seed sizes suggests that ecological advantages differ across species and possibly growing conditions.

Even small C gains may confer seedlings a performance advantage in the forest understory (Merckx *et al.*, 2024), and expand their niches (Tedersoo *et al.*, 2020). By influencing the performance of seedlings, which comprise a bottleneck in population dynamics (Harper 1977) and the future forests, biogenic C gains of seedlings through PMH may impact regeneration success, and subsequently community composition (Mangan *et al.*, 2010; Parihar *et al.*, 2020). Such indirect effects on community composition may have pervasive implications on ecosystem function, specifically for C uptake and storage, although the direct contribution from fungi to overall C cycling processes in tropical forests is likely insignificant (Merckx *et al.*, 2024).

Plant-fungus-plant C exchange dynamics among woody plants within CMNs have been primarily studied in ectomycorrhizal systems in temperate and boreal climates (e.g. Simard *et al.*, 1997; Avital *et al.*, 2022; Cahanovitc *et al.*, 2022; Klein *et al.*, 2023) and only few studies consider woody AM species (e.g. Lerat *et al.*, 2002; Avital *et al.*, 2022). Direct C transfer and resource transfer within such CMNs remains highly debated (Figueiredo *et al.*, 2021; Henriksson *et al.*, 2023; Karst *et al.*, 2023 Klein *et al.*, 2023; Luo *et al.*, 2023), because it is challenging to firmly establish a connection between plants (see Rillig *et al.*, (2024) for a critical evaluation of the definition of CMNs). Shared mycorrhizal fungi in CMNs have been suggested to link over- and understorey carbon and nutrient exchanges (Balandier *et al.*, 2022), and to play a fundamental role in balancing plant interactions within communities harbouring mycoheterotrophs (Selosse *et al.*, 2006; Tedersoo *et al.*, 2020). While plant-fungus-plant C transfer has been known for herbaceous AM plant species (Sheldrake *et al.*, 2017; Giesemann *et al.*, 2021), our results indirectly imply that

some C transfer through CMNs must also occur among woody AM species in tropical lowland forest (Merckx *et al.*, 2024).

This study showed for the first time that PMH occurs in woody seedlings of AM dominated tropical lowland forests. Additional studies are called for to explore how widespread PHM is in (tropical) woody species, and across forests under different environmental conditions (e.g. different soils, seasonality) and to assess how extensive the transfer of biogenic C though PHM is. The actual ecological advantages that PMH provides for woody species in tropical forests remain to be investigated, as well as the consequences for community composition and ecosystem function.

To confirm fungal connections and C transfer, valuable insights could be gained from tracer experiments (Klein *et al.*, 2016) and stable isotope signatures of fungal hyphae extracted from plant roots (Klink *et al.*, 2020; Gomes *et al.*, 2023; Zahn *et al.*, 2023) together with DNA sequencing for fungal partner identification.

Conclusion

Based on stable isotope signatures, we detected PMH nutrition among seedlings of almost 30 % of the investigated *Paris*-type AM woody species in the understory of neo-tropical lowland forests and thus provide the first evidence for a fungus-to-plant organic C transfer in tropical woody AM species. We revealed that *Paris*-type AM, considered a prerequisite for biogenic C gain, occurs in seedlings of about 80 % of the investigated genera, and is thus considerably more widespread in tropical woody plants than previously recognised.

Given the far-reaching possible ecological implications of PMH in tree seedlings of tropical (as well as other) forests, we urgently need to gain further insights.

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AUTHORS' CONTRIBUTIONS

BMJE conceived the idea for the study. BMJE, GG and FEZ developed the research design. All authors were involved in the sample collection. Species identification was done by BC. The sample preparation and analyses were conducted by FEZ. GG supervised and quality-controlled the isotope abundance analyses. FEZ analysed the data and wrote the first manuscript draft with inputs from BMJE and GG. All authors commented and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

SUPPORTING INFORMATION

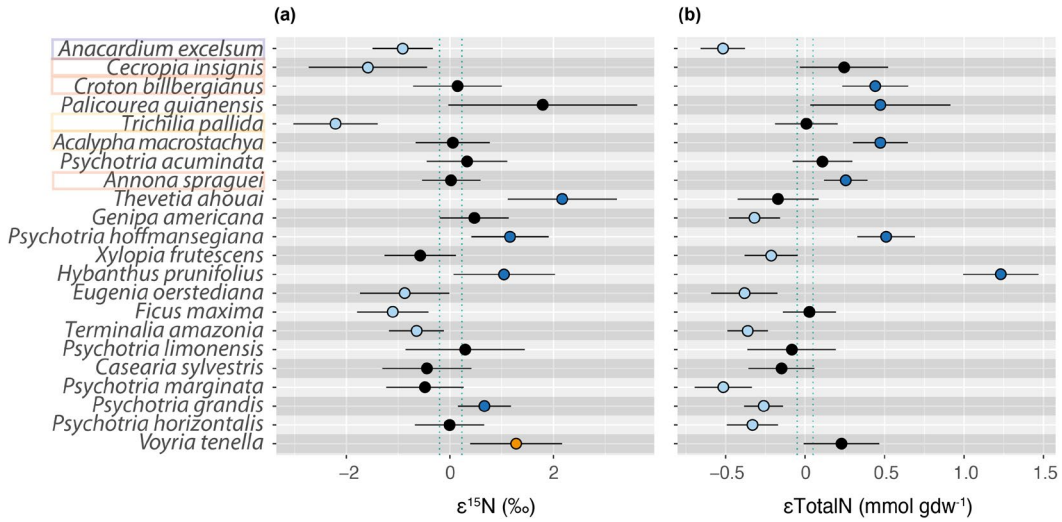

Fig. S1 Estimated enrichment factors $\varepsilon^{15}N$ (a) and Total N concentration (b) and 95% CI of individual '*Paris*-type' species in comparison to pooled 'non *Paris*-type' reference plant species (95% CI displayed by dotted vertical lines). Dark and light blue dots indicate significant enrichment or depletion of '*Paris*-type' species in comparison to 'non *Paris*-type' reference plants, respectively. Orange dots indicate significant enrichment for FMH *Voyria*.

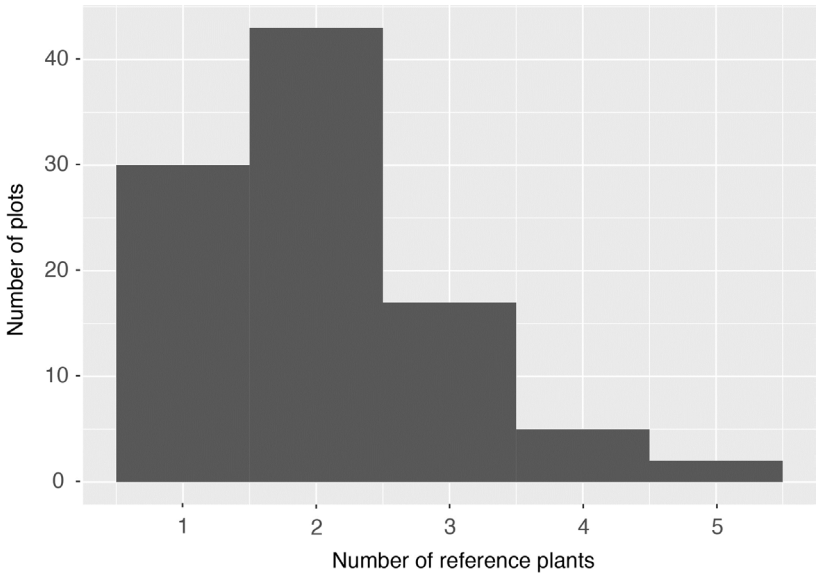

Fig. S2 Distribution of 'non *Paris*-type' reference plant number across plots.

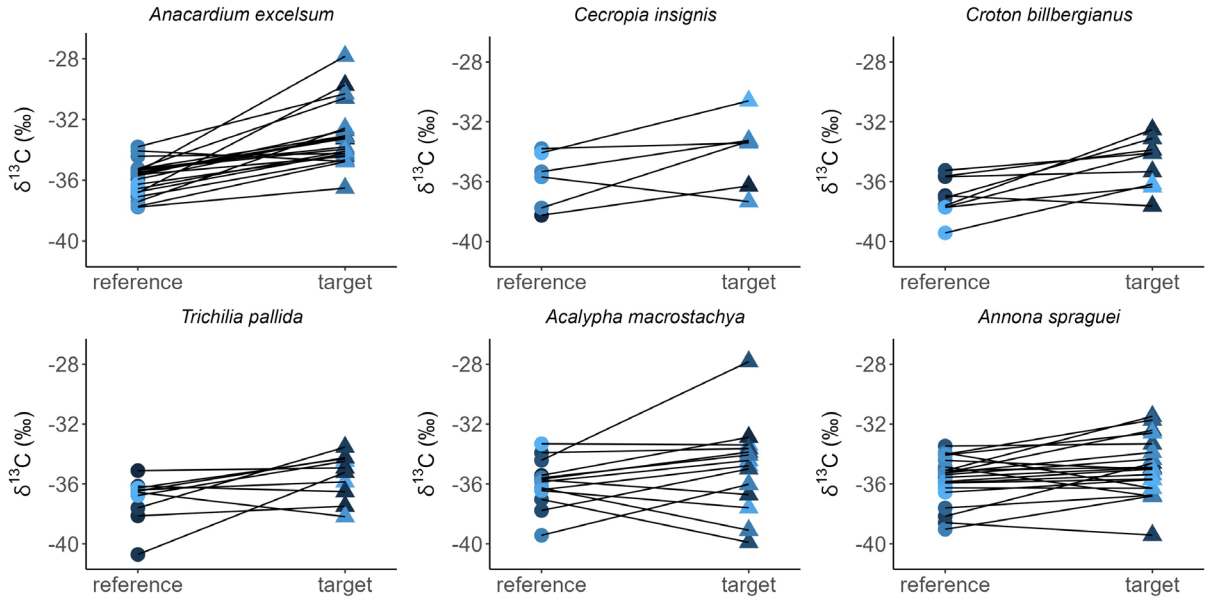

Fig. S3 Comparison of the individual $\delta^{13}C$ values of the six *Paris*-type' target species with significant ^{13}C enrichment compared to the mean $\delta^{13}C$ of all 'non *Paris*-type' reference plants in a respective plot for each *Paris*-type' target species. Within each subgraph colours indicate the different sampling plots. The lines connect the individual $\delta^{13}C$ values of a *Paris*-type' target species to the mean $\delta^{13}C$ of all 'non *Paris*-type' reference plants in a respective plot and illustrate the calculation principle of the enrichment factor $\varepsilon^{13}C = \delta S - \delta REF$, where δS is a single $\delta^{13}C$ value of an individual plant (i.e. '*Paris*-type' target), and δREF is the mean value across all 'non *Paris*-type' reference plants in the respective plot.

Table S1 Morphotype determination based on Literature and Microscopic observations.

	Species	Family	Habit	AM morphotype determined in this study	Genus level AM morphotype (Dickson et al., 2007)	Genus level AM morphotype in Dickson et al., 2007 refers to	
Paris-type target	*Annona spraguei* Saff.	Annonaceae	tree (5 to 20 m)	P	P	*Annona glabra*	●
	Casearia sylvestris Sw.	Salicaceae	tree (5 to 15 m)	P	P/A	*Casearia sp.*	●
	Eugenia oerstediana O. Berg	Myrtaceae	tree (5 to 10 m)	P	P/A	*Eugenia sp.*	●
	Ficus maxima Mill.	Moraceae	tree (10 to 20 m)	P	P	*Ficus sp.*	●
	Genipa americana L.*	Rubiaceae	tree (10 to 25 m)	P	P	*Genipa americana*	●
	Hybanthus prunfolius (Humb. & Bonpl. ex Schult.) Schulze-Menz	Violaceae	tree or shrub (2 to 6 m)	P	A	*Hybanthus floribundus*	○
	Palicourea guianensis Aubl.	Rubiaceae	Shrub (2 to 4 m)	P	A	*Palicourea rigida*	○
	Psychotria acuminata Benth.	Rubiaceae	shrub (2 to 3 m)	P	A	*2 Psychotria sp.*	○
	Psychotria grandis Sw.	Rubiaceae	tree or shrub (2 to 8 m)	P	A	*2 Psychotria sp.*	○
	Psychotria hoffmannseggiana (Roem. & Schult.) Müll. Arg.	Rubiaceae	shrub (1 to 3 m)	P	A	*2 Psychotria sp.*	○
	Psychotria horizontalis Sw.	Rubiaceae	tree or shrub (2 to 4 m)	P	A	*2 Psychotria sp.*	○
	Psychotria limonensis K. Krause	Rubiaceae	shrub (2 to 4 m)	P	A	*2 Psychotria sp.*	○
	Psychotria marginata Sw.	Rubiaceae	shrub or treelet (2 to 4 m)	P	A	*2 Psychotria sp.*	○
	Terminalia amazonia (J.F. Gmel.) Exell	Combretaceae	tree (20 to 40 m)	P	A	*Terminalia dichotama*	○
	Thevetia ahouai (L.) A. DC.	Apocynaceae	tree (2 to 8 m)	P	P	*Thevetia peruviana*	●
	Trichilia pallida Sw.	Meliaceae	tree (10 to 20 m)	P	P	*2 Trichilia sp.*	●
	Xylopia frutescens Aubl.	Annonaceae	tree (5 to 10 m)	P	P	*Xylopia aromatica*	●
	Acalypha macrostachya Jacq.	Euphorbiacea	tree or shrub (2 to 8 m)	P/A	A	*Acalypha indica*	○
	Anacardium excelsum (Bertero & Balb. ex Kunth) Skeels	Anacardiaceae	tree (20 to 40 m)	P/A	A	*Anacardium occidentale*	○
	Cecropia insignis Liebm.	Urticaceae	tree (10 to 30 m)	P/A	P	*Cecropia peltata*	●
	Croton billbergianus Müll. Arg.	Euphorbiacea	tree (5 to 10 m)	P/A	P/A	*Croton sp.*	●

Table S1 continued

	Species	Family	Habit	AM morphotype determined in this study	Genus level AM morphotype (Dickson et al., 2007)	Genus level AM morphotype in Dickson et al., 2007 refers to	
non-*Paris*-type reference	*Piper grande* Vahl	Piperaceae	shrub (3 to 5 m)	A	A	*3 Piper sp.*	●
	Piper hispidum Sw.	Piperaceae	shrub (3 to 6 m)	A	A	*3 Piper sp.*	●
	Piper reticulatum L.	Piperaceae	tree or shrub (3 to 6 m)	A	A	*3 Piper sp.*	●
	Acalypha diversifolia Jacq.	Euphorbiacea	shrub or treelet (2 to 6 m)	**NM**	A	*Acalypha indica*	●
	Piper aequale Vahl	Piperaceae	shrub (1 to 3 m)	**NM**	A	*3 Piper sp.*	●
	Piper arieianum C. DC.	Piperaceae	shrub (1 to 1.5 m)	**NM**	A	*3 Piper sp.*	●
	Piper cordulatum C. DC.	Piperaceae	shrub (1 to 2.5 m)	**NM**	A	*3 Piper sp.*	●
	Piper culebranum C. DC.	Piperaceae	shrub or treelet (1.5 to 4 m)	**NM**	A	*3 Piper sp.*	●
	Piper darienense C. DC.	Piperaceae	shrub (1 m)	**NM**	A	*3 Piper sp.*	●
	Piper friedrichsthalii C. DC.	Piperaceae	shrub (6 m)	**NM**	A	*3 Piper sp.*	●
	Piper marginatum Jacq.	Piperaceae	shrub or treelet (2 to 3 m)	**NM**	A	*3 Piper sp.*	●
	Piper arboreum Aubl.	Piperaceae	shrub (2 to 5 m)	**NM/A**	A	*3 Piper sp.*	●
NA	*Calophyllum longifolium* Willd.	Calophyllaceae	tree (20 to 40 m)	NA	A	*Calophyllum antillanum*	○
	Cordia alliodora (Ruiz & Pav.) Cham.	Cordiaceae	tree (5 to 25 m)	NA	P	*Cordia currassivica*	○
	Piper peltatum L.	Piperaceae	shrub (2 to 4 m)	NA	A	*3 Piper sp.*	○
	Piper dolichotrichum Yunck.	Piperaceae	climber	NA	A	*3 Piper sp.*	○
	Piper perlasense Yunck.	Piperaceae	shrub (2 to 3 m)	NA	A	*3 Piper sp.*	○
	Psychotria deflexa DC.	Rubiaceae	shrub (2 to 3 m)	NA	A	*2 Psychotria sp.*	○
	Psychotria glomerulata (Donn. Sm.) Steyerm.	Rubiaceae	shrub (1 m)	NA	A	*2 Psychotria sp.*	○
	Trichilia tuberculata (Triana & Planch.) C. DC.	Meliaceae	tree (20 to 30 m)	NA	P	*2 Trichilia sp.*	○
FMH	*Voyria tenella* Hook.	Gentianaceae	herb	**FMH**	**P**		

*same species in Dickson et al. 2007; **filled circle**: Accordance of microscopy & literature and **unfilled circle**: discrepancy; P: *Paris*-type, A: *Arum*-type, NM: Non-mycorrhizal; FMH: Fully mycoheterotroph.

Table S2 Mean and single $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$, $\delta^{15}N$, Total N concentration values, enrichment factors $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$, εTotalN, i.e., isotope shifts of individual plants relative to the mean value of autotrophic reference plants, of investigated seedlings and *Voyria*.

Species	*A posteriori* classification	*n*	$\delta^{13}C$ [‰]		$\delta^{2}H$ [‰]		$\delta^{18}O$ [‰]		$\delta^{15}N$ [‰]		TotalN [mmol gdw^{-1}]	
			mean	*sd*	*mean*	*sd*	*mean*	*sd*	*mean*	*sd*	*mean*	*sd*
Acalypha diversifolia Jacq.	reference	49	-35.54	1.82	-65.95	5.99	23.52	1.44	-0.08	2.05	1.74	0.30
Acalypha macrostachya Jacq.	target	14	-35.02	3.07	-77.85	5.64	20.31	2.11	-0.37	1.63	2.46	0.44
Anacardium excelsum (Bertero & Balb. ex Kunth) Skeels	target	22	-33.18	2.07	-62.57	7.84	24.05	1.21	-2.00	2.55	1.35	0.32
Annona spraguei Saff.	target	24	-34.92	1.92	-74.63	9.64	23.70	1.53	-1.33	1.42	2.09	0.32
Calophyllum longifolium Willd.	excluded	4	-33.83	0.72	-60.31	8.15	24.74	2.13	-3.20	1.89	NA	NA
Casearia sylvestris Sw.	target	9	-36.54	2.38	-80.49	8.86	24.53	2.15	0.85	1.49	1.85	0.29
Cecropia insignis Liebm.	target	6	-33.37	2.01	-82.25	12.72	23.34	1.25	-0.76	2.44	2.17	0.59
Cordia alliodora (Ruiz & Pav.) Cham.	excluded	12	-34.92	1.21	-75.89	7.99	21.92	1.23	-1.81	1.84	NA	NA
Croton billbergianus Müll. Arg.	target	10	-34.80	1.68	-79.24	4.54	23.19	1.31	-0.63	1.26	2.28	0.41
Eugenia oerstediana O. Berg	target	12	-36.28	0.89	-79.35	6.92	22.26	1.04	-2.79	1.78	1.32	0.11
Ficus maxima Mill.	target	14	-36.47	2.89	-76.86	6.92	23.75	1.36	-2.39	2.06	1.94	0.31
Genipa americana L.	target	15	-35.60	3.15	-69.15	7.32	23.44	1.76	-0.49	2.82	1.56	0.27
Hybanthus prunifolius (Humb. & Bonpl. ex Schult.) Schulze-Menz	target	11	-36.48	1.63	-69.69	4.52	22.96	1.25	1.37	0.80	2.97	0.56
Palicourea guianensis Aubl.	target	5	-35.11	0.62	-60.42	0.59	23.94	1.87	1.21	1.98	2.48	0.37
Piper aequale Vahl	reference	11	-36.62	1.84	-70.62	9.75	23.11	2.03	-1.11	1.68	1.87	0.32
Piper arboreum Aubl.	reference	7	-37.64	2.11	-75.47	10.18	22.96	1.47	0.44	1.35	2.38	0.34
Piper arieianum C. DC.	reference	3	-35.51	0.58	-76.03	10.38	25.16	1.67	1.21	3.28	2.32	0.46
Piper cordulatum C. DC.	reference	49	-36.60	1.42	-68.86	8.88	21.25	2.76	-0.37	2.26	1.88	0.36
Piper culebranum C. DC.	reference	28	-36.29	1.73	-68.67	5.22	24.26	1.64	-0.31	1.99	2.14	0.31
Piper darienense C. DC.	reference	1	-34.36	NA	-53.74	NA	22.56	NA	-2.82	NA	1.95	NA
Piper dolichotrichum Yunck.	excluded	1	-32.13	NA	-90.30	NA	18.50	NA	3.07	NA	NA	NA
Piper friedrichsthalii C. DC.	reference	2	-35.75	0.97	-65.66	6.68	25.86	0.65	1.13	0.98	2.04	0.21
Piper grande Vahl	reference	1	-35.42	NA	-56.66	NA	24.86	NA	-0.40	NA	1.90	NA
Piper hispidum Sw.	reference	1	-35.04	NA	-69.16	NA	26.04	NA	-0.24	NA	2.07	NA
Piper marginatum Jacq.	reference	1	-33.96	NA	-63.71	NA	26.96	NA	3.54	NA	2.92	NA
Piper peltatum L.	excluded	1	-35.07	NA	-72.73	NA	25.81	NA	-2.08	NA	NA	NA
Piper perlasense Yunck.	excluded	1	-35.69	NA	-72.76	NA	24.46	NA	0.69	NA	NA	NA
Piper reticulatum L.	reference	44	-35.54	1.74	-69.26	9.41	24.43	1.82	-0.82	1.98	1.97	0.44
Psychotria acuminata Benth.	target	13	-36.07	2.51	-71.74	5.89	20.73	1.73	-0.66	2.00	1.91	0.26
Psychotria deflexa DC.	excluded	5	-35.78	1.10	-59.52	3.80	21.09	1.62	0.17	2.46	NA	NA
Psychotria glomerulata (Donn. Sm.) Steyerm.	excluded	1	-36.88	NA	-70.33	NA	21.51	NA	0.52	NA	NA	NA
Psychotria grandis Sw.	target	26	-36.67	2.42	-72.82	5.76	21.54	1.39	-1.03	1.81	1.55	0.17
Psychotria hoffmannseggiana (Roem. & Schult.) Müll. Arg.	target	15	-35.94	2.00	-69.19	5.01	20.95	0.87	0.09	1.25	2.20	0.31
Psychotria horizontalis Sw.	target	19	-36.26	1.17	-77.29	4.70	21.05	1.34	-1.04	1.94	1.50	0.32
Psychotria limonensis K. Krause	target	6	-36.77	0.60	-78.32	5.04	21.83	2.41	0.47	1.43	1.86	0.29
Psychotria marginata Sw.	target	16	-37.13	1.11	-75.44	2.87	18.77	1.95	-1.47	2.23	1.32	0.23
Terminalia amazonia (J.F. Gmel.) Exell	target	25	-36.37	2.63	-69.06	5.58	23.66	1.30	-1.41	2.37	1.44	0.37
Thevetia ahouai (L.) A. DC.	target	6	-35.88	1.51	-72.11	7.07	23.82	1.37	-0.04	2.03	1.67	0.16
Trichilia pallida Sw.	target	10	-35.48	1.51	-76.06	8.40	23.02	1.79	-2.79	2.19	1.95	0.20
Trichilia tuberculata (Triana & Planch.) C. DC.	excluded	24	-36.08	1.30	-77.02	8.57	21.75	1.80	-1.71	2.07	NA	NA
Xylopia frutescens Aubl.	target	14	-35.27	1.62	-80.35	3.13	22.98	1.64	-1.46	1.40	1.64	0.17
Voyria tenella Hook.	FMH	9	-29.74	1.01	-54.14	2.08	18.68	0.42	2.68	1.71	2.45	0.17

Table S2 continued

Species	*A posteriori* classification	*n*	$\varepsilon^{13}C$ [‰]		$\varepsilon^{2}H$ [‰]		$\varepsilon^{18}O$ [‰]		$\varepsilon^{15}N$ [‰]		εTotalN [mmol gdw^{-1}]	
			mean	*sd*	*mean*	*sd*	*mean*	*sd*	*mean*	*sd*	*mean*	*sd*
Acalypha diversifolia Jacq.	reference	49	0.31	0.93	0.80	4.36	-0.37	0.95	0.15	0.69	-0.15	0.21
Acalypha macrostachya Jacq.	target	14	0.96	2.62	-10.04	5.90	-3.04	1.81	-0.07	1.28	0.47	0.46
Anacardium excelsum (Bertero & Balb. ex Kunth) Skeels	target	22	2.72	2.05	7.02	9.96	0.64	1.60	-0.80	2.33	-0.55	0.39
Annona spraguei Saff.	target	24	0.87	1.53	-7.71	9.42	0.07	1.20	-0.06	1.13	0.26	0.45
Calophyllum longifolium Willd.	excluded	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Casearia sylvestris Sw.	target	9	-0.36	2.63	-10.15	13.17	0.27	2.22	-0.71	2.24	-0.14	0.55
Cecropia insignis Liebm.	target	6	2.47	1.56	-7.75	16.37	-0.55	1.12	-1.57	1.45	0.24	0.57
Cordia alliodora (Ruiz & Pav.) Cham.	excluded	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Croton billbergianus Müll. Arg.	target	10	2.21	1.88	-9.94	5.36	-0.67	1.13	0.32	0.81	0.43	0.58
Eugenia oerstediana O. Berg	target	12	-0.06	2.06	-12.71	8.67	0.23	2.07	-0.51	0.82	-0.34	0.19
Ficus maxima Mill.	target	14	-0.02	2.62	-7.14	7.71	-0.06	1.13	-1.09	1.44	0.02	0.30
Genipa americana L.	target	15	0.31	3.17	-1.45	5.99	0.10	0.98	0.51	3.05	-0.33	0.30
Hybanthus prunifolius (Humb. & Bonpl. ex Schult.) Schulze-Menz	target	11	0.43	2.20	-6.45	8.65	0.28	1.54	1.06	2.99	1.28	0.54
Palicourea guianensis Aubl.	target	5	1.94	0.39	9.57	8.22	1.54	2.40	1.65	0.34	0.44	0.31
Piper aequale Vahl	reference	11	-0.24	1.08	-0.20	2.29	-0.09	0.50	0.12	0.72	0.03	0.26
Piper arboreum Aubl.	reference	7	-0.91	1.81	-7.27	10.29	-0.52	0.50	-0.21	0.72	0.27	0.29
Piper arieianum C. DC.	reference	3	-0.88	0.77	-1.37	2.34	-0.13	0.12	-0.09	0.09	0.17	0.29
Piper cordulatum C. DC.	reference	49	-0.19	0.83	0.94	5.34	-0.09	1.26	-0.22	0.76	-0.06	0.16
Piper culebranum C. DC.	reference	28	-0.32	1.06	-0.50	3.36	0.30	0.63	0.05	0.75	0.16	0.24
Piper darienense C. DC.	reference	1	0.00	NA	0.00	NA	0.00	NA	0.00	NA	0.00	NA
Piper dolichotrichum Yunck.	excluded	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Piper friedrichsthalii C. DC.	reference	2	0.34	0.94	-0.24	0.71	0.89	1.31	0.40	0.76	0.12	0.09
Piper grande Vahl	reference	1	1.60	NA	8.69	NA	0.50	NA	-0.84	NA	0.04	NA
Piper hispidum Sw.	reference	1	0.05	NA	-8.01	NA	-0.66	NA	0.94	NA	0.04	NA
Piper marginatum Jacq.	reference	1	3.38	NA	3.25	NA	2.00	NA	1.05	NA	0.36	NA
Piper peltatum L.	excluded	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Piper perlasense Yunck.	excluded	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Piper reticulatum L.	reference	44	0.20	0.84	-0.41	4.66	0.36	0.79	0.01	0.61	0.05	0.25
Psychotria acuminata Benth.	target	13	1.06	2.74	-4.68	7.00	-1.70	2.04	0.36	1.90	0.11	0.33
Psychotria deflexa DC.	excluded	5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Psychotria glomerulata (Donn. Sm.) Steyerm.	excluded	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Psychotria grandis Sw.	target	26	-0.33	2.59	-4.52	8.26	-2.29	1.53	0.77	1.48	-0.25	0.30
Psychotria hoffmannseggiana (Roem. & Schult.) Müll. Arg.	target	15	0.36	2.40	-1.71	9.60	-1.89	1.88	1.02	2.22	0.54	0.32
Psychotria horizontalis Sw.	target	19	-0.45	1.40	-8.26	6.39	-2.95	1.14	-0.24	1.68	-0.33	0.25
Psychotria limonensis K. Krause	target	6	-0.14	0.89	-9.50	2.10	-0.34	4.47	0.24	0.93	-0.10	0.31
Psychotria marginata Sw.	target	16	-0.36	2.32	-7.95	5.88	-3.81	1.95	-0.41	1.17	-0.49	0.26
Terminalia amazonia (J.F. Gmel.) Exell	target	25	-0.10	2.23	1.19	7.67	-0.11	1.58	-0.45	1.29	-0.34	0.40
Thevetia ahouai (L.) A. DC.	target	6	1.26	2.45	-7.00	9.53	-1.09	1.53	2.09	2.48	-0.20	0.25
Trichilia pallida Sw.	target	10	1.54	2.12	-10.70	7.11	-1.04	1.86	-2.28	0.83	0.02	0.30
Trichilia tuberculata (Triana & Planch.) C. DC.	excluded	24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Xylopia frutescens Aubl.	target	14	0.47	1.34	-11.06	5.64	-0.36	1.14	-0.49	1.47	-0.21	0.43
Voyria tenella Hook.	FMH	9	6.11	1.37	22.57	9.16	0.39	0.69	1.50	1.84	0.23	0.26

Table S3 Summaries of linear mixed models (estimated using REML and nloptwrap optimizer, R function: lmer) predicting $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN, respectively with *Group* ('*Paris*-type', 'non *Paris*-type', 'FMH') for woody tree and shrub species (and *Voyria*). For fixed effects *Group*['non *Paris*-type'] is the baseline. All models include 'plot' as random effect. Model diagnostic was applied. 95% Confidence Intervals (CIs) and *P*-values were computed using a Wald t-distribution approximation.

	$\varepsilon^{13}C$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(458)*	*P*
(Intercept)	0.01	0.30	-0.58 – 0.60	0.04	0.972
class [Paris-type]	0.58	0.35	-0.11 – 1.27	1.66	0.098
class [FMH]	6.30	0.97	4.40 – 8.20	6.51	**<0.001**
Random effects	n	σ^2			
Species	37	2.7		*Marginal R^2*	*0.171*
Plot	97			*AICc*	*1882.088*

	$\varepsilon^{2}H$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(457)*	*P*
(Intercept)	-0.75	1.59	-3.88 – 2.37	-0.47	0.635
class [Paris-type]	-5.42	1.88	-9.11 – -1.73	-2.88	**0.004**
Group [FMH]	24.31	5.16	14.18 – 34.44	4.71	**<0.001**
Random effects	n	σ^2			
Species	34	39.78		*Marginal R^2*	*0.230*
Plot	97			*AICc*	*3140.871*

	$\varepsilon^{18}O$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(458)*	*P*
(Intercept)	0.08	0.37	-0.65 – 0.81	0.22	0.827
class [Paris-type]	-0.93	0.44	-1.80 – -0.06	-2.11	**0.035**
Group [FMH]	0.7	1.2	-1.66 – 3.07	0.58	0.559
Random effects	n	σ^2			
Species	34	1.6		*Marginal R^2*	*0.073*
Plot	97			*AICc*	*1673.733*

	$\varepsilon^{15}N$				
Fixed effects	*Estimate*	*SE*	*CI*	*t(457)*	*P*
(Intercept)	0.02	0.29	-0.55 – 0.58	0.06	0.953
class [Paris-type]	-0.1	0.33	-0.75 – 0.55	-0.31	0.757
Group [FMH]	1.17	0.90	-0.59 – 2.94	1.31	0.192
Random effects	n	σ^2			
Species	34	1.48		*Marginal R^2*	*0.013*
Plot	97			*AICc*	*1644.789*

	εTotalN				
Fixed effects	*Estimate*	*SE*	*CI*	*t(456)*	*P*
(Intercept)	0.11	0.11	-0.11 – 0.34	0.99	0.324
class [Paris-type]	-0.1	0.14	-0.37 – 0.17	-0.72	0.472
Group [FMH]	0.08	0.39	-0.68 – 0.84	0.21	0.835
Random effects	n	σ^2			
Species	34	0.08		*Marginal R^2*	*0.021*
Plot	97			*AICc*	*318.357*

Table S4 Pairwise comparisons of *Group* effect ('*Paris*-type', 'non *Paris*-type', 'FMH')on $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN of woody tree and shrub species obtained from linear mixed models' *post hoc* tests comparing differences in means. *P*-values were adjusted using Bonferroni-Holm correction. For underlying models see Table S3.

$\varepsilon^{13}C$

contrast	estimate	SE	df	t.ratio	*P*
'non Paris-type' vs. 'Paris-type'	-0.56	0.36	26.00	-1.50	0.140
'non Paris-type' vs. 'FMH'	-6.36	0.98	33.00	-6.50	**<0.001**
'Paris-type' vs. 'FMH'	-5.81	0.96	34.00	-6.10	**<0.001**

$\varepsilon^{2}H$

contrast	estimate	SE	df	t.ratio	*P*
'non Paris-type' vs. 'Paris-type'	5.4	1.9	30	2.9	**0.008**
'non Paris-type' vs. 'FMH'	-24.3	5.2	31	-4.7	**<0.001**
'Paris-type' vs. 'FMH'	-29.7	5.1	31	-5.9	**<0.001**

$\varepsilon^{18}O$

contrast	estimate	SE	df	t.ratio	*P*
'non Paris-type' vs. 'Paris-type'	0.93	0.45	32	2.1	0.130
'non Paris-type' vs. 'FMH'	-0.7	1.21	29	-0.6	0.570
'Paris-type' vs. 'FMH'	-1.64	1.18	29	-1.4	0.260

$\varepsilon^{15}N$

contrast	estimate	SE	df	t.ratio	*P*
reference - target	0.10	0.33	29	0.3	0.760
reference - FMH	-1.20	0.90	31	-1.3	0.310
target - FMH	-1.30	0.88	31	-1.5	0.310

εTotal N

contrast	estimate	SE	df	t.ratio	*P*
'non Paris-type' vs. 'Paris-type'	0.11	0.14	33	0.8	0.790
'non Paris-type' vs. 'FMH'	-0.10	0.39	31	-0.3	0.790
'Paris-type' vs. 'FMH'	-0.21	0.38	30	-0.6	0.790

Table S5 Summaries of linear mixed models (estimated using REML and nloptwrap optimizer, R function: lmer) comparing $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN, respectively of individual potentially PMH '*Paris*-type' target species (and 'FMH' *Voyria*) to pooled 'non *Paris*-type' reference plants. All models include 'plot' as random effect. Model diagnostic was applied. 95% Confidence Intervals (CIs) and *P*-values were computed using a Wald t-distribution approximation.

	$\varepsilon^{13}C$						$\varepsilon^{2}H$					
Predictors	*Estimates*	*std. Error*	*CI*	*Stat.*	*P*	*df*	*Estimates*	*std. Error*	*CI*	*Stat.*	*P*	*df*
(Intercept)	0.02	0.14	-0.25 – 0.30	0.17	0.868	439	0.09	0.54	-0.98 – 1.15	0.16	0.873	438
Spec [ACALMA]	***1.05***	0.49	0.08 – 2.02	2.13	**0.034**	439	-9.87	1.92	-13.64 – -6.10	-5.15	**<0.001**	438
Spec [ANACEX]	***2.61***	0.40	1.81 – 3.40	6.44	**<0.001**	439	***6.63***	1.57	3.55 – 9.72	4.23	**<0.001**	438
Spec [ANNOSP]	***0.87***	0.39	0.10 – 1.65	2.21	**0.027**	439	-7.80	1.53	-10.81 – -4.79	-5.10	**<0.001**	438
Spec [CASESY]	-0.36	0.59	-1.51 – 0.80	-0.60	0.546	439	-10.92	2.29	-15.41 – -6.43	-4.78	**<0.001**	438
Spec [CECRIN]	***2.49***	0.78	0.94 – 4.03	3.17	**0.002**	439	-8.36	3.04	-14.34 – -2.38	-2.75	**0.006**	438
Spec [CROTBI]	***2.27***	0.59	1.11 – 3.43	3.84	**<0.001**	439	-10.74	2.29	-15.24 – -6.25	-4.69	**<0.001**	438
Spec [EUGEOE]	-0.09	0.59	-1.25 – 1.08	-0.15	0.884	439	-12.40	2.31	-16.93 – -7.87	-5.38	**<0.001**	438
Spec [FICUMA]	-0.13	0.48	-1.07 – 0.81	-0.27	0.787	439	-7.63	1.85	-11.26 – -3.99	-4.12	**<0.001**	438
Spec [GENIAM]	0.46	0.46	-0.44 – 1.37	1.01	0.315	439	-1.40	1.79	-4.92 – 2.11	-0.78	0.433	438
Spec [HYBAPR]	0.35	0.67	-0.97 – 1.67	0.52	0.604	439	-6.42	2.61	-11.55 – -1.29	-2.46	**0.014**	438
Spec [PALIGU]	1.55	1.24	-0.89 – 3.98	1.25	0.213	439	8.17	4.81	-1.29 – 17.63	1.70	0.090	438
Spec [PSYCAC]	0.91	0.54	-0.14 – 1.97	1.70	0.090	439	-4.75	2.08	-8.84 – -0.66	-2.28	**0.023**	438
Spec [PSYCGR]	-0.45	0.36	-1.16 – 0.26	-1.26	0.210	439	-5.10	1.40	-7.84 – -2.35	-3.65	**<0.001**	438
Spec [PSYCH1]	0.43	0.52	-0.58 – 1.45	0.84	0.401	439	-2.31	2.00	-6.25 – 1.63	-1.15	0.250	438
Spec [PSYCHO]	-0.54	0.46	-1.45 – 0.37	-1.16	0.246	439	-9.08	1.80	-12.61 – -5.55	-5.06	**<0.001**	438
Spec [PSYCLI]	-0.18	0.79	-1.73 – 1.36	-0.23	0.819	439	-9.19	3.05	-15.19 – -3.20	-3.01	**0.003**	438
Spec [PSYCMA]	-0.36	0.52	-1.37 – 0.65	-0.70	0.486	439	-7.97	2.00	-11.91 – -4.04	-3.99	**<0.001**	438
Spec [TERMAM]	-0.15	0.37	-0.88 – 0.58	-0.40	0.687	439	0.81	1.45	-2.03 – 3.65	0.56	0.574	438
Spec [THEVAH]	0.80	0.72	-0.62 – 2.21	1.11	0.269	439	-6.69	2.80	-12.18 – -1.19	-2.39	**0.017**	438
Spec [TRI2PA]	***1.23***	0.56	0.13 – 2.33	2.19	**0.029**	439	-10.35	2.17	-14.62 – -6.07	-4.76	**<0.001**	438
Spec [Voyria]	***6.35***	0.60	5.16 – 7.53	10.55	**<0.001**	439	***23.20***	2.48	18.33 – 28.06	9.37	**<0.001**	438
Spec [XYL1FR]	0.38	0.48	-0.55 – 1.32	0.80	0.423	439	-12.08	1.85	-15.71 – -8.44	-6.53	**<0.001**	438
Random Effects												
σ^2	2.76						41.33					
τ_{00}	0.45 $_{plot}$						7.20 $_{plot}$					
ICC	0.14						0.15					
N	97 $_{plot}$						97 $_{plot}$					
Observations	464						464					
Marginal R^2 / Conditional R^2	0.282 / 0.383						0.419 / 0.506					
AICc	1861.765						3051.586					

Table S5 continued

	$\varepsilon^{18}O$						$\varepsilon^{15}N$					
Predictors	*Estimates*	*std. Error*	*CI*	*Stat.*	*P*	*df*	*Estimates*	*std. Error*	*CI*	*Stat.*	*P*	*df*
(Intercept)	0.01	0.11	-0.20 – 0.22	0.06	0.955	439	0.02	0.11	-0.20 – 0.23	0.14	0.891	439
Spec [ACALMA]	-3.02	0.39	-3.77 – -2.26	-7.83	**<0.001**	439	0.04	0.37	-0.68 – 0.76	0.11	0.913	439
Spec [ANACEX]	0.59	0.32	-0.03 – 1.21	1.87	0.062	439	-0.93	0.30	-1.52 – -0.34	-3.08	**0.002**	439
Spec [ANNOSP]	0.05	0.31	-0.55 – 0.66	0.17	0.868	439	0.01	0.29	-0.57 – 0.59	0.04	0.970	439
Spec [CASESY]	0.29	0.46	-0.61 – 1.19	0.64	0.524	439	-0.46	0.44	-1.32 – 0.40	-1.05	0.295	439
Spec [CECRIN]	-0.63	0.61	-1.83 – 0.57	-1.04	0.300	439	-1.60	0.58	-2.75 – -0.45	-2.74	**0.006**	439
Spec [CROTBI]	-0.68	0.46	-1.59 – 0.22	-1.49	0.137	439	0.13	0.44	-0.73 – 0.99	0.30	0.767	439
Spec [EUGEOE]	0.09	0.46	-0.82 – 1.00	0.19	0.846	439	-0.89	0.44	-1.76 – -0.02	-2.00	**0.046**	439
Spec [FICUMA]	-0.06	0.37	-0.79 – 0.67	-0.16	0.871	439	-1.12	0.36	-1.82 – -0.42	-3.15	**0.002**	439
Spec [GENIAM]	0.11	0.36	-0.59 – 0.82	0.31	0.754	439	0.46	0.34	-0.22 – 1.13	1.33	0.185	439
Spec [HYBAPR]	0.29	0.52	-0.74 – 1.32	0.56	0.579	439	***1.03***	0.50	0.04 – 2.02	2.05	**0.041**	439
Spec [PALIGU]	1.46	0.97	-0.43 – 3.36	1.52	0.130	439	1.78	0.93	-0.05 – 3.60	1.92	0.056	439
Spec [PSYCAC]	-1.71	0.42	-2.53 – -0.89	-4.10	**<0.001**	439	0.32	0.40	-0.47 – 1.10	0.79	0.430	439
Spec [PSYCGR]	-2.29	0.28	-2.84 – -1.74	-8.18	**<0.001**	439	***0.65***	0.27	0.13 – 1.18	2.44	**0.015**	439
Spec [PSYCH1]	-1.85	0.40	-2.64 – -1.06	-4.61	**<0.001**	439	***1.15***	0.39	0.39 – 1.91	2.97	**0.003**	439
Spec [PSYCHO]	-2.91	0.36	-3.61 – -2.20	-8.06	**<0.001**	439	-0.02	0.34	-0.70 – 0.66	-0.06	0.949	439
Spec [PSYCLI]	-0.59	0.61	-1.79 – 0.61	-0.96	0.337	439	0.28	0.59	-0.87 – 1.43	0.48	0.634	439
Spec [PSYCMA]	-3.76	0.40	-4.55 – -2.98	-9.38	**<0.001**	439	-0.50	0.38	-1.25 – 0.26	-1.30	0.196	439
Spec [TERMAM]	-0.13	0.29	-0.70 – 0.44	-0.43	0.666	439	-0.66	0.28	-1.21 – -0.12	-2.38	**0.018**	439
Spec [THEVAH]	-0.88	0.56	-1.98 – 0.22	-1.57	0.118	439	***2.16***	0.54	1.10 – 3.21	4.01	**<0.001**	439
Spec [TRI2PA]	-0.98	0.44	-1.83 – -0.12	-2.24	**0.026**	439	-2.22	0.42	-3.05 – -1.40	-5.33	**<0.001**	439
Spec [Voyria]	0.82	0.47	-0.10 – 1.74	1.75	0.080	439	***1.26***	0.45	0.37 – 2.15	2.79	**0.006**	439
Spec [XYL1FR]	-0.40	0.37	-1.13 – 0.33	-1.08	0.282	439	-0.59	0.35	-1.28 – 0.11	-1.65	0.099	439
Random Effects												
σ^2	1.68						1.49					
τ_{00}	0.25 $_{plot}$						0.42 $_{plot}$					
ICC	0.13						0.22					
N	97 $_{plot}$						97 $_{plot}$					
Observations	464						464					
Marginal R^2 / Conditional R^2	0.381 / 0.461						0.192 / 0.370					
AICc	1639.328						1615.418					

Table S5 continued

	εTotalN					
Predictors	*Estimates*	*std. Error*	*CI*	*Stat.*	*P*	*df*
(Intercept)	0.00	0.03	-0.05 – 0.05	0.10	0.920	437
Spec [ACALMA]	***0.47***	0.09	0.29 – 0.65	5.25	**<0.001**	437
Spec [ANACEX]	-0.52	0.07	-0.66 – -0.38	-7.08	**<0.001**	437
Spec [ANNOSP]	***0.25***	0.07	0.11 – 0.39	3.54	**<0.001**	437
Spec [CASESY]	-0.15	0.11	-0.36 – 0.06	-1.42	0.156	437
Spec [CECRIN]	0.24	0.14	-0.04 – 0.52	1.70	0.090	437
Spec [CROTBI]	***0.44***	0.11	0.23 – 0.65	4.10	**<0.001**	437
Spec [EUGEOE]	-0.38	0.11	-0.60 – -0.17	-3.57	**<0.001**	437
Spec [FICUMA]	0.03	0.09	-0.15 – 0.20	0.29	0.772	437
Spec [GENIAM]	-0.32	0.08	-0.49 – -0.16	-3.84	**<0.001**	437
Spec [HYBAPR]	***1.23***	0.12	0.99 – 1.47	10.08	**<0.001**	437
Spec [PALIGU]	***0.47***	0.22	0.03 – 0.91	2.09	**0.037**	437
Spec [PSYCAC]	0.11	0.10	-0.08 – 0.30	1.10	0.274	437
Spec [PSYCGR]	-0.26	0.07	-0.39 – -0.13	-4.03	**<0.001**	437
Spec [PSYCH1]	***0.51***	0.09	0.32 – 0.69	5.41	**<0.001**	437
Spec [PSYCHO]	-0.33	0.08	-0.50 – -0.17	-3.98	**<0.001**	437
Spec [PSYCLI]	-0.09	0.14	-0.37 – 0.19	-0.61	0.542	437
Spec [PSYCMA]	-0.52	0.09	-0.70 – -0.33	-5.53	**<0.001**	437
Spec [TERMAM]	-0.36	0.07	-0.50 – -0.23	-5.39	**<0.001**	437
Spec [THEVAH]	-0.17	0.13	-0.43 – 0.08	-1.32	0.187	437
Spec [TRI2PA]	0.01	0.10	-0.19 – 0.21	0.06	0.956	437
Spec [Voyria]	0.23	0.12	-0.01 – 0.47	1.86	0.064	437
Spec [XYL1FR]	-0.22	0.09	-0.39 – -0.05	-2.50	**0.013**	437
Random Effects						
σ^2	0.09					
τ_{00}	0.01_{plot}					
ICC	0.13					
N	97_{plot}					
Observations	464					
Marginal R^2 / Conditional R^2	0.450 / 0.523					
AICc	353.629					

MANUSCRIPT 6 – PERSPECTIVE

Mycoheterotrophy in the wood-wide web

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ABSTRACT

The prevalence and potential functions of common mycorrhizal networks, or the 'wood-wide web', resulting from the simultaneous interaction of mycorrhizal fungi and roots of different neighbouring plants has been increasingly capturing the interest of science and society, sometimes leading to hyperbole and misinterpretation. Several recent reviews conclude that popular claims regarding the widespread nature of these networks in forests and their role in the transfer of resources and information between plants are "disconnected from evidence" [1]. Here we argue that mycoheterotrophic plants associated with ecto- or arbuscular mycorrhizal fungi require resource transfer through common mycorrhizal networks and thus are natural evidence for the occurrence and function of these networks, offering a largely overlooked window into this methodologically challenging underground phenomenon. The wide evolutionary and geographic distribution of mycoheterotrophs and their interactions with a broad phylogenetic range of mycorrhizal fungi indicate that common mycorrhizal networks are prevalent, particularly in forests, and result in net carbon transfer among diverse plants through shared mycorrhizal fungi. Based on the available scientific evidence, we propose a continuum of carbon transfer options within common mycorrhizal networks, and we discuss how knowledge on the biology of mycoheterotrophic plants can be instrumental for the study of mycorrhizal-mediated transfers between plants.

Box 1. Key features

• **Fully mycoheterotrophic plants (c. 580 spp.)** do not photosynthesize and rely on carbon from mycorrhizal fungi for their entire development, largely underground. They belong to liverworts (Aneuraceae), gymnosperms (Podocarpaceae), monocots (Petrosaviaceae, Burmanniaceae, Thismiaceae, Triuridaceae, Iridaceae, Corsiaceae, Orchidaceae) and eudicots (Polygalaceae, Gentianaceae, Ericaceae). Many species appear to be locally rare, and sometimes are considered indicators for undisturbed common mycorrhizal networks.

• **Initially mycoheterotrophic plants (c. 28,000 spp.)** are non-photosynthetic plants completely dependent on fungi for carbon in their early developmental stages. They

belong to clubmosses (Lycopodiaceae), ferns (Ophioglossaceae, Psilotaceae, Gleicheniaceae), monocots (Orchidaceae), and eudicots (Ericaceae).

• **Partially mycoheterotrophic plants** combine autotrophy and mycoheterotrophy as adults. This has been experimentally tested for only a few plant species so far, but stable isotope signatures of carbon suggest that partial mycoheterotrophy occurs in a wide range of ferns, monocots, and eudicots.

• **The fungi targeted by mycoheterotrophic plants** belong to a wide range of ectomycorrhizal fungi (Atheliales, Boletales, Agaricales, Russulales, Thelephorales, Hymenochaetales, Gomphales, Cantharellales, Sebacinales, Leotiomycetes, Dothideomycetes, Pezizomycetes), arbuscular mycorrhizal fungi (Glomerales, Diversisporales), and saprotrophic fungi (mostly Agaricales). Fully mycoheterotrophic plants often show **higher specificity** towards narrow, ecto- or arbuscular, mycorrhizal fungal lineages than green plants.

• Mycoheterotrophy evolved over 40 times across **arbuscular, ecto-, and orchid mycorrhizal symbioses**. In a mycoheterotrophic interaction, plant-to-fungus flow of carbon is reversed relative to the net plant-to-fungus C flow that is characteristic of ecto- and arbuscular mycorrhizas. Because mycoheterotrophs have often evolved from photosynthetic ecto- or arbuscular mycorrhizal plants, they are considered as examples of mutualism breakdown.

• **Symbiotic germination**, in which 'dust-like' seeds or plant spores with little or no reserves rely on fungal carbon for germination, is often considered as a prerequisite for the evolution of initial and full mycoheterotrophy.

• **Common mycorrhizal networks** are formed when the same individual of mycorrhizal fungus physically links the roots of two or more different individual plants, belonging to the same or different species. These networks have the potential to transfer molecules or signals among plants and this has been the focus of many investigations.

• **Wood-wide web** is a term coined by *Nature* in 1997. While its initial use referred to resource transfer between specific trees and tree seedlings through a common mycorrhizal network, popular media has considerably broadened this concept to a fungal network through which most trees in a forest share resources and information.

The roots or root-like structures of most land plants are colonized by mycorrhizal fungi, which help plants to take up growth-limiting soil nutrients in exchange for photosynthetically fixed carbon [2]. These ancient interactions generally have low specificity and consequently, 'common mycorrhizal networks' can be formed when a mycorrhizal fungus simultaneously colonizes the roots of different plant individuals [3-5]. Based on indications that these networks can transfer resources between trees [6], common mycorrhizal networks in forests were labelled the 'wood-wide web', a concept which has since been expanded into a widespread fungal network that allows trees to exchange nutritional resources and even information [7-10]. Although the existence of common mycorrhizal networks is not in doubt, researchers have recently pointed out that for many of these popular claims scientific support is still lacking [1,11-14] and identified a bias in citing positive effects of common mycorrhizal networks in the scientific literature [1]. Therefore, the widespread occurrence and significance of common mycorrhizal networks has remained controversial, particularly in the scientific community [1,11-14].

Yet, as mentioned in all recent evaluations of the occurrence and potential functions of common mycorrhizal networks [1,12-14], there is one phenomenon in which the establishment and function of common mycorrhizal networks is undisputed: mycoheterotrophy. Here, non-photosynthetic mycorrhizal plants represent diverse 'positive controls' for the potential ecological and evolutionary consequences of common mycorrhizal networks. The existence of mycoheterotrophic plant species, hundreds of which obtain carbon from surrounding green plants through shared ecto- or arbuscular mycorrhizal fungi, is natural evidence for both the persistent formation of common mycorrhizal networks and their ability to act as a significant carbon source for plants. However, because most mycoheterotrophic plants tend to be small understory herbs only seen during flowering and fruiting, they are usually ignored or considered as exceptions to the mutualistic carbon-for-nutrients exchange typical of the mycorrhizal symbiosis [15], and therefore have received little attention in the controversies surrounding common mycorrhizal networks. We argue that these fascinating plants, which played a key role in the discovery of mycorrhizas [16] and are at the centre of questions about mycorrhizal cheating [17], continue to play their role as the 'sphinxes of mycorrhizal research' [18] – natural examples of mycorrhiza-mediated carbon uptake in plants within common mycorrhizal networks. To underline their unique importance, we briefly summarize the current knowledge on mycoheterotrophic plants, discuss how their

biology contributes to our understanding of the occurrence and functions of common mycorrhizal networks, and highlight how they can play a key role to advance our knowledge in this controversial field.

Mycoheterotrophy

Mycoheterotrophy is a plant trophic mode defined by the ability to obtain carbon from root- and/or rhizoid-associated fungi [19,20]. The most obvious examples are the c. 580 species of diverse, leafless, achlorophyllous plants, known as full mycoheterotrophs (Fig. 1) [21], including dicots, monocots, a gymnosperm and a bryophyte. These evolved from photosynthetic mycorrhizal ancestors at least 40 times across land plants (Fig 2b) [20] and their obligate mycoheterotrophic mode of life has mostly been deduced from the absence of photosynthesis [22], the absence of a direct (i.e. haustorial) link to any host plant (hence they are not holoparasites [23]), and dense fungal colonization in their roots or rhizoids (Fig. 2a) [16,19].

Fig. 1 The intricate root matrix of a forest ecosystem on the slopes of Mount Pirongia in New Zealand. Brown roots are those of *Beilschmiedia tawa* (Lauraceae, magnoliids), the dominant tree species at this location. White-yellowish roots are those of *Thismia hillii* (Thismiaceae, monocots), a perennial non-photosynthetic, mycoheterotrophic plant which is linked to *B. tawa* by a common mycorrhizal network of *Rhizophagus* sp. (Glomeraceae) arbuscular mycorrhizal fungi [122]. A thin layer of leaf litter was removed before taking this picture. Bar = 1 cm. Picture by V.S.F.T.

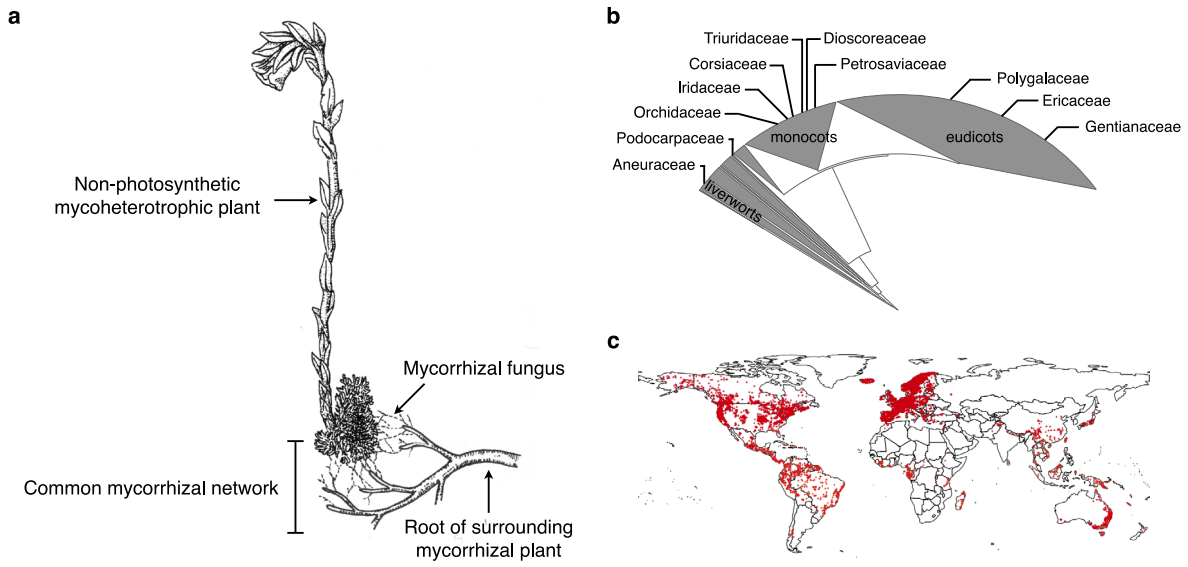

Fig. 2 Mycoheterotrophy in plants and its phylogenetic and geographic distribution. (a) Full mycoheterotrophy as evidence for interplant net carbon transfer through common mycorrhizal networks, with the example of the non-photosynthetic *Monotropa uniflora* (Ericaceae) linked to tree roots via ectomycorrhizal Russulaceae fungi; (b) Occurrence of achlorophyllous mycoheterotrophic plants in families across the plant tree of life; (c) Global distribution of observations of fully mycoheterotrophic angiosperms growing on arbuscular or ectomycorrhizal fungi based on available data from natural history collections (data from [63]).

The fungi of mycoheterotrophic plants have been repeatedly identified as fungi which form ecto- or arbuscular mycorrhiza with green plants [25,26], or as free-living wood or litter-decaying fungi [26]. Several DNA barcoding studies found support for ecto- or arbuscular mycorrhizal fungi simultaneously colonizing the roots of mycoheterotrophic plants and those of surrounding plants [25,27,28]. Ecto- and arbuscular mycorrhizal fungi are obligate biotrophs with little or no capabilities for saprotrophy [29,30] or plant cell wall degradation [31]; therefore, mycoheterotrophy requires a carbon source such as a nearby photosynthetic host for the non-photosynthetic plant's fungi. These neighboring plants provide all carbon required by the mycorrhizal fungi [2,32] and via these fungi carbon is provided to the mycoheterotrophic plant, thus creating a tripartite symbiosis. Natural abundances of stable carbon isotopes show that (fully) mycoheterotrophic plants have isotope signatures alike those of their mycorrhizal fungi [33-35], which are enriched in heavy carbon and nitrogen isotopes compared to photosynthetic understory plants growing at the same location [36,37]. Tracer experiments using ^{13}C or ^{14}C and mycoheterotrophic plants growing on ectomycorrhizal fungi have provided additional

evidence that fungi provide a pathway for transfer of carbon from green plants to mycoheterotrophs [38-42].

Mycoheterotrophy is not limited to non-photosynthetic plants. Initial mycoheterotrophs obtain carbon during germination and their non-photosynthetic early developmental stages, and then they rely on photosynthesis later in development [19]. All c. 28,000 species of orchids are considered initial mycoheterotrophs (some on ectomycorrhizal fungi, but most on saprotrophic fungi, which do not form common mycorrhizal networks), though outside orchids this mode of life is also known in Ericaceae and several genera of ferns and clubmosses, whose gametophytes are non-photosynthetic and colonised by ecto- or arbuscular mycorrhizal fungi [20,43]. Importantly, initially mycoheterotrophic plants reveal that reliance on carbon from mycorrhizal fungi connected to photosynthetic plants is dynamic and can change over plant development [44].

In addition to initial mycoheterotrophs, some adult photosynthetic plants are known to obtain carbon from fungi, a mode of life known as 'partial mycoheterotrophy' [45] or 'mixotrophy' [46]. Carbon dioxide assimilation measurements indicate that the green orchid *Corallorhiza trifida* obtains 85% of its carbon from ectomycorrhizal fungi colonising its roots [47]. In Orchidaceae and Ericaceae, natural abundances of stable isotopes, intermediate between those of fully mycoheterotrophic species and autotrophic plant species, provide further support for the existence of partial mycoheterotrophy [36,48,49]. In these partially mycoheterotrophic mature plants, the proportional gain of carbon from mycorrhizal fungi has been estimated to vary from near 0 to 84% [36]. Isotope studies indicate that carbon uptake in partial mycoheterotrophs can vary according to light levels, season, soil nutrients, and developmental stage [50-54]. In some species, the ability to obtain significant amounts of carbon from mycorrhizal fungi is supported by the natural occurrence of 'albino' plants and populations, which persist despite their non-functional photosynthesis [46,55-57]. Furthermore, analysis of natural abundances of stable isotopes suggests that partial mycoheterotrophy might be common in understory, photosynthetic, arbuscular mycorrhizal plants [58].

The autotrophy-mycoheterotrophy continuum

Partially mycoheterotrophic plants are often closely related to fully mycoheterotrophic species, and embedded in lineages where initial mycoheterotrophy is observed or suspected [21]. Evolutionary reconstructions support a stepwise evolution of mycoheterotrophy, from initial through partial to full mycoheterotrophy [59,60]. Overall, these observations support a model in which carbon uptake from mycorrhizal fungi is dynamic and can vary over ecological, evolutionary, and plant developmental scales leading to a continuum of carbon transfer options within common mycorrhizal networks, both ecological and evolutionary (Fig. 3). This continuum provides a functional framework to address questions regarding common mycorrhizal networks and their potential for interplant resource transfer. In particular, plants and fungi at the mycoheterotrophy end point are expected to reveal common biological characteristics, which are instrumental for mycorrhizal-mediated carbon transfer between green plants. Here we focus on carbon as the resource transferred, though others (e.g. mineral nutrients) may be relevant to common mycorrhizal networks [61,62], as mycoheterotrophy is likely to primarily inform carbon transfer via common mycorrhizal networks.

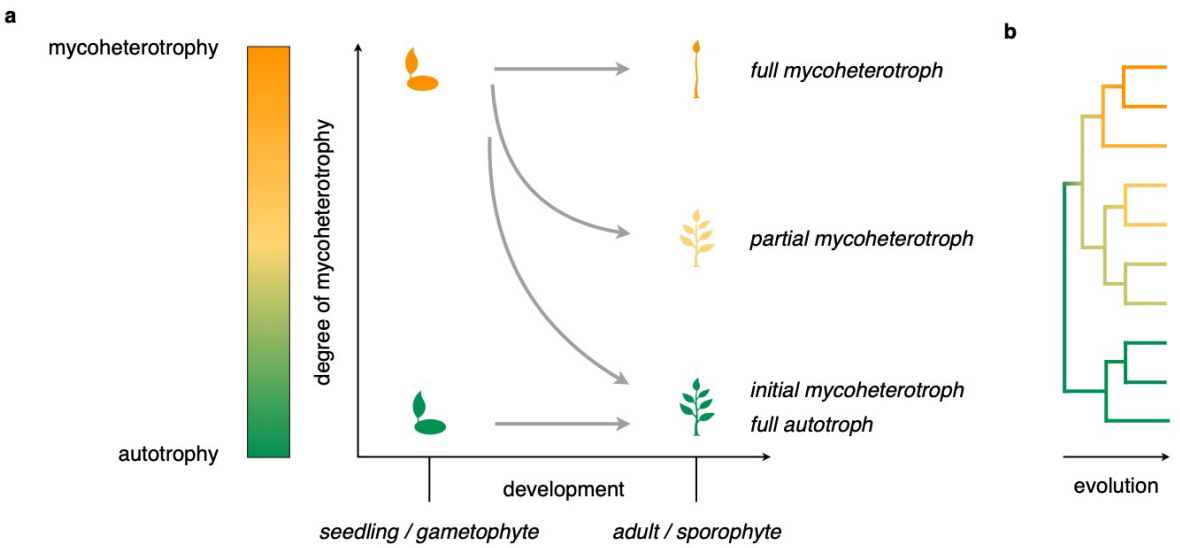

Fig. 3 The autotrophy-mycoheterotrophy continuum of mycorrhizal plants (a) over plant development, and (b) over evolutionary time scale (based on [21]).

Common mycorrhizal networks are widespread

Karst et al. [1] questioned the claim that common mycorrhizal networks are widespread in forests. To evaluate this claim they exclusively focussed on evidence from trees and highlighted that, with current technology, it is difficult to confirm that continuous, non-transient mycelial connections exist between trees in the field. They concluded that support for the widespread occurrence of common mycorrhizal networks is limited, owing to the paucity of information on common mycorrhizal network structure, and especially dynamics, in the field.

However, because fully mycoheterotrophic plants growing on ecto- or arbuscular mycorrhizal fungi provide natural evidence for the occurrence of common mycorrhizal networks, their distributions and those of their associated fungi offer additional information on the prevalence of common mycorrhizal networks. Locally, fully mycoheterotrophic plants are often rare (or difficult to observe), yet globally they have a wide distribution that is closely associated with the occurrence of forests (Fig. 2c) [63]. Unlike typical mycorrhizal-generalist photosynthetic plants, individual species of fully mycoheterotrophic plants often show high specificity in their interactions with mycorrhizal fungi, including extreme specificity to a single ecto- or arbuscular mycorrhizal lineage [64]. However, mycorrhizal specificity is not a requirement for mycoheterotrophy [65,66]. The fungi themselves belong to a phylogenetically wide range of arbuscular and ectomycorrhizal fungi – in fact only few major lineages of mycorrhizal fungi have not (yet) been found to be targeted by full mycoheterotrophs [67]. In the arbuscular mycorrhizal symbiosis, full mycoheterotrophs preferentially associate with Glomeraceae [68,69], which is the most abundant family of arbuscular mycorrhizal fungi in forests globally [70]. Several fully mycoheterotrophic Ericaceae species have also been found to associate with ectomycorrhizal fungi that are common in temperate forests [71]. Therefore, the global occurrence of fully mycoheterotrophic plants growing on mycorrhizal fungi and the wide range and distribution of fungi supporting these interactions strongly indicate that the potential for formation of common mycorrhizal networks and carbon transfer is widespread.

Full mycoheterotrophs can take up several years to develop, from germination to fruiting [43,72] and mycoheterotrophic fern gametophytes live up to several years [73], showing that the physical link between mycorrhizal fungi – often a single fungus – and a

neighbouring photosynthetic host plant is either maintained or continuously renewed over years. These observations further demonstrate the capacity of common mycorrhizal networks to persistently link the roots of different plant species under natural circumstances. Not surprisingly, mycoheterotrophs are considered indicators of undisturbed mycorrhizal networks such as old-growth forests [74-77].

Common mycorrhizal networks facilitate carbon transfer between plants

The hypothesis that carbon is transferred between plants through common mycorrhizal networks is central to the current debate on the wood-wide web [1,11-14]. Although multiple pulse-chase experiments have shown that labelled carbon is transferred from a donor tree to a receiver tree or sapling [5,78-81], it remains under dispute whether carbon was transferred through a soil or mycorrhizal pathway [1,12]. However, to experimentally rule out that carbon is transferred through the soil, rather than through a common mycorrhizal network, plant root systems would have to be separated by an air gap which physically separates the soil, but still allows for fungal hyphae to cross. Since mycorrhizal fungi grow through soil and soil solution, it is challenging to maintain ecological relevance with this setup. Also, because fungal hyphae are coated with aqueous films and fungal cell walls use apoplastic transport, this would not provide absolute proof for active carbon transport through a common mycorrhizal network.

Nevertheless, without carbon transfer via common mycorrhizal networks, mycoheterotrophic plants cannot exist. Because fully mycoheterotrophic plants provide evidence for carbon uptake from mycorrhizal fungi by plants (Fig. 2a), both the plants and their mycorrhizal fungi offer us clues into the mycorrhizal pathway. There is clear evidence that a wide range of photosynthetic plants also obtain carbon via such pathway, both in the arbuscular and ectomycorrhizal symbioses [36,59,82,83], and that the fungi involved also sustain achlorophyllous mycoheterotrophic plants, which themselves evolved from partially and initially mycoheterotrophic ancestors [21,60]. Given the widespread existence of fully, initially, and partially mycoheterotrophic plants, the question is not whether net carbon transfer between green plants occurs through common mycorrhizal networks, but rather how significant it is.

Mycoheterotrophic plants can also provide clues about the mechanisms by which plants get carbon from mycorrhizal fungi [14]. Although relatively few studies have focussed on fungus-to-plant transport in mycoheterotrophic plants, they provide an important

framework to explore mycorrhizal-mediated carbon uptake in green plants. It is often assumed that mycoheterotrophic plants obtain carbon from the 'digestion' of degenerating fungal hyphae or active lysis of hyphae, but given the quantity of fungal biomass in the roots of fully mycoheterotrophic plants, this mechanism has been considered insufficient to account for the full carbon demand of the plant [19]. Indeed, Kuga et al. [84] showed that carbon transfer from a *Ceratobasidium* fungus to the orchid *Spiranthes sinensis* occurs both through active transport and fungal degradation. Moreover, the fungal-induced growth of mycoheterotrophic protocorms (underground seedlings) of the orchid *Dactylorhiza purpurella* precedes lysis of fungal hyphae [85]. Also, the rapid transfer of ^{14}C from fungus to the orchid *Goodyera repens* – plant-respired $^{14}CO_2$ was detected within 7 hours [86] – indicates active carbon transfer from fungus to plant across intact membranes.

Furthermore, carbon-labelling and genomic studies of mycoheterotrophic orchids further revealed that trehalose is actively transported from fungus to plant and is likely the main carbon source supporting mycoheterotrophy for these species [32,87-89], but see [90]. *In vitro* germination experiments with ectomycorrhizal Ericaceae, which are initially mycoheterotrophic, provide clear evidence that the developing plants can use trehalose as their only carbon source [43]. Trehalose is an important component of carbohydrate conversion and biosynthesis in algae, early-branching land plants, and fungi, including ectomycorrhizal fungi and arbuscular mycorrhizal fungi, while it is sucrose that plays these roles in vascular plants [91-93]. The presence of a metabolic pathway in vascular plants for utilizing fungal trehalose as a carbon source may thus be an important component for mycorrhizal-mediated carbon uptake.

Plants benefit from carbon transfer through common mycorrhizal networks

An outstanding challenge is to assess the effect of carbon transfer through common mycorrhizal networks on plants and plant communities [1,12-13]. The difficulty to quantify carbon transfer through common mycorrhizal networks has been highlighted as a major reason for this challenge [11,12]. Based on isotope mixing models, carbon gain in some partially mycoheterotrophic orchids and Ericaceae species is considerable and may constitute more than half of the total carbon budget of the plant [94]. Similar levels of carbon gain have been reported for candidate partial mycoheterotrophs associated with arbuscular mycorrhizal fungi [58]. The autotrophy and mycoheterotrophy endpoints in

these two-source mixing models are averages of whole-plant signatures. These averages usually show some variation [95], and therefore the resulting estimates are prone to large uncertainties [96]. Importantly, this technique only provides a unidirectional estimate of fungus-to-plant carbon fluxes. Although a photosynthetic plant may receive carbon from mycorrhizal fungi, it may still be a net carbon donor to these fungi [6].

In pulse-trace experiments on trees and tree saplings, only relatively small gains have been detected in aboveground plant tissue (<10% of carbon acquired in plant tissue during the experiment [97-99], but see [100]), although factors such as low labelling intensity, duration of the experiment, and heterogeneity of carbon partitioning may lead to considerable underestimates of net carbon gain [101]. A pulse-trace experiment on *Cephalanthera damasonium,* a partially mycoheterotrophic orchid, highlighted the importance of the latter process; even though the investigated plants perform photosynthesis, the resulting photosynthates are not used for the growth of perennial underground organs [54]. Similarly, Simard et al. [6] estimated that 13% to 45% of the total fungal carbon acquired in the roots was translocated to foliar tissue in tree saplings. Therefore, mycorrhizal-mediated carbon gain can be considerably underestimated based on measurements of above-ground tissue. Finally, dual-labelling experiments allow for the measurement of bidirectional carbon transfer between two plants connect by a common mycorrhizal network, and thus the inference of net carbon transfer between plants when carbon transfer in one direction is larger than in the opposite direction. Only small levels (<10% of the total amount of carbon fixed by both plants) of net transfer have been reported using this technique [6,79], but always over short experiments (carbon 'pulse' periods of several hours followed by a 'chase' period of 7 to 9 days).

In the ectomycorrhizal symbiosis, it has been suggested that small amounts of carbon gain might be a by-product of nitrogen uptake [97]. But several studies indicate that nitrogen is transferred from the fungus to the plant in a non-organic form [102-105]. Similarly, in the arbuscular mycorrhizal symbiosis, both phosphorous and nitrogen are transferred from the fungus to the plant without carbon [106,107]. Therefore, carbon uptake is unlikely to be solely a by-product of nutrient uptake. Yet, it is clear that carbon uptake by plants from mycorrhizal fungi may only constitute a minute fraction of the total plant biomass in forest ecosystems or even in individual plants [97]. Also, because no study has reported a positive effect on plant growth or performance due to carbon gain from

common mycorrhizal networks, this phenomenon has been considered 'physiologically insignificant' [14]. Henriksson et al. [13] argued that if carbon uptake from mycorrhizal fungi would influence tree establishment and survival, seedling abundance and growth should be higher within the zone of active roots and associated mycorrhizal fungi of large trees than outside this zone – a concept known as the 'mother tree hypothesis' [13,14]. In line with this view, a slight carbon gain through mycorrhizal fungi is only effective if it outweighs interplant competition for light, nutrients and space, as well as growth-supressing negative plant-soil feedbacks. However, based on our knowledge of the dynamics of mycoheterotrophy in relation to environmental factors and plant development, even a minute amount of carbon during a particular developmental stage can be a determining factor in the success of plant establishment and development if it outweighs potential costs [108]. Therefore, we propose focusing on the question of whether small amounts of carbon gain can lead to niche expansion of species or a competitive advantage, rather than comparing overall plant performance under different environmental conditions. Similarly, on an ecosystem level, the absolute amount of plant carbon uptake from common mycorrhizal networks has likely an insignificant contribution to overall carbon cycling processes. Nevertheless, if widespread, mycoheterotrophy could be a determining factor in the composition of the forest understory vegetation, which not only includes the next generation of canopy trees but can also make up the majority of plant species in forest stands [109], is a substantial component of the carbon sink of forests [110], and is an overlooked reservoir of biodiversity [111]. Therefore, the timing and the ecological drivers of carbon transfer, rather than its relative contribution to plant biomass, may be of central importance to plant communities. Nevertheless, some researchers will continue to focus on the need for conclusive physiological evidence of significant net plant-to-plant transfer via fungi, while others will continue to focus on demonstrating fitness effects for the plants receiving any such transfer from neighbouring plants via shared fungi. Mycoheterotrophic plants represent a natural reference point, so far untapped, for both of those research lines.

Cheating the mycorrhizal symbiosis

The occurrence of carbon uptake by plants from mycorrhizal fungi has been further questioned from an evolutionary perspective: "Why should mycorrhizal fungi export carbon at all when the evolutionary stability of the symbiosis is based on fungal import of

plant carbon in exchange for nutrients such as nitrogen?" [13]. This view ignores that cheating is ubiquitous in mutualisms, as predicted by theory [112], and the mycorrhizal mutualism is no exception as there are clear indications that both mycorrhizal plants and fungi behave as cheaters [18,59,71,113,114]. Experiments indicate that mycorrhizal nutrient exchange dynamics are better understood as community-wide interactions between multiple players rather than as strict exchanges between individual plants and their symbionts [115,116]. In addition, our knowledge from mycoheterotrophy stresses the importance of temporal dynamics of resource exchange in the mycorrhizal symbiosis, which can change in rate and direction over time. While plants can be mycoheterotrophic during early developmental stages, they may be autotrophic and thus mutualistic during later stages. This may stabilize the symbiosis through selection for net overall fitness benefits for both partners over their lifetimes [117]. Thus, the multiple independent evolutionary shifts towards mycoheterotrophy within mycorrhizal plant lineages as part of the autotrophy-mycoheterotrophy continuum provide unequivocal examples for the evolutionary stability of mycoheterotrophy without the need to invoke fungal altruism.

Mycoheterotrophic plants as positive controls for carbon transfer in common mycorrhizal networks

By explicitly positioning the current debate on the occurrence and functions of common mycorrhizal networks in the context of the autotrophy-mycoheterotrophy continuum of the mycorrhizal symbiosis (Fig. 3), fully mycoheterotrophic plants emerge as positive controls for mycorrhizal-mediated transfer of carbon between plants. While at the autotrophic side of the continuum small gains of carbon are difficult to measure and their effects hard to assess, plants positioned at the mycoheterotrophic side provide essential clues on the ecology and physiology of fungus-to-plant carbon transfer. These clues can help uncover the true extent of resource transfer in common mycorrhizal networks and their importance in plant biology and biodiversity.

In particular, future studies on the genomics and metabolomics of mycoheterotrophic plants are needed to reveal the mechanisms, composition, quantity and chronology of metabolite transfers from fungus to plant, characterize the metabolic pathways involved in storage and allocation by the plant of the carbon and nutrients received from fungal partners, determine any metabolite fluxes from plant to fungus and their roles in establishing, maintaining or repaying the fungal association [96]. Further investigation of

common molecular, morphological, developmental and biochemical characteristics of mycoheterotrophic plants and their mycorrhizal fungi are necessary to reveal potential partially or initially mycoheterotrophic plant lineages beyond those that include fully mycoheterotrophic species [118-120], and to identify potential predispositions for fungus-to-plant carbon transfer through phylogenetic comparative analyses. Recent advances in the cultivation of fully and initially mycoheterotrophic plants provide important opportunities to study these aspects [43].

Continued molecular identification and genome sequencing of the mycorrhizal fungi associated with mycoheterotrophic plants and their interaction patterns in mycorrhizal networks are necessary to assess the fungal diversity involved in mycorrhizal-mediated resource transfer [28] and will eventually reveal the genetic and metabolic pathways of the fungal partner. Finally, key insight into the regulation of the mycorrhizal symbiosis will likely develop from uncovering the environmental conditions under which mycoheterotrophy occurs [121]. Labelling studies at forests sites where partially mycoheterotrophic plants occur will provide insights into these aspects. Subsequently, these genomic, metabolomic, morphological, biochemical, symbiotic, and environmental data will allow for targeted detection of mycorrhizal-mediated carbon gain in plants, including trees and their saplings.

Conclusions

Mycoheterotrophy provides natural evidence for the prevalence of common arbuscular and ectomycorrhizal networks and for their ability to mediate significant net carbon transfer among plants. The widespread occurrence of mycoheterotrophy, involving diverse plants and mycorrhizal fungi, suggests that persistent common mycorrhizal networks are common, particularly in forests, and can support net carbon transfer between green plants. How common the latter phenomenon is remains to be determined. The autotrophy-mycoheterotrophy continuum of mycorrhizal plants provides a functional framework to address this question. In particular, the biology of plants and fungi involved in the mycoheterotrophy end of the continuum offers essential tools to test for mycorrhizal-mediated resource transfer between plants. Mycoheterotrophy on mycorrhizal fungi and its widespread occurrence challenge the dogma of carbon-for-nutrients transfer in the mycorrhizal symbiosis as well as the assumption that all green plants are strict autotrophs. Hence, this phenomenon offers exciting opportunities for the

investigation of common mycorrhizal networks and their function. In this sense, mycoheterotrophic plants may be exceptions that both prove and challenge rules.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

The focus of this Perspective was conceived by all of the authors. V.S.F.T.M. led the writing, with contributions from S.I.F.G., D.W., C.V., H.J., F.E.Z., G.G., and M.I.B.

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