Partial mycoheterotrophy in orchids

Dissertation

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Partial mycoheterotrophy in orchids

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Für meinen Vater Hans Jürgen Kaufmann († 26. September 2018)

"They are wonderful creatures, these Orchids."

Charles Darwin, 1880

"Plants do not have roots, they have mycorrhizae."

unknown

Illustration of the orchid *Epipactis neglecta*, the truffle *Tuber excavatum* and the common beech *Fagus sylvatica* on the cover page by Hanna Kaiser (2016)

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ACKNOWLEDGEMENTS

ZUSAMMENFASSUNG

Mutualismen zählen zu den wichtigsten und in der Ökologie am häufigsten untersuchten, zwischenartlichen Interaktionen. Die Mykorrhiza dient hierbei als Paradebeispiel für einen Mutualismus. Dadurch, dass die absolute Mehrheit der Gefäßpflanzen in irgendeine Form der Mykorrhiza involviert ist, ist diese Form des Mutualismus die vielleicht weit verbreitetste im Pflanzenreich. In der Mykorrhiza gehen bodenlebende Pilze und Pflanzenwurzeln miteinander eine enge, wechselseitige Beziehung ein, in der sie mineralische Bodennährstoffe und Wasser gegen Kohlenhydrate aus des während der Fotosynthese fixierten CO_2 austauschen. Jedoch muss die engmaschige Beziehung zwischen beiden beteiligten Partnern nicht notwendigerweise gleichermaßen von Vorteil für beide involvierte Seiten sein.

Voll mykoheterotrophe Pflanzen besitzen kein Chlorophyll in ihren ohnehin sehr reduzierten Schuppenblättern und Stängeln, sie haben jedoch die Fähigkeit ausgebildet, Kohlenstoff von ihren Pilzpartnern zu beziehen und somit die reguläre Richtung des Kohlenstoff-Flusses in Mykorrhizabeziehungen vollkommen umzukehren. Anfänglich mykoheterotrophe Pflanzen sind während der Keimungsphase und bis zum Etablieren der jungen Keimlinge von der Nährstoffversorgung durch ihre Pilzpartner abhängig, als erwachsene Pflanzen gelten sie dann jedoch zunächst augenscheinlich als autotroph. Partiell mykoheterotrophe Pflanzen hingegen besitzen jedoch die Fähigkeit, simultan zum Kohlenstoff-Gewinn aus der Fotosynthese auch Nährstoffe von ihren Mykorrhiza-Pilzen zu beziehen. Möglicherweise sind partiell mykoheterotrophe Pflanzen genau dazu durch ihre anfängliche mykoheterotrophe Keimungsphase befähigt und somit geradezu vorbelastet, einen Teil ihres Nährstoffbedarfs auch im adulten Stadium durch ihre Pilzpartner zu decken. Das Vorhandensein von Chlorophyll in Blättern oder Stängeln mag partiell mykoheterotrophe Pflanzen dabei recht unauffällig erscheinen lassen. Es ist jedoch möglich, partiell und voll mykoheterotrophe Pflanzen mithilfe von Stabilisotopenverhältnissen natürlicher Häufigkeiten ($\delta^{13}C$, $\delta^{15}N$ und $\delta^{2}H$) und Stickstoff-Konzentrationen von wirklich autotrophen Pflanzenarten abzugrenzen und zu unterscheiden. Partiell und voll mykoheterotrophe Pflanzen sind dabei in ^{13}C, ^{15}N oder ^{2}H gegenüber autotrophen Pflanzen angereichert. Die Stabilisotopenanalyse zur Aufklärung trophischer Strategien wird idealerweise durch molekulare Methoden zur Identifizierung der Pilzpartner ergänzt.

Partiell mykoheterotrophe Arten wurden schon in einigen Pflanzenfamilien nachgewiesen, jedoch ist die Familie der Orchidaceae diesbezüglich in der Vergangenheit am gründlichsten untersucht worden. Dies liegt möglicherweise an der reinen Artenzahl in dieser artenreichsten Pflanzenfamilie (ca. 28.000 Arten), ganz sicher jedoch daran, dass Orchideen aus den staubfeinen, endospermlosen Samen unter natürlichen Bedingungen überhaupt nur in Anwesenheit eines Mykorrhiza-Pilzes keimen können. Die Orchideen stellen durch die Vielzahl trophischer Strategien von der

anfänglichen Mykoheterotrophie, über Autotrophie, verschiedenen Ausprägungen der partiellen bis hin zur vollen Mykoheterotrophie, eine ausgesprochen interessante Pflanzenfamilie dar. Zusätzlich ist der Mykorrhiza-Typ der Orchideen-Mykorrhiza auf die Orchidaceae beschränkt, jedoch wird diese von vielen unterschiedlichen Pilzgruppen (Ektomykorrhiza-Pilze, Holz- und Streuzersetzer, Rhizoctonia-Pilze) in den Orchideen-Wurzeln gebildet. Vergangene Forschungsvorhaben haben sich eher auf Charakteristika voll und partiell mykoheterotropher Orchideen konzentriert, die mit Ektomykorrhiza-Pilzen, Holz- und Streuzersetzern vergesellschaftet sind. Auch dieser Forschungsstand wird in der vorliegenden Arbeit zusammengefasst, allerdings befasst sich diese Arbeit größtenteils mit der partiell mykoheterotrophen Ernährung von Orchideen-Arten, die mit Rhizoctonia-Pilzen vergesellschaftet sind.

Das Hauptaugenmerk dieser Dissertation, in der neben der Synopse auch fünf Manuskripte inbegriffen sind, liegt darin, das generelle ökologische Verständnis der partiellen Mykoheterotrophie besonders in der Familie der Orchidaceae entscheidend zu verbessern. Fünf Fragestellungen wurden dabei hauptsächlich verfolgt, nämlich (1) Muster ökophysiologischer Traits bei Arten in den Ericaceae und Orchidaceae auf der Familienebene zu identifizieren, (2) die Treiber hinter der isotopischen Anreicherung in der Gattung der Ständelwurzen (*Epipactis*) zu bestimmen, (3) die Ernährungsweise Rhizoctonia-assoziierter Orchideen eindeutig zuzuordnen und (4) den prozentualen Anteil organischen Substanz-Gewinns durch die Mykorrhiza-Pilze zu quantifizieren und zuletzt (5) eine mögliche Lichtabhängigkeit der partiellen Mykoheterotrophie bei Orchideen-Arten, die mit Rhizoctonia-Pilzen vergesellschaftet sind, herauszustellen.

(1) Familienspezifische Muster in den Stabilisotopenverhältnissen und den Stickstoff-Konzentrationen in partiell und voll mykoheterotrophen Ericaceae und Orchidaceae in Assoziation mit Ektomykorrhiza-Pilzen konnten durch das Auswerten eines großen zusammengeführten Datensatzes erfolgreich identifiziert werden. (2) Der beobachtete Gradient in der ^{15}N-Anreicherung in der Gattung *Epipactis* konnte durch den Typ der jeweils mykorrhizierten Pilze aufgeklärt werden: Die konsistent höchsten ^{15}N-Anreicherungen wurden bei *Epipactis*-Arten festgestellt, die mit Ascomyceten der Gattung *Tuber* (Echte Trüffel) mykorrhiziert waren. (3) Mit dem neuen Ansatz der Bestimmung der H-Isotopenhäufigkeit (δ^2H-Ansatz) konnten viele Rhizoctonia-vergesellschaftete Orchideenarten offener Wiesenstandorte als partiell mykoheterotroph identifiziert werden. Darüber hinaus ist die trophische Strategie der partiellen Mykoheterotrophie wohl auch viel weiter verbreitet, als zunächst angenommen. (4) In einem nächsten Schritt wurde der Gewinn organischer Substanz einiger Rhizoctonia-assoziierter Orchideen auf ca. 20 % Ernährung durch die Pilzpartner quantifiziert. Dies geschah mithilfe eines methodologisch verbesserten Mischungsmodell-Ansatzes, nämlich unter Einbeziehung der C- und H-Isotopenhäufigkeiten voll mykoheterotropher, *in situ* gewonnener Orchideen-Keimlinge in die Berechnungen. (5) Zuletzt konnte erfolgreich gezeigt werden, dass die Ausprägung der partiellen Mykoheterotrophie auch bei zwei

Orchideenarten, die hauptsächlich mit Rhizoctonia-Pilzen mykorrhiziert sind (*Neottia ovata* und *Ophrys insectifera*), abhängig ist von der Lichtintensität am Standort.

Die Ergebnisse dieser Dissertation tragen zur Verbesserung des Verständnisses der komplexen Ökologie der partiellen Mykoheterotrophie besonders in der Familie der Orchidaceae bei, eröffnen jedoch auch noch neue Wege ihrer Erforschung.

ABSTRACT

Interspecific mutualisms are some of the most important and widely studied species interactions in ecology. Mycorrhizae qualify as the classical and prime example of mutualisms as they are very abundant: The absolute majority of vascular land plants engage in mycorrhizae. In mycorrhizae, soil fungi and plant roots participate in a bidirectional exchange of mineral soil nutrients and water for carbohydrates fixed from atmospheric CO_2 through photosynthesis. However, the association between mycorrhizal fungi and plants does not necessarily need to be mutually beneficial for both partners involved.

Fully mycoheterotrophic plants lack chlorophyll but have the ability to obtain carbon from fungi and thereby reverse the usual direction of plant-to-fungus carbon flow in mycorrhizae. Initially mycoheterotrophic plants rely on carbohydrates supplied by fungi for successful germination and seedling establishment but then become (putatively) autotrophic as mature plants. Partially mycoheterotrophic plants may be predisposed to an exploitation of their mycorrhizal fungi also at adulthood due to their first, initially mycoheterotrophic ontogenetic stages. The presence of green leaves or likewise stems may be misleading but partially mycoheterotrophic plants have the ability to obtain carbohydrates both from photosynthesis and by exploiting their mycorrhizal fungi. Stable isotope natural abundances ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$) together with N concentrations are the state-of-the-art tools to differentiate partial and full mycoheterotrophs from autotrophic plants. Partially and fully mycoheterotrophic plants can be differentiated from autotrophic plants due to their ^{13}C, ^{15}N or ^{2}H enrichments. The stable isotope approach for the clarification of trophic strategies is ideally complemented by the molecular identification of mycorrhizal fungi.

Partially mycoheterotrophic species have been detected in several plant families. However, the orchid family has been studied most intensively regarding trophic strategies, probably due to the sheer species richness in the family (ca. 28,000 species). Most certainly yet because orchid seedlings obligatorily require mycorrhizal fungi for germination from the dust-like seeds and subsequent successful seedling establishment in nature. The Orchidaceae hence represent an interesting plant family as its species feature all trophic strategies from obligate initial mycoheterotrophy, putative autotrophy over different degrees of partial to full mycoheterotrophy. Additionally, many different groups of soil fungi such as ectomycorrhizal, wood- or litter-decomposing fungi or the elusive rhizoctonia fungi are able to form orchid mycorrhiza, an endomycorrhiza unique to the Orchidaceae family, in the roots of orchid species. Research has mainly focused on partially and fully mycoheterotrophic orchids associated with ectomycorrhizal or saprotrophic, wood- or litter-decomposing fungi and is summarised in this thesis. Among

others, especially questions on the partially mycoheterotrophic nutrition of rhizoctonia-associated orchids remained open.

This thesis, which includes five manuscripts, aims to contribute to the general understanding of the complex ecology of the trophic mode of partial mycoheterotrophy, mainly in the Orchidaceae plant family. Five main objectives were pursued in this thesis, namely to investigate (1) the dependency of ecophysiological traits on the plant familial identity in Ericaceae and Orchidaceae, (2) the drivers of isotopic enrichment in the orchid genus *Epipactis*, (3) the presence of partial mycoheterotrophy in meadow orchids associated with rhizoctonia fungi, (4) the percentage of organic matter gain in rhizoctonia-associated orchids using an improved methodological approach and (5) the light-dependency of partial mycoheterotrophy in rhizoctonia-associated orchids.

The key findings are outlined in the synopsis and in detail presented in five manuscripts. (1) I collaborated on identifying family-specific patterns in stable isotope abundances and nitrogen concentrations in partially and fully mycoheterotrophic species in the Ericaceae and Orchidaceae associated with ectomycorrhizal fungi by synthesising the to date largest available dataset on their ecophysiologies. (2) This manuscript elucidates that the observed gradient in ^{15}N enrichment in partially mycoheterotrophic species within the orchid genus *Epipactis* is driven by the type of mycorrhizal fungi associated with these species, while *Epipactis* species associated with ectomycorrhizal Ascomycota of the true truffle genus *Tuber* exhibited the highest ^{15}N enrichments. (3) Partial mycoheterotrophy as nutritional mode for a suite of investigated meadow orchid species was successfully inferred based on the δ^2H approach and facilitated to conclude that partial mycoheterotrophy in rhizoctonia-associated orchid species of open habitats plays a far greater role than hitherto expected. (4) In a next step it succeeded to improve the linear two-source mixing model using *in situ* grown orchid seedlings as fully mycoheterotrphic endpoint in the calculations to quantify the organic matter gain derived by mature, rhizoctonia-associated orchids from mycorrhizae to reach approximately 20 %. (5) Finally, the degree of partial mycoheterotrophy in two rhizoctonia-associated orchid species (*Neottia ovata* and *Ophrys insectifera*) was shown to be dependent on the light availability at the microhabitat.

The results of this thesis contribute to improve the understanding of the complex ecology of partial mycoheterotrophy especially in the orchid family but also pave new research avenues.

SYNOPSIS

Structure of this thesis

My thesis starts by introducing the *mycorrhizal symbiosis* as a prime example of an interspecific mutualism, followed by the concept of *mycoheterotrophy* and *partial mycoheterotrophy*, the *evolution of mycorrhizae* and the *Orchidaceae* plant family as interesting case study to investigate the prevalent mycoheterotrophic strategies due to the interesting characteristics of these plant species such as e.g. their phylogenetic age, the diversity of habitats in which they occur, and their diverse above- and belowground specialisations. Afterward I portray the peculiarities of the *orchid mycorrhiza* and discuss its controversial classification as a mutualism. These sections are followed by a summary of the stable isotope profiles of *putatively autotrophic, initially, partially and fully mycoheterotrophic orchids* in which I also address knowledge gaps. In the following I list the *objectives* of my thesis and summarise my main results in the *synthesis* and elaborate on how they contribute to the state of the art in this research area. In an *outlook* I suggest how future research might address uncertainties and close knowledge gaps that remained open.

I close the synopsis with the individual *extended summaries* of the five manuscripts addressing different objectives on the ecology of partial mycoheterotrophy included in this thesis and a *declaration of my own contributions* to each manuscript are presented thereafter. *Oral presentations, posters and invited talks* that I have contributed to national and international conferences during my thesis as the lead-author as well as *prizes* I was awarded, are listed chronologically in Table 1. *Additional publications* that are not included in this thesis but I wrote or contributed to during my PhD are listed thereafter and are in part printed in *Appendix I* (Gebauer & Schiebold 2017) and *II* (Ogura-Tsujita *et al.* 2018). Finally, the *manuscripts* are presented as published (manuscript 1 – manuscript 4) or in their latest form (manuscript 5) together with their respective supplementary information.

As the thesis addresses readers from different research fields, a box of definitions and explanations of specific terms that are further explained throughout the text in more detail, is provided at the beginning of the *synopsis* (Box 1). Three additional boxes providing more detail on *mycorrhizae* (Box 2), an *excursus on mixotrophy* (Box 3) and another *excursus on dust seeds* (Box 4) are optional choices for the reader.

Box 1 *Definitions and explanations of terms used in this thesis*

Symbiosis: a neutral term referring to the regular coexistence of dissimilar organisms in intimate interspecific interactions.

Mutualism: a symbiosis with benefits for both partners in the association.

Mycorrhiza: originating from Greek *mýkēs* "fungus" and *rhiza* "root" (literally "fungus root"); Usually mutually beneficial association between soil fungi and plant roots characterising the bidirectional exchange of nutrients and water from the fungus for photosynthetically-fixed carbon from the plant. The vast majority of vascular plant species (85 %) are mycorrhizal (s. Box 3).

Mycoheterotrophy: the ability of a plant to obtain carbon (C) from fungi and thus reversing the usual direction of plant-to-fungus C flow in mycorrhizae.

Initially mycoheterotrophic plants rely on carbohydrates supplied by fungi for successful germination and seedling establishment but then become (putatively) autotrophic as mature plants. **Fully mycoheterotrophic** plants also lack chlorophyll at maturity and rely on nutrients supplied by associated fungi during their entire life cycle.

Partial mycoheterotrophy (also mixotrophy, s. separate section for ambiguity of this term): the ability of a plant to obtain carbohydrates both from photosynthesis and mycorrhizal fungi. Plants may be mistaken as autotrophs due to the presence of chlorophyll in their leaves or stems.

Stable isotope natural abundances $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ together with N concentrations are the state-of-the-art tool to differentiate partially (PMH) and fully mycoheterotrophic (FMH) species from autotrophic plants. The analysis of food webs and the clarification of trophic strategies using stable isotope signatures have a long tradition in ecology: "You are what you eat – plus a few permil" (DeNiro & Epstein 1976) holds true as $\delta^{13}C$ and $\delta^{15}N$ increase with each trophic level of a food chain. PMH or FMH plants obtaining nutrients from mycorrhizal fungi by can thus be identified as they enriched in ^{13}C, ^{15}N or ^{2}H in comparison to autotrophic plants.

Stable isotope abundance data (δ) can be converted into **enrichment factors** (ε) through normalisation to enable comparisons of C, N and H stable isotope abundances across sites: $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single value of an autotrophic, a PMH or FMH plant, and δ_{REF} is the mean value of all autotrophic reference plants by plot.

The **linear two-source mixing model** can be used to identify the percentage of fungal-derived nutrients in PMH plants: % $x_{df} = (\varepsilon_{PMH}/\varepsilon_{MH}) \times 100$, where ε_{PMH} is the enrichment factor of a PMH plant and ε_{MH} the mean enrichment factor of a full mycoheterotroph.

Species interactions: the mycorrhizal symbiosis

Interspecific mutualisms are some of the most important and widely studied species interactions in ecology (Hoeksema & Bruna 2000). Alongside intricate plant-pollinator interactions, mycorrhizae are considered the classical and prime example of mutualisms. In mycorrhizae, translating literally to *fungus root* from Greek, soil fungi and plant roots engage in a bidirectional exchange of mineral soil nutrients and water for carbohydrates fixed from atmospheric CO_2 through photosynthesis (Smith & Read 2008). Also intricate *mycorrhiza-like* associations between soil fungi and non-vascular plants lacking true roots, such as bryophytes, exist. Mycorrhizae are very widespread interspecific mutualisms: Current estimates classify 85 % of all terrestrial plants representing 92% of all plant families as mycorrhizal (Wang & Qiu 2006; Strullu-Derrien *et al.* 2018). The association between mycorrhizal fungi and plants is usually regarded as mutually beneficial for both partners (Smith & Read 2008). However, mycorrhizal associations might become asymmetric when one of the partners draws much more benefit from the interaction than the other (Hoeksema & Bruna 2000). Hence, partners in mycorrhizae can be placed along a *mutualism-parasitism continuum* (Johnson, Graham & Smith 1997).

Mycoheterotrophy

It is a widespread conception that plants in general are photoautotrophs that fix inorganic carbon from atmospheric CO_2 with energy from light and convert it into organic materials. However, some plants have completely lost the ability to photosynthesise and are able to obtain all nutrients from fungi. Due to a cascade of photosynthetic gene losses until final severe disruptions of the plastid genome on the evolutional pathway to full mycoheterotrophy, the photosynthetic ability is irreversibly lost (Graham, Lam & Merckx 2017). This contrasts with truly hemiparasitic and holoparasitic plants that tap on other plants by means of an haustorium – a unique organ functioning in attachment, penetration, and solute transfer from host to parasite (Shen *et al.* 2006; Eriksson & Kainulainen 2011). A number growing from at least 514 achlorophyllous plant species due to new species discoveries or rediscoveries with at the minimum 46 independent evolutionary origins (Leake 1994; Merckx 2013) are fully mycoheterotrophic (FMH) and have reversed the usual mycorrhizal carbon flow: They obtain all their resources from their associated fungi which can be mycorrhizal fungi usually forming arbuscular mycorrhizae or ectomycorrhizae with surrounding autotrophic plants, or saprotrophic fungi decomposing wood or litter (Bidartondo *et al.* 2004; Ogura-Tsujita *et al.* 2009; Cameron & Bolin 2010; Merckx *et al.* 2010; Merckx 2013). FMH plants were long misconceived as being saprophytic, translating to be living directly on soil organic matter, but today their obligate association with fungi is beyond

question (Merckx 2013). It was Erik Björkmann who first demonstrated the flow of carbon (C) and phosphorus from *Picea* to *Hypopitys* through a shared mycorrhizal network of the ectomycorrhizal fungus *Tricholoma* using radioisotopes and he also coined the term *epiparasitism* for this symbiosis (Björkman 1960). The term *epiparasitism* is occasionally used as a synonym for full mycoheterotrophy (Cullings, Szaro & Bruns 1996; Bidartondo *et al.* 2002; Leake 2004). Full mycoheterotrophy is an adaptation to low light conditions that especially plants growing on the forest floor experience. It is not surprising that the largest numbers of mycoheterotrophic species are distributed in tropical forests that are characterised by dense canopies and only very few light arriving on the forest floor (Leake 1994; Selosse & Roy 2009). In fact, the resources that FMH obtain from their associated fungi originate from the photosynthesis of autotrophic plants that allocate C to their mycorrhizal fungi – a tripartite matter flux between trees and FMH plants via a shared mycorrhizal network takes place (Ogura-Tsujita *et al.* 2009; Bougoure, Brundrett & Grierson 2010; Hynson *et al.* 2013). FMH plants have an absolutely striking appearance (Fig. 1) as their aboveground structures only serve for reproduction and dispersal (Merckx 2013). However, due to the lack of chlorophyll they can also be inconspicuous, especially if they blend with fallen leaf litter on the forest floor and may thus be easily overlooked. Raised awareness for these plants due to ongoing research have led to many new species being described (Suetsugu 2017; Chantanaorrapint & Suddee 2018; Dixon & Christenhusz 2018) or rediscovered that were believed to have already faced extinction (Sochor *et al.* 2018). In a review from 1994, Jonathan Leake coined the term *myco-heterotrophy* for plants that can be identified by their achlorophyllous appearance and meet their C and nutrient demands exclusively via fungi (Leake 1994). FMH plants can thus be placed at the parasitic end of the *mutualism–parasitism continuum*.

Fig. 1 (from left to right) The fully mycoheterotrophic species *Thismia thaitongiana* Chantanaorr. & Suddee, *sp. nov.* (Burmanniaceae, Thailand; image courtesy of S. Chantanaorrapint published in Chantanaorrapint & Suddee 2018); *Rhizanthella johnstonii* K.W.Dixon & Christenh., *sp. nov.* (Orchidaceae species flowering under-ground, Western Australia; image courtesy of K. Dixon published in Dixon & Christenhusz 2018) and *Monotropa hypopitys* L. (Ericaceae, Germany).

Partial mycoheterotrophy – a trophic continuum

A group of green-leaved plants have retained the ability to photosynthesise, but simultaneously meet a proportion of their nutrition via associations with mycorrhizal fungi (Gebauer & Meyer 2003). These plants are partially mycoheterotrophic (PMH) and in fact, the sheer presence of chlorophyll may not be sufficient to classify them as autotrophic (Smith & Read 2008). Partial mycoheterotrophy has been detected in green Orchidaceae species, Burmanniaceae, Ericaceae and Gentianaceae (Zimmer *et al.* 2007; Hynson *et al.* 2009; Cameron & Bolin 2010; Merckx *et al.* 2013b; Bolin *et al.* 2015). The trophic strategy of partial mycoheterotrophy can exhibit all intermediate stages between the extreme endpoints of autotrophy and full mycoheterotrophy (Fig. 2; Merckx 2013).
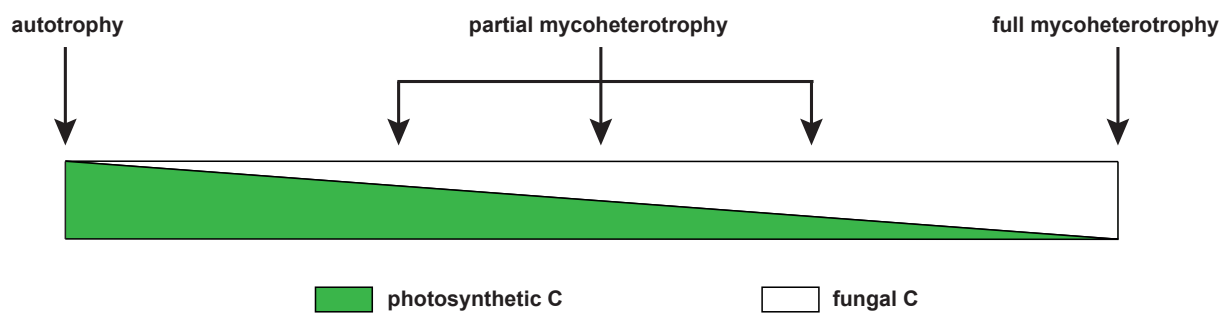
Fig. 2 Trophic gradient from autotrophy to mycoheterotrophy via different degrees of partial mycoheterotrophy expressed by the amount of photosynthetically fixed carbon (C) or C exploited from mycorrhizal fungi used by a plant. Schematic representation reproduced from Merckx (2013).

As a partially mycoheterotrophic plant obtains C from two sources, photosynthesis and mycorrhizal fungi, this trophic mode has also been termed *mixotrophy* (Julou *et al.* 2005; Abadie *et al.* 2006; Selosse & Roy 2009; Yagame *et al.* 2012; Roy *et al.* 2013; Selosse *et al.* 2016; Jacquemyn *et al.* 2017b; Lallemand *et al.* 2017) (s. Box 2). Partial mycoheterotrophy is a facultative nutritional mode where nutrient exploitation from mycorrhizal fungi is supplemental to photosynthesis (Eiler 2006), e.g. the degree of partial mycoheterotrophy and thus the reliance on fungal carbon obtained by a plant can be dynamically increased when the light level is low (Preiss, Adam & Gebauer 2010). Whether the exploitation of fungal carbon can be fine-tuned by all PMH plants associating with different types of mycorrhizal fungi remains to be investigated. Partial mycoheterotrophy can be considered as a backup for the photosynthetic apparatus in times of low light intensity. All so far known PMH plants exhibit a change of trophic strategies during their development. In addition to all fully mycoheterotrophic plants, all species in the Orchidaceae and the subfamily Pyroloideae in the Ericaceae produce *dust seeds* that are characterised by an undifferentiated embryo and a lack of endosperm and

Box 2 *Excursus − partial mycoheterotrophy vs. mixotrophy*

Quite frequently the term *mixotrophy* is used as a synonym or instead the in Merckx (2013) well-defined technical term *partial mycoheterotrophy* (Julou *et al.* 2005; Abadie *et al.* 2006; Selosse & Roy 2009; Yagame *et al.* 2012; Roy *et al.* 2013; Selosse *et al.* 2016; Selosse, Charpin & Not 2017; Jacquemyn *et al.* 2017b; Lallemand *et al.* 2017). Generally, mixotrophy is defined as the ability of an organism to be autotrophic and heterotrophic at the same time (Stoecker *et al.* 1988). This definition might fit within the same framework that is applied to partial mycoheterotrophy on the first sight. However, in the ecological literature the term mixotrophy is primarily employed for protists that are photo– and phagotrophic at the same time, and thus combine photoautotrophy with heterotrophy (Stoecker 1998). The mixotrophic lifestyle was first described for protozoa (ciliates and flagellates) in marine habitats that are primarily heterotrophic but have photosynthetic abilities by harbouring algal endosymbionts (Stoecker *et al.* 1988).
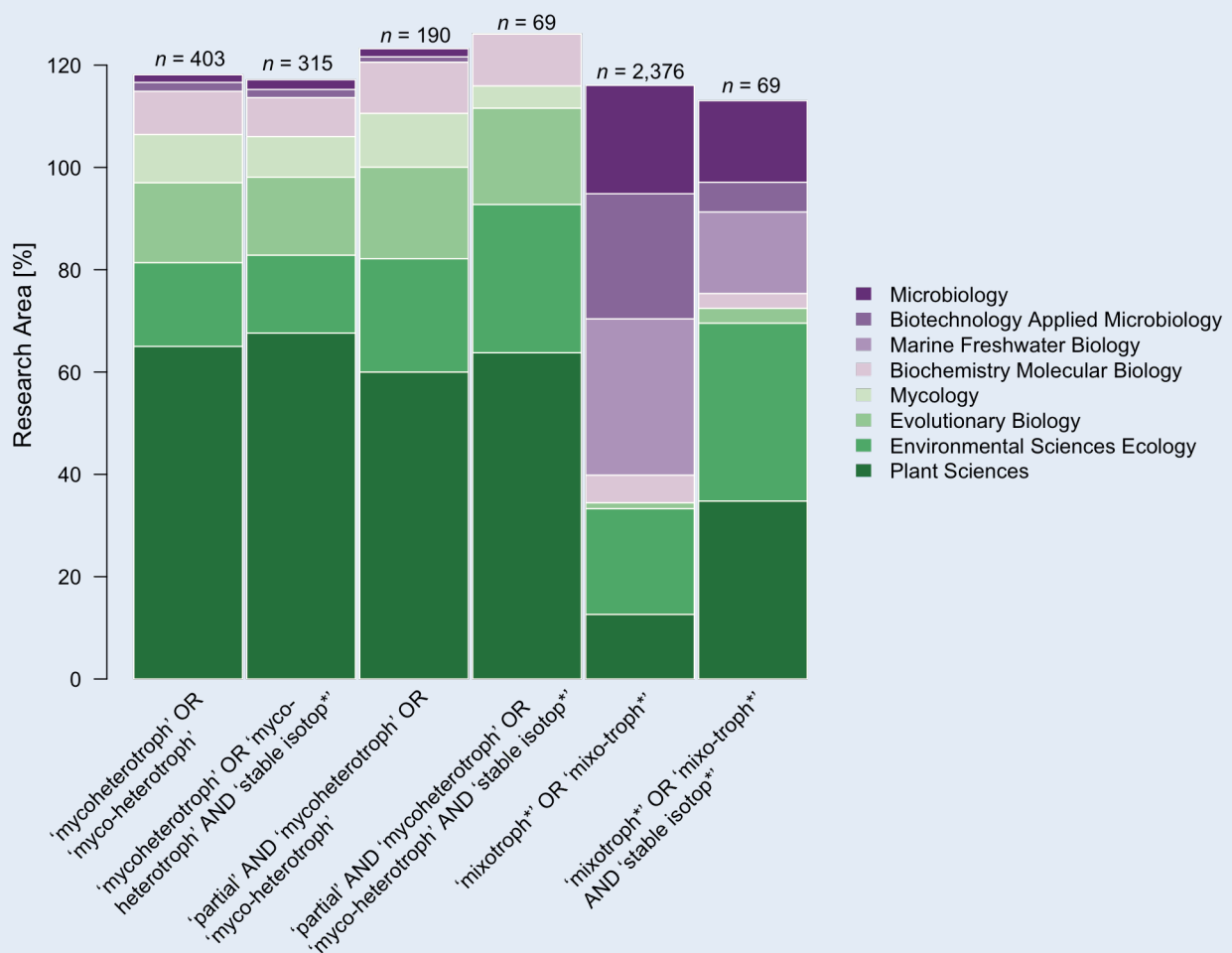
Fig. 3 Results of a literature survey using the web-based search engine Web of Science conducted on 1st of March 2018 with different sets of key words used in the field "Topic" shown below the individual stacked bars. Number of hits per search string are shown above the stacked bars. The studies are differentiated according to eight major research areas [%]. The summed proportions can exceed 100 % as some studies are assigned to more than one research area.

Box 2 (continued) *Excursus − partial mycoheterotrophy vs. mixotrophy*

A literature survey using the web-based search engine "Web of Science" (http://apps.webofknowledge.com/) conducted on 1st of March 2018 with different sets of key words (Fig. 6) used in the field "Topic" confirmed the ambiguity of the term mixotrophy. Search strings containing any combination of "mycoheterotroph*" or "myco-heterotroph*" with or without "partial*" yielded up to 403 hits in the research areas *Plant Sciences*, *Environmental Sciences Ecology*, *Evolutionary Ecology* and *Mycology*. These search strings in combination with "stable isotop*" reduced the number of hits but the main research areas stayed unchanged. Using "mixotroph*" or "mixo-troph*" as search string almost sextupled the number of hits to 2,376 and yielded dramatically different results concerning the associated research areas. The major research areas associated with the hits were *Marine Freshwater Biology*, *Biotechnology Applied Microbiology* and *Microbiology* followed by *Environmental Sciences Ecology* emphasising the restriction to the original research areas were the term mixotrophy was originally employed. Using the search string in combination with "stable isotop*" effectively reduced the number of hits as well as the main research areas to *Environmental Sciences Ecology*, *Plant Sciences*, *Marine Freshwater Biology and Microbiology* then approaching the results of the search strings with "mycoheterotroph*" or "myco-heterotroph*". Concluding, the technical term *partial mycoheterotrophy* should be preferred over the ambiguous expression *mixotrophy* if the trophic mode of plants simultaneously using photosynthesis and nutrient gain via mycorrhizal fungi is described to reach the desired readership in a designated research area.

are dependent on colonisation by a mycorrhizal fungus and supply of carbohydrates to facilitate germination and seedling establishment and are thus initially mycoheterotrophic (IMH) (Merckx 2013). At adulthood these IMH plants either stay FMH (e.g. *Neottia nidus-avis*, s. Fig. 4) or they become (putatively) autotrophic, or PMH where they conduct photosynthesis and supplement their nutrition with nutrients obtained from mycorrhizal fungi. The sheer species richness in the Orchidaceae makes initial mycoheterotrophy the most widespread fungi-mediated trophic strategy. For partial mycoheterotrophs, or initial mycoheterotrophs that experience a change of trophic strategies from mycoheterotrophy at juvenile stages to putative autotrophy at adulthood during ontogeny, the categorisation along the *mutualism-parasitism continuum* is comparably more challenging than for FMH plants. However, it is not fully resolved whether the associated fungi eventually benefit from this association in so far unexplored ways (Selosse & Roy 2009; Graham *et al.* 2017).

Stable isotope natural abundance analysis together with the molecular identification of mycorrhizal fungi via ITS- or Next-Generation Sequencing have become

the standard tools to identify PMH and FMH plants, and most of this research has been conducted in Orchidaceae and Ericaceae (Leake & Cameron, 2010). Analysis of food webs and clarification of trophic strategies with $\delta^{13}C$ and $\delta^{15}N$ stable isotope natural abundances have a long tradition in ecology (DeNiro & Epstein 1976, 1981). DeNiro and Epstein coined the term "you are what you eat – plus a few permil" (DeNiro & Epstein 1976) to stress the systematic increase in the relative abundance of the heavy isotopes ^{13}C and ^{15}N at each trophic level of a food web. Fungi, being heterotrophic organisms, are enriched in ^{13}C as well as in ^{15}N in comparison to their substrates and to autotrophic plants (Gebauer & Dietrich 1993; Gleixner *et al.* 1993; Trudell, Rygiewicz & Edmonds 2003). FMH and PMH plants of various degrees take up fungal material either via hyphal lysis and / or transfer across intact membranes of the fungal hyphae and plant roots and incorporate this material into their biomass (Smith & Read, 2008). Following the systematic increase in the relative abundance of ^{13}C and ^{15}N at each trophic level in a food web (DeNiro and Epstein 1978 & 1981), tissues of FMH and PMH plants consequently reflect the isotopic signature of their associated fungi. The pioneering works on the ecophysiology of mycoheterotrophy using stable isotope natural abundances were published in 2003 (Gebauer & Meyer 2003; Trudell *et al.* 2003). Both research teams concluded that FMH plants were significantly enriched in ^{13}C and ^{15}N compared to surrounding autotrophic plant species. Gebauer and Meyer (2003) also realised that some green-leaved, putatively autotrophic and in fact PMH orchids exhibited an intermediate ^{13}C enrichment relative to FMH and autotrophic plant species. PMH plant species occupy an intermediate position in ^{13}C enrichment between FMH and autotrophic plant species as they incorporate both ^{13}C-enriched resources from mycorrhizal fungi and CO_2 from photosynthesis that is relatively depleted in ^{13}C. Contrasting, FMH species rely on their mycorrhizal fungi, and thus ^{13}C-enriched substances, as sole resource.

Using stable isotope natural abundance analysis, a growing number of PMH species have been identified (Gebauer & Meyer 2003; Girlanda *et al.* 2006; Cameron & Bolin 2010; Merckx *et al.* 2010; Hynson *et al.* 2013; Bolin *et al.* 2015; Hynson, Schiebold & Gebauer 2016, manuscript 1). Isotopic enrichment in ^{13}C of PMH orchids was not only shown to be intermediate between autotrophic plants from the same microhabitats (Gebauer & Meyer 2003) but also that this parameter varies with light availability (Preiss *et al.* 2010) and leaf chlorophyll concentration (Stöckel, Meyer & Gebauer 2011). The proportions of C gained by PMH species from ectomycorrhizal fungi have successfully been quantified with a linear two-source mixing-model (Gebauer & Meyer 2003; Preiss & Gebauer 2008; Hynson *et al.* 2013). Carbon gain in the orchid species *Cephalanthera damasonium*, for example, can range from 33 % in an open forest dominated by Scots Pine *Pinus sylvestris* to about 85 % in a dark forest dominated by the common beech *Fagus sylvatica* (Gebauer 2005; Hynson *et al.* 2013). For orchids associating with rhizoctonia fungi this attempt proved unsuccessful and quantifications are still open for new methodological approaches. Far less clear is the explanation of

variations in ^{15}N enrichment found for fully, partially and initially mycoheterotrophic plants, but also for putatively autotrophic species (Gebauer & Meyer 2003; Abadie *et al.* 2006; Tedersoo *et al.* 2007; Preiss & Gebauer 2008; Selosse & Roy 2009; Liebel *et al.* 2010; Hynson *et al.* 2013). Additionally, the ^{15}N enrichment was described to be not linearly related to the degree of heterotrophic C gain (Leake & Cameron 2010; Merckx 2013).

Evolution of mycorrhizae

The mycorrhizal symbiosis is probably the phylogenetically oldest interspecific mutualism. It is a widely accepted concept that mutualistic interactions with soil fungi facilitated the colonisation of the terrestrial land by plants. Fungi had colonised the terrestrial environment long before the first land plants emerged. The earliest, rootless, terrestrial nonvascular plants coevolved with Glomeromycota fungi that are believed to be the earliest symbiotic fungi (Wang & Qiu 2006). Forming mycorrhiza-like structures with these early land plants, fungi enhanced access to soil mineral nutrients and water in exchange for photosynthetically fixed carbon (van der Heijden *et al.* 2015). Additionally, Glomeromycota that formed the ancestral mycorrhiza resembling modern arbuscular mycorrhiza (AM), depend entirely on their plant partners for photosynthetically produced carbohydrates, making them strictly biotrophic and thus heterotrophic (Pirozynski & Malloch 1975; Field *et al.* 2015). Judging from the plant side, mycotrophism made terrestrial plant life possible (Pirozynski & Malloch 1975).

The oldest fossil of lower land plants harbouring fungal hyphae of Glomeromycota and Mucoromycotina exhibiting striking similarities to modern AM is the *Rhynie Chert* that was dated back to the early-Devonian (407 Myr) (Smith & Read 2008; Strullu-Derrien *et al.* 2014; Field *et al.* 2015). A recent study pointing at the fossil gap in early land plants, used Bayesian relaxed molecular clock models pushed the emergence of land plants further back to the middle Cambrian (c. 515 Myr) (Morris *et al.* 2018) and thus also the emergence of the first mycorrhiza. All other types of mycorrhizal symbioses (s. Box 3) such as ectomycorrhiza (ECM), ericoid mycorrhiza (ErM) and orchid mycorrhiza (OrM) are believed to be of a more recent origin and to have evolved through later switches from arbuscular mycorrhiza-forming Glomeromycota and Mucoromycotina to other fungal lineages in the Cretaceaous (van der Heijden *et al.* 2015; Wang & Qiu 2006; Brundrett & Tedersoo 2018). Probably owing to multiple independent evolutionary losses of AM, only 8 % of all plants are non-mycorrhizal (NM) such as the Brassicaceae, Caryophyllaceae, Proteaceae and Cyperaceae among others with only very scattered fungal colonisation in the plants roots (Smith & Read 2008; Brundrett & Tedersoo 2018).

Box 3 *Mycorrhizae*

Mycorrhiza types: Depending on the morphology of root tissues and involved plant lineages, plant species engage the four main mycorrhiza types arbuscular mycorrhiza (72 %), ectomycorrhiza (2 %), ericoid mycorrhiza (1.5 %) or orchid mycorrhiza (10 %). In all types of mycorrhiza fungal hyphae penetrate the plant roots and structures for resource exchange are formed between mycorrhizal fungi and plant roots.

Arbuscular mycorrhiza (AM): AM, being the most ancestral type of mycorrhiza, is also the predominant mycorrhiza type with 72 % of plant species forming AM (Wang & Qiu 2006; Brundrett & Tedersoo 2018). The variety of plants associating with AM fungi is extremely diverse including numerous plant families in angiosperms, gymnosperms and even pteridophytes and both woody and herbaceous species (Smith & Read 2008). The AM "symbiosis is so ubiquitous that it is easier to list the plant families in which it is not known to occur than to compile a list of families in which it has been found" (Gerdemann 1968 in Smith & Read 2008). In AM, fungal hyphae of the Glomeromycotina grow in the apoplastic space between plant cells, they penetrate the cells in which they form arbusculues or vesicles, depending of the subtype of the endomycorrhiza (Smith & Read 2008).

Ectomycorrhiza (ECM): ECM is abundant in only 2 % of plant species being mostly woody perennials in the Pinaceae, Fagaceae and Dipterocarpaceae, but formed by many species of Ascomycota and Basidiomycota. However, this relatively small percentage of ectomycorrhizal plants species is relativised by the dominance of these plant families in the boreal forests of the northern hemisphere, temperate forests in both northern and southern hemispheres and tropical forests in South-East Asia, respectively, and their economic values as main timber producers (Smith & Read 2008). In ECM, sheaths of fungal hyphae enclose the plant roots, penetrate the intercellular space of the cortical cells but not the cells themselves to form a hyphal network, the *Hartig net* (Smith & Read 2008).

Ericoid mycorrhiza (ErM): ErM is only abundant in 1.5 % of the total plant species organised in the order Ericales (families Ericaceae, Epacridaceae and Empetraceae) that are dominant in heathland ecosystems in the northern hemisphere, southern Africa and Australasia (Smith & Read 2008; Brundrett & Tedersoo 2018). Several Ascomycota and Basidiomycota form this type of endomycorrhiza where fungal hyphae penetrate the cortical cells.

Orchid mycorrhiza (OrM): OrM is an endomycorrhiza restricted to the Orchidaceae plant family where intracellular colonisation of the plants' cortex cells and the formation of pelotons (hyphae coils) are characteristic. In contrast to ECM and ErM probably having many independent origins in numerous unrelated plant lineages, OM appears to have evolved only once: OrM is the common mycorrhiza type for all 28,000 species in the Orchidaceae plant family. Due to the shear species richness with accounting to every 10th plant species being an orchid, OrM is also a very abundant mycorrhiza type (10 %) (s. separate section *Orchid mycorrhiza*) .

The orchid family – Orchidaceae

Evolution of the Orchidaceae

Closely followed by the Compositae, the Orchidaceae is considered as the largest angiosperm family with ca. 28,000 species in 736 genera, making one in every ten plant species an orchid (Chase *et al.* 2015; Christenhusz & Byng 2016). The Orchidaceae is a polyphyletic family, placed basal in the order Asparagales and the Orchidaceae might be a sister clade to the rest of the Asparagales (e.g. with families Amaryllidaceae, Asparagaceae). The Orchidaceae is subdivided into five subfamilies: Epidendroideae, Orchidoideae, Cypripedioideae, Vanilloideae and Apostasioideae. In the phylogeny the Apostasioideae is placed as sister to all other subfamilies, then Vanilloideae and Cypripedioideae sister to Orchidoideae and Epidendroideae (Givnish *et al.* 2015). Despite several indications suggesting an phylogenetic older age of the Orchidaceae such as its enormous species richness, their worldwide distribution, and the basal placement in the order Asparagales, the Orchidaceae family was hypothesised to be of evolutionary young origin (Ramírez *et al.* 2007). The hypothesis of a young origin of the Orchidaceae was widely accepted due to the prevalence of modern traits such as highly specialised pollination mechanisms and epiphytism, and the absence of orchids in the fossil record. With the first orchid fossil found preserved in Dominican amber, Ramírez *et al.* (2007) rejected the hypothesis of a phylogenetic young age of the Orchidaceae for an ancient origin: Tightly packed pollen units (pollinia) of the orchid species *Meliorchis caribea* (gen. et sp. nov.) where found attached to an individual of the extinct stingless bee, *Proplebeia dominicana* Wille & Chandler. This fossil retrieved from Dominican amber dated back to 15 – 20 Myr, was not only the first fossil orchid find but also the first fossil observation of a plant-pollinator interaction. Using a calibrated molecular phylogenetic tree, Ramírez *et al.* (2007) estimated the most recent common ancestor of extant orchids to have lived in the Late Cretaceous (76 – 84 Myr) during the Cretaceous Terrestrial Revolution (KTR). During KTR (125 – 80 Myr), ferns and gymnosperms were replaced by angiosperms and flowering plants radiated explosively alongside rapidly diversifying pollinators, herbivores and their predators (Lloyd *et al.* 2008; Barba-Montoya *et al.* 2018). Ramírez *et al.'s* (2007) minimum estimates of the most recent common ancestor of extant Orchidaceae were pushed back even further to ca. 112 Myr (Givnish *et al.* 2015) and even back to 128 Myr through sequencing of the genome of *Apostasia shenzhenica* from the basal subfamily Apostasioideae and the reconstruction of an ancient orchid gene toolkit (Zhang *et al.* 2017). Two recent studies concluded that angiosperms appeared much earlier (crown-Angiosperms 256 – 149 Myr) than indicated by the fossil record allowing to push the origin of Orchidaceae even further back (Barba-Montoya *et al.* 2018; Katz 2018).

Ecology of the Orchidaceae

Orchids have a world-wide distribution, and they occur in a variety of habitats only being absent from the polar regions and the driest deserts (Merckx *et al.* 2013a; Chase *et al.* 2015). Orchids are an exceptional plant family as they successfully colonised epiphytic, lithophytic, terrestrial and even subterranean habitats. The absolute majority of orchids (> 80 %) grow as epiphytes using tropical forest trees as phorophytes (Givnish *et al.* 2015; Rasmussen & Rasmussen 2018). In fact, epiphytic orchids account for the diversity of 69 % of all epiphytic vascular plant species world-wide (Zotz & Winkler 2013). Orchids are well adapted to the challenges of the epiphytic habitat with its seasonally dry conditions by taking up water with the *velamen radicum*, a spongy epidermis covering their aerial roots, featuring thick leaves or pseudobulbs to store water and conducting CAM (crassulacean acid metabolism)-photosynthesis (Zhang *et al.* 2017; Zotz, Schickenberg & Albach 2017). CAM-photosynthesis in epiphytic and lithophytic orchid species is reflected in their bulk tissue $\delta^{13}C$ values ranging around – 14 ‰ due to a small fractionation between atmospheric CO_2 and plant biomass during CO_2 fixation catalysed by PEP carboxylase (O'Leary 1981; Winter *et al.* 1983). The orchids inhabiting temperate regions are terrestrial (Fig. 4) forming relatively few, usually fleshy and unbranched roots sharing all characteristics of plants with a high dependency on mycorrhizal fungi (Peterson, Massicotte & Melville 2004). Generally, terrestrial orchids conduct C_3-photosynthesis which is mirrored in more negative $\delta^{13}C$ values due to a higher fractionation between atmospheric CO_2 and plant biomass during CO_2 fixation catalysed by Rubisco (Winter *et al.* 1983; Zotz & Ziegler 1997; Fry 2006). About 450 species and subspecies of terrestrial orchids in the three subfamilies Cypripedioideae, Epidendroideae and Orchidoideae occur in Europe with a diversity centre in the Mediterranean region (Baumann, Künkele & Lorenz 2006). The lady's slippers orchid *Cypripedium calceolus* is the single northern European species in the Cypripedioideae. About 100 species, subspecies and varieties are distributed in Germany in nutrient-poor habitats with mainly calcareous substrates such as wet meadows, fens, dry calcareous grasslands, alpine meadows and woodlands dominated by either the common beech *Fagus sylvatica*, Norway spruce *Picea abies* or Scots pine *Pinus sylvestris* (Kretzschmar 2013; AHO Bayern 2014).

Fig. 4 Photographs of the 32 orchid species that were sampled for stable isotope abundance analyses during this PhD thesis (from left to right, top to bottom): *Neottia nidus-avis* (L.) Rich., *Cephalanthera damasonium* (Mill.) Druce, *Cephalanthera rubra* (L.) Rich., *Corallorhiza trifida* Châtel., *Epipactis helleborine* (L.) Crantz, *Epipactis helleborine* subsp. *neerlandica* (Verm.) Buttler, *Epipactis helleborine* subsp. *orbicularis* (K.Richt.) E.Klein (syn. *Epipactis distans* Arv.-Touv.), *Epipactis leptochila* (Godfery) Godfery, *Epipactis leptochila* subsp. *neglecta* Kümpel (syn. *Epipactis neglecta* (Kümpel) Kümpel), *Epipactis microphylla* (Ehrh.) Sw., *Epipactis muelleri* Godfery, *Epipactis palustris* (L.) Crantz, *Epipactis purpurata* Sm., *Liparis loeselii* (L.) Rich., *Malaxis monophyllos* (L.) Sw., *Neottia cordata* (L.) Rich. Image courtesy for *E. leptochila*, *E. leptochila* subsp. *neglecta*, *E. microphylla* and *N. cordata* by Florian Fraaß. Image courtesy for *M. monophyllos* by Heiko Liebel.

Fig. 4 (continued) Photographs of the 32 orchid species that were sampled for stable isotope abundance analyses during this PhD thesis (from left to right, top to bottom): *Neottia ovata* (L.) Bluff & Fingerh., *Dactylorhiza incarnata* (L.) Soó, *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh., *Dactylorhiza viridis* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Gymnadenia conopsea* (L.) R.Br., *Gymnadenia odoratissima* (L.) Rich., *Gymnadenia nigra* (L.) Rchb.f., *Herminium monorchis* (L.) R.Br., *Himantoglossum hircinum* (L.) Spreng., *Neotinea ustulata* (L.) R.M.Bateman, Pridgeon & M.W.Chase (syn. *Orchis ustulata* L.), *Ophrys insectifera* L., *Orchis militaris* L., *Platanthera bifolia* (L.) Rich., *Pseudorchis albida* (L.) Á.Löve & D.Löve, *Spiranthes aestivalis* (Poir.) Rich., *Traunsteinera globosa* (L.) Rchb. Image courtesy for *D. majalis* by Florian Fraaß and for *G. odoratissima* by Andreas Schweiger.

Orchidaceae – specialisations above- and belowground

Orchids are well-known and even famous for their pollination syndromes bearing high levels of specialisation. For example, deceptive bee orchids in the genus *Ophrys* lack nectar as floral reward and trick their pollinators by both mimicking the appearance and the scent of female insects. The male insects are attracted by this seductive combination of floral volatiles and the display of the flower and tricked into pseudocopulation with the deceptive orchid by in fact attempting to mate with a female insect (Schiestl *et al.* 2003; Vereecken *et al.* 2012). By doing so, the pollinia (packets of pollen grains) are attached to the insect's body and transported to another individual of the same orchid species during the next floral visit. After successful pollination a capsule is formed by the orchid enclosing numerous tiny, endospermless seeds containing only very small amounts of nutrients and an undiffertiated embryo (Arditti & Ghani 2000). Independent of their life-form, all orchid species share the trait of producing large numbers of 'dust-like' seeds which is an atypical trait for the rest of the families in the order Asparagales (s. Box 4 *Excursus – Dust seeds*). For successful germination, all orchid seeds need to be obligatorily colonised by mycorrhizal fungi and provisioned with nutrients in the achlorophyllous protocorm stage until the later chlorophyllous seedling is capable of photosynthesis. Protocorms are the non-photosynthetic, fully mycoheterotrophic, pre-seedling stages formed after germination of dust seeds through colonisation by a mycorrhizal fungus. Consequently, the trophic strategy of initial mycoheterotrophy is a characteristic feature common to all Orchidaceae (Alexander & Hadley 1985; Leake 1994; Rasmussen 1995; Rasmussen & Whigham 1998; Merckx 2013). At adulthood these

initially mycoheterotrophic orchids stay either achlorophyllous, continue to obtain all nutrients by exploiting their mycorrhizal fungi and are fully mycoheterotrophic (e.g. *Neottia nidus-avis*, Fig. 4), or they photosynthesise and thus become (putatively) autotrophic or partially mycoheterotrophic. Hence, the dependency of orchids on fungi is very high but can also be pushed further along. The potato orchid *Gastrodia pubilabiata* Sawa was already known to be fully mycoheterotrophic such as the complete genus *Gastrodia* (Ogura-Tsujita *et al.* 2009; Dearnaley & Bougoure 2010). However, the dependency of this species on fungi continues with its deceptive pollination syndrome. The floral scent of *G. pubilabiata* mimics fermented fruit and rotting mushrooms to attract fruit flies to lay their eggs into the flowers (brood-site deception) and by doing so, the fruit flies pollinate the flowers (Suetsugu 2018). The attraction of *G. pubilabiata* for fruit flies is even increased if rotting mushrooms are placed next to the plants.

All orchid species are characterised by intricate interactions with both their pollinators and mycorrhizal fungi, meaning that orchids are engaged in at least two types of mutualism; that is probably generally a very widespread situation in nature (Hoeksema & Bruna 2000). However, the evolution of pollinia, the abundance of specialised pollination syndromes driven by the orchids' extraordinary floral diversity, the reduction of seed mass through the reduction or total absence of endosperm, orchid mycorrhiza, an epiphytic life-form per se or associated traits such as CAM photosynthesis, and the predominant distribution in the tropics have all been proposed as drivers of the extraordinary species richness of orchids (Givnish *et al.* 2015 and references herein; Zhang *et al.* 2017).

Orchidaceae – Dust seeds

Probably owing to their charismatic appearance and their sheer species richness the Orchidaceae have been studied most intensively of all plant families with initially mycoheterotrophic species. Already in 1899, Noël Bernard was the first researcher to recognise the necessery presence of fungal hosts for successful orchid seed germination. Hence, the complex of requirements determining seed germination and seedling establishment involving orchid mycobionts are intensively studied until today (Rasmussen *et al.* 2015). Orchid seeds are very small ranging in a length from c. 0.05 to 6.0 mm, extremely light with individual seed weights from 0.31 µg to 24 µg and produced in great numbers. Temperate orchid species have a tendency towards larger seeds than tropical orchids (9.84 ± 10.22 µg seed^{-1} vs. 2.56 ± 1.76 µg seed^{-1}). An individual seed capsule can contain 4 million or only 20 – 50 seeds (Arditti & Ghani 2000). The transparent testa encloses the embryo that sometimes consists only of a few cells and food reserves such as oil droplets and starch grains; as the mature orchid seeds lack endosperm (Rasmussen 1995; Arditti & Ghani 2000; Eriksson & Kainulainen 2011).

Box 4 *Excursus – Dust seeds*

The variation in individual seed size among angiosperms is immense and differentiates over ten orders of magnitude. Dust seeds are the smallest existing seeds in angiosperms and are thus positioned at the lower end of the size range (Harper, Lovell & Moore 1970; Eriksson & Kainulainen 2011). In their review Arditti & Ghani (2000) explained the etymology of the term *dust seed* for extremely small, simply dust-like seeds as originating from an odd translation from the German expression *staubförmiger Samen* to English. The dust or minute seeds contain the embryo and only a limited amount of endosperm tissue or highly concentrated nutrient reserves such as proteins or lipids that are surrounded by the testa, a thin seed coat (Rasmussen 1995; Arditti & Ghani 2000; Eriksson & Kainulainen 2011). Embryo and reserves only fill a small proportion of the total seed volume limited by the testa and thus the seed is often balloon-shaped and can float in the air for long periods favouring anemochory as dispersal strategy. Furthermore, the outer surface of the testa is hydrophobic and makes the air-filled seed buoyant (Arditti & Ghani 2000). Due to their very limited reserves, plant species with dust seeds are dependent on external sources of organic carbon for seed germination and seedling establishment and are thus parasitic during recruitment. Parasitism directly on plants or fungi, initial mycoheterotrophy, for organic carbon acquisition during these early stages of ontogeny can be differentiated. Plants that parasitise other plants such as hemiparasitic and holoparasitic species in the Orobanchaceae are characterised by a haustorium – a unique organ that functions in attachment, penetration, and solute transfer (Shen *et al.* 2006; Eriksson & Kainulainen 2011). In contrast, "an 'initially mycoheterotrophic' plant ('initial mycoheterotroph') is fully dependent on associated fungi for its carbon supply during the early stages of development" (Merckx 2013). The feature of dust seeds has evolved independently at least 12 times in the plant families Burmanniaceae, Corsiaceae, Orchidaceae, Triuridaceae, Petrosaviaceae, Ericaceae, Gentianaceae, Polygalaceae, Orobanchaceae, Rubiaceae, Buddlejaceae and Gesneriaceae (Eriksson & Kainulainen 2011). The driver of dust seed evolution is most likely the selection for maximal fecundity at the cost of reducing maternal resources per seed. Seed size minimisation through evolution of an undifferentiated embryo and endosperm reduction passed a threshold where external hosts were required as organic carbon source. Raven (1999) proposed a minimum dry weight of 5 µg per seed to produce photosynthetically self-sufficient seedlings without a heterotrophic carbon source (Eriksson & Kainulainen 2011). For the Rubiaceae, Buddlejaceae and Gesneriaceae parasitic behaviour during recruitment has not been described yet. Some species in the Burmanniaceae and Polygalaceae are initially or fully mycoheterotrophic on arbuscular mycorrhizae, all Corsiaceae, Petrosaviaceae and Triurdiaceae species are fully mycoheterotrophic (Eriksson & Kainulainen 2011; Merckx *et al.* 2013a). Most species in the subfamily Pyroloideae in the Ericaceae family and all Orchidaceae species are initial mycoheterotrophs (Alexander & Hadley 1985; Leake 1994; Rasmussen 1995; Rasmussen & Whigham 1998; Merckx *et al.* 2013a). In lower plants, sporophytes are temporary parasitic on the gametophyte during their early development. If these gametophytes are mycoheterotrophic, the sporophytes of these lower plant species can be considered initial mycoheterotrophs as well (Leake, Cameron & Beerling 2008; Merckx 2013).

Already Charles Darwin concluded that there must be limitations in the germination of orchid seeds and seedling establishment as the number of produced and disseminated orchid seeds exceeds the number of mature orchid individuals by far: "[Orchid] species… are sparingly distributed; yet, if their seeds or seedlings were not largely destroyed, any one of them would immediately cover the whole land… The number of the individuals which come to maturity does not seem to be at all closely determined by the number of seeds which each species produces" (Darwin 1877 in Arditti & Ghani 2000). Today a well-known reason for this discrepancy in the number of orchid seeds and established mature plants is the presence of and infection by germination mycobionts, and thus the successful supply of nutrients in the fully mycoheterotrophic initial life stage. The early life stages of terrestrial orchids are elusive bottleneck stages as the protocorms remain underground and fully mycoheterotrophic for months or even years (Rasmussen & Whigham 1998). The seedlings only reach the aboveground photosynthetic seedling stage after having been both successfully colonised by mycorrhizal fungi for germination and the protocorms having been provisioned with carbohydrates. The unusual mode of obtaining energy by means of mycorrhiza makes a strong selection pressure acting on the underground stages very likely (Rasmussen 1995), especially if mycorrhizal specificity occurs during symbiotic development (Bidartondo & Read 2008).

Orchid mycorrhiza

The Orchidaceae plant family has a consistent mycorrhizal status as the type of orchid mycorrhiza (OrM) is confined to the Orchidaceae (Brundrett & Tedersoo 2018). Accounting for the number of plant species associated with a certain mycorrhizal type, OrM is the second most abundant mycorrhizal type after the arbuscular mycorrhiza (AM): 72 % of all vascular plant species form a mycorrhiza of the AM type, 10 % of all vascular plant species are orchids and thus form OrM.

OrM is an endomycorrhiza in which fungal hyphae colonise the orchid root cells from the root surface or from neighbouring cells (Brundrett & Tedersoo 2018). The unique feature of OrM is that the fungal hyphae form complex hyphal coils, so-called pelotons, inside the cortical cells between cell wall and membrane of an orchid root (Fig. 5). Most likely, either the intact fungal coils are the location for nutrient exchange by transfer through membranes between orchid and fungus (Cameron *et al.* 2008; Dearnaley, Martos & Selosse 2012) and / or the digestion of collapsed hyphal pelotons in later stages by the plant. Besides the morphology, OrM is clearly distinct from other mycorrhizal types as it is essential for both orchid seed germination and seedling establishment (Rasmussen 1995) making all orchid species initially mycoheterotrophic.
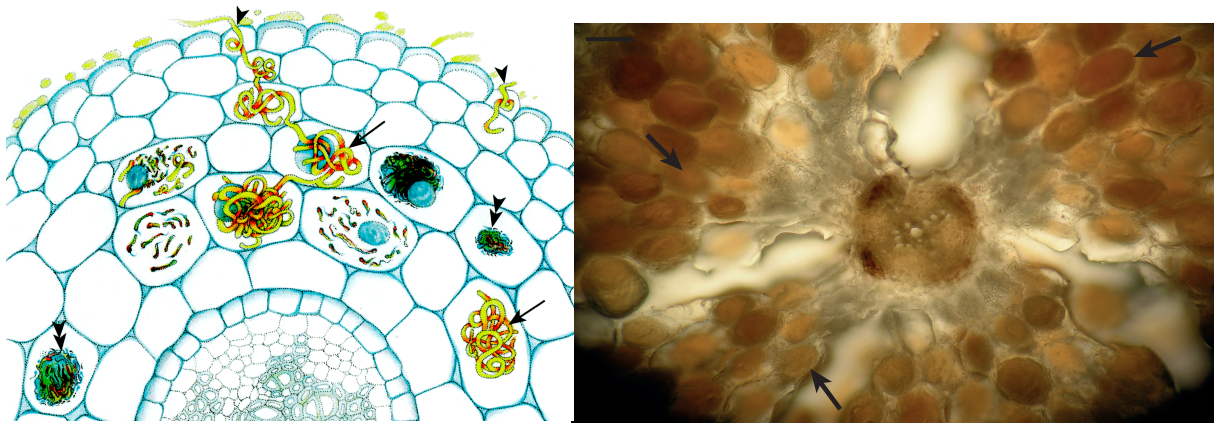
Fig. 5 Orchid mycorrhiza. (left) Illustration of a transverse section of an orchid root showing the colonisation typical of orchid mycorrhizae. Fungal hyphae enter the orchid root through the epidermis (arrowheads), pelotons (hyphal coils) form in cortical cells (arrow) and are lysed (double arrowheads), reproduced from Peterson, Massicotte & Melville (2004); (right) Light micrograph showing a transverse section of a root of Epipactis neglecta. Fungal colonisation by the truffle Tuber excavatum is visible as exodermal, outer and inner cortical cells filled with pelotons (arrows). Scale bar = 100 μm. Photograph by Julienne M.-I. Schweiger, published in Schiebold *et al.* (2017).

Inferring from the age of the most recent common ancestor of extant Orchidaceae of ca. 112 Myr, 128 Myr or even further back (Givnish *et al.* 2015; Zhang *et al.* 2017; Barba-Montoya *et al.* 2018; Katz 2018) and assuming the trait of dust-like seeds to have been present already in these first orchids, orchid mycorrhiza must have co-occurred to facilitate germination of these ancient orchids and thus, must be of a similar phylogenetic age.

Fungal species involved in the OrM between terrestrial green-leaved orchids inhabiting open habitats and fungi are typically those classically named "rhizoctonias", a polyphyletic group of basidiomycetes. Rhizoctonias are part of the three basidiomycete families Tulasnellaceae (genus *Tulasnella*), Ceratobasidiaceae (genus *Ceratobasidium*) (both from the order Cantharellales) and Serendipitaceae (formerly called Sebacinales clade B) (Jacquemyn, Duffy & Selosse 2017a). These fungal families forming OrM all have in common to be cryptic, in the sense that they are free-living and do not form fruiting bodies. They are thought to be saprotrophic fungi that decompose diverse carbon sources in the soil but have also been found as endophytic fungi in non-orchid plants where they are nonmycorrhizal or even exhibit a plant-parasitic behaviour (Rasmussen 1995; Smith & Read 2008; Veldre *et al.* 2013; Riess *et al.* 2014; Kohler *et al.* 2015; Jacquemyn *et al.* 2017a). Contrasting, achlorophyllous, fully mycoheterotrophic (FMH) orchid species have been shown to associate with fungi known to simultaneously form ectomycorrhizae with forest trees (Zelmer & Currah 1995; Taylor & Bruns 1997; McKendrick *et al.* 2000). These associations between FMH orchids and ectomycorrhizal fungi are usually very specific in the sense that the achlorophyllous orchids associate

with narrow phylogenetic groups of fungi (Taylor *et al.* 2002), e.g. the bird's nest orchid *Neottia nidus-avis* and the genus *Hexalectris* (crested coralroots) both associating with *Sebacina* (clade A) (McKendrick *et al.* 2002; Kennedy, Taylor & Watson 2011). In the tropics and sub-tropics saprotrophic non-rhizoctonia fungi decomposing wood or litter from the families Psathyrellaceae and Mycenaceae seem to be frequent OrM fungi especially of FMH orchid species but also in the case of the photosynthtic species *Cremastra appendiculata* (D. Don) Makino (Ogura-Tsujita *et al.* 2009 & 2018; Yagame *et al.* 2013; Lee, Yang & Gebauer 2015). Mycorrhizal fungi found in roots of green-leaved orchids growing in forests, near trees or occasionally even in orchids of open habitats that usually form OrM with rhizoctonias, encompass a large diversity of ectomycorrhizal fungi (Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Selosse *et al.* 2004; Abadie *et al.* 2006; Selosse & Roy 2009; Liebel *et al.* 2010; Gonneau *et al.* 2014). Examples are the basidiomycete genera *Cortinarius*, *Inocybe* and *Tomentella* and the ascomycete genera *Wilcoxina* and *Tuber* (Bidartondo *et al.* 2004; Selosse *et al.* 2004).

The extraction of fungal pelotons from orchid root cross-sections to identify mycorrhizal fungi with molecular techniques is rather unproblematic. In contrast, retrieving underground orchid seedlings and identifying their germination mycobionts was very challenging until the development of the Rasmussen–Whigham technique (Rasmussen & Whigham 1993): Orchid seeds are placed in bags made of mesh that is permeable for fungal hyphae but not for plant roots. These bags can be buried *in situ* in the soil, they later be easily retrieved to monitor germination and also to extract fungal DNA from the orchid protocorms (Baskin & Baskin 2014). Germination mycobionts can be overlapping with the mycorrhizal fungi of mature plants or be a subset of them (Peterson, Massicotte & Melville 2004; Rasmussen *et al.* 2015; Schweiger, Bidartondo & Gebauer 2018).

Is orchid mycorrhiza a mutualism?

Arbuscular mycorrhiza (AM) is the most ancestral type of mycorrhiza that enabled the emergence of land plants possibly already in the middle Cambrian (c. 515 Myr) (Morris *et al.* 2018). AM is also the predominant mycorrhiza type of 72 % of all plant species with an immense phylogenetic breadth including numerous plant families in angiosperms, gymnosperms and even pteridophytes and both woody and herbaceous species (Wang & Qiu 2006; Smith & Read 2008; Brundrett & Tedersoo 2018). It has been widely questioned why it came to a mutualism breakdown in many cases during evolution, in more details why plant lineages ceased to form AM. In their study, Werner *et al.* (2018) have found that the AM mutualism is evolutionary highly stable and retained in most plant lineages (Fig. 6). This also includes cases where plants engage with more than one symbiont type (e.g. AM and ECM in woody species, or AM and N_2-fixation in the Fabaceae family) (Wang & Qiu 2006). However, if it came to an AM mutualism

breakdown, either a symbiont switching took place where AM fungi were replaced with novel symbionts such as other mycorrhizal fungi (Basidiomycota and Ascomycota, in ECM, ErM and OM) or N_2-fixing partners (*Rhizobium* in legumes or *Frankia* root nodules of *Alnus*, *Eleagnus* or *Hippophae*). Alternatively, a mutualism was generally abandoned and substituted by non-symbiotic nutrient acquisition strategies evolved (e.g. carnivory, cluster roots or parasitism) (Fig. 6).

It is still unresolved whether OrM can be classified as a mutualism in analogy to, e.g. arbuscular mycorrhiza or ectomycorrhiza in the sense that assimilated carbon is allocated from the plant to the fungus. All orchids are initially mycoheterotrophic during germination and seedling establishment. However, after the development of chlorophyll in aboveground structures, adult orchids are capable of photosynthesis and could reward their mycorrhizal fungi with assimilated carbon for the advance nutrients during germination and seedling establishment. In a laboratory experiment Cameron *et al.* (2008) have shown an 'up-flow' of carbon from fungus to plant in the photosynthesising orchid *Goodyera repens* with a simultaneous 'down-flow' vice versa. After all, the allocation of assimilates from plant to mycorrhizal fungus was worth the fivefold amount of carbon. However, Cameron *et al.*'s study is so far the single work pointing in this direction and their results have not been verified *in situ* yet. In mutualisms between two partners one partner often appears to benefit much more from the interspecific interaction than the other partner. This might be the case in orchid mycorrhiza, where the plant obviously benefits as the mycorrhizal fungi facilitate its germination and seedling establishment. Hence, the orchid saves resources that it would otherwise need to allocate into the seeds (endosperm), it can produce numerous seeds that may be dispersed over long distances and the orchid can allocate resources into storage organs (rhizomes and tuberous roots) instead to facilitate dormancy enabling the survival of unfavourable years and maximising competition (Schiebold *et al.* 2018; Shefferson *et al.* 2018). It remains to explore possible benefits for orchid mycorrhizal fungi that could encompass providing a habitat for the fungus in the orchid roots or the provisioning with vitamins or other substances (Selosse & Roy 2009).

It has been proposed that mutualisms, usually defined as being beneficial for the involved partners, may best be regarded as reciprocal exploitations that, still, provide net benefits to each partner (Herre *et al.* 1999 in Merckx 2013). Consequently, mutualisms might be vulnerable to exploitation and are susceptible for an invasion by cheaters that use the benefits but do not pay back (Taylor & Bruns 1997; Bronstein 2001; Sachs & Simms 2006). In the case of partially and fully mycoheterotrophic orchids associating with fungi that usually form ectomycorrhizae with surrounding forest trees (Bidartondo *et al.* 2004) might best be explained by the concept of *cooperation* (Sachs *et al.* 2004). Tree species and ectomycorrhizal fungi partake in a cooperation that is a two-way interaction involving the bidirectional exchange of nutrients and water from the fungus for photosynthetically-fixed carbon from the plant, hence the classic mycorrhizal
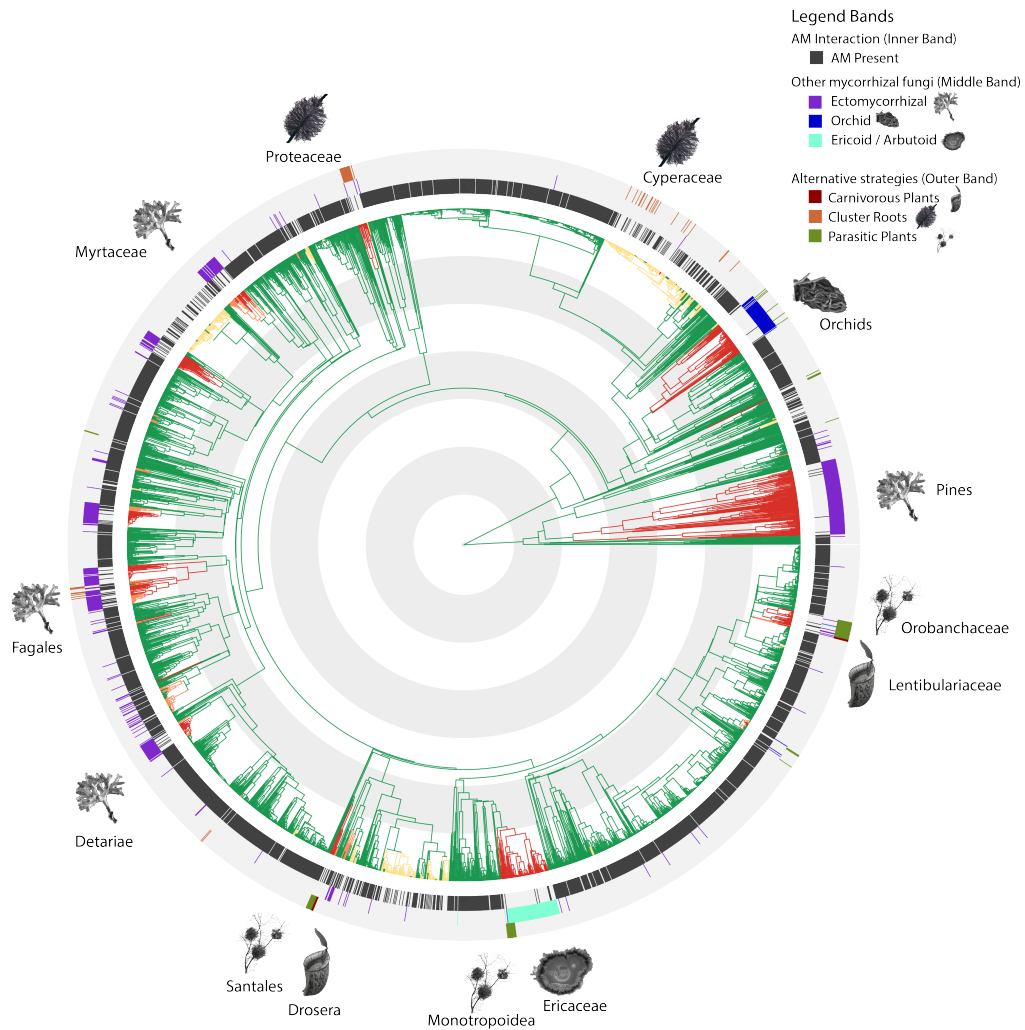
Fig. 6 Ancestral state reconstruction of the evolution of plant-AM status and alternative resource aquisition strategies. (green) the ancestral state is AM presence with the presence of Glomeromycota fungi; (red) replacement of the AM fungi with novel symbionts (symbiont switching) such as other mycorrhizal fungi (Basidiomycota and Ascomycota, in ECM, ErM and OM) or N$_2$-fixing partners (*Rhizobium* or *Frankia*), or mutualism abandonment with the evolution of alternative non-symbiotic nutrient acquisition strategies (e.g. carnivory, cluster roots or parasitism). Key clades (e.g. Orchidaceae in the Asparagales) having lost AM fungal interactions during evolution are marked with pictograms of the respective alternative nutrient acquisition strategies. Figure reproduced with the kind permission of Gijsbert D. A. Werner (Werner *et al.* 2018).

mutualism. Partially mycoheterotrophic (PMH) orchids engaging in a tripartite relationship through the shared mycorrhizal network now benefit from the mutualism between trees and mycorrhizal fungi through byproduct benefits. PMH orchids might tap on the excess carbon that is allocated from the trees to the ectomycorrhizal fungi to supplement their photosynthetic carbon fixation in low light conditions. The costs for the mycorrhizal fungi remain unknown (Selosse & Roy 2009). However, the costs for the autotrophic plants might be negligibly small in relation to a forest stand's net primary production (NPP) but essential for the PMH or FMH plant species. It was assessed that

in a mixed forest dominated by the common beech *Fagus sylvatica* only 0.0007 % of the NPP of the forest trees exceeding 12.100 kg C ha^{-1} a^{-1} is allocated from the trees to the PMH and FMH plants via the shared network of ectomycorrhizal fungi (Adam 2009). However, the byproduct benefits drawn by the partially mycoheterotrophic orchid might be so overly exploited that they may take the form of *harvesting* and thus provoke the development of fully mycoheterotrophic species (Sachs *et al.* 2004). Due to the sheer abundance of mycorrhizal associations it is not only imaginable that orchids have invaded the mutualism between trees and ectomycorrhizal fungi by harvesting, but also many other plant species disrupting other mycorrhizal associations. 72 % of all vascular plant species are engaged in the AM type of mycorrhiza and due to its ancient origin and sheer abundance this association makes it especially vulnerable to cheating, too (Merckx 2013). A growing number of at least 514 achlorophyllous plant species with at the minimum 46 independent evolutionary origins are fully mycoheterotrophic and many of them exploit (or *harvest*) arbuscular mycorrhizae (AM) such as some genera in the families Burmanniaceae (*Thismia*) and Gentianaceae (*Voyria*) or even entire families (Corsiaceae, Petrosaviaceae and Triurdiaceae) (Merckx *et al.* 2013a).

Trophic strategies in orchids – the stable isotope abundances of putatively autotrophic, initially, partially and fully mycoheterotrophic orchids

The Orchidaceae is an interesting plant family as its species feature all trophic strategies from obligate initial mycoheterotrophy, putative autotrophy over different degrees of partial mycoheterotrophy to full mycoheterotrophy. Additionally, many different groups of mycorrhizal and even usually nonmycorrhizal saprotrophic fungi are able to form orchid mycorrhiza (OrM) in roots of Orchidaceae species. Full mycoheterotrophy has arisen independently in at least 17 plant families (Merckx 2013), but the Orchidaceae family followed by the Ericaceae has clearly received the most attention from researchers (Bidartondo 2005; Hynson & Bruns 2010; Dearnaley *et al.* 2012). The number of fully mycoheterotrophic (FMH) orchid species is growing continuously from a minimum of 235 species in 43 genera (Merckx *et al.* 2013a) due to ongoing taxonomic work and recent discoveries of new FMH orchid species (Suetsugu 2017; Dixon & Christenhusz 2018; Suetsugu *et al.* 2018). Since Gebauer & Meyer (2003) published the first study suggesting a reference sampling system allowing enrichment factor calculations to gather site-independent and thus comparable stable isotope data of mycoheterotrophic plants, a growing number of publications following these criteria have been reported.

Many FMH Orchidaceae species of the subfamilies Epidendroideae and Vanilloideae originating from the tropics and subtropics are associated with saprotrophic wood- or litter-decomposing fungi. Some FMH orchid species of the last-mentioned group and the temperate FMH orchids including rarer albino forms of usually green-leaved Epidendroideae are associated with ectomycorrhizal fungi that supply them with all nutrients. Generally, the identity of the associated fungus forming OrM with an orchid species can have a substantial influence on the isotopic composition of an orchid and is mirrored in it (Fig. 7a). FMH orchids associated with wood-decomposing fungi are more enriched in ^{13}C than orchids mycorrhizal with fungi that decompose litter (Martos *et al.* 2009; Lee *et al.* 2015). This can be explained by the isotopic signatures of the individual substrates that these different guilds of litter- and wood-decomposing fungi use, namely that wood as non-photosynthetic tissue is typically more enriched in ^{13}C than leaves (Gebauer & Schulze 1991; Cernusak *et al.* 2009). Another interesting case is *Erythrorchis altissima*, the largest FMH orchid species in the world (Ogura-Tsujita *et al.* 2018; Appendix II). This climbing Vanilloideae is mainly associated with wood-decaying saprotrophic non-rhizoctonia fungi and could be found as a liana on dead and living trunks of the host tree species *Castanopsis sieboldii* and *Distylium racemosum*. FMH orchids mycorrhizal with ECM fungi are more enriched in ^{15}N than FMH orchids associating with litter- and wood-decomposing fungi (Fig. 7a) due to the general pattern

a

b
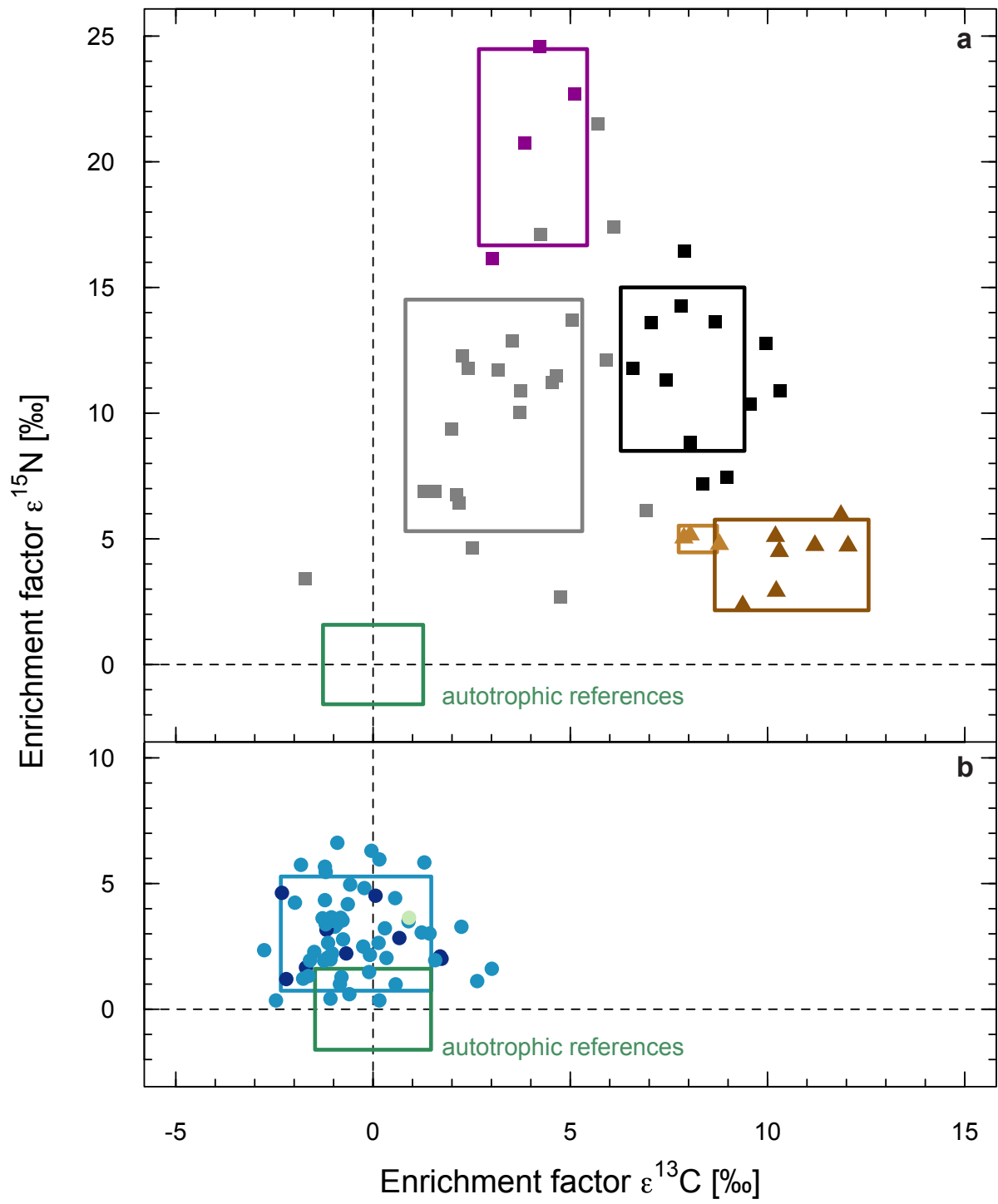
■ FMH ECM
▲ FMH SAP litter
▲ FMH SAP wood
■ PMH ECM
■ PMH ECM Ascomycota
● green Cypripedioideae rhizoctonia
● green Epidendroideae rhizoctonia
● green Orchidoideae rhizoctonia

Fig. 7 Mean enrichment factors ε^{13}C and ε^{15}N of 112 fully (FMH) and partially (PMH) mycoheterotrophic Orchidaceae species extracted from published literature until January 2018 (except an hitherto unavailable dataset by Lallemand *et al.*, 2018). The boxes represent ± 1 SD of the mean ε values for the different groups of PMH, FMH and green Orchidaceae species and autotrophic references. For details on the 109 reviewed orchid species please refer to Table S1.
This figure is an extension to the original figure published as a case study in the chapter "Orchid Mycorrhizal Associations" in the book "Conservation Methods of Terrestrial Orchids" (Fig. 3.1 in Gebauer & Schiebold 2017; Appendix I).
a) FMH orchid species associated with ectomycorrhizal fungi (FMH ECM) ($n = 12$; 150 data points with $n = 3$-62 per species), FMH orchid species associated with saprotrophic litter-decomposing fungi (FMH SAP litter) ($n = 3$; 15 data points with $n = 5$ per species), FMH orchid species associated with saprotrophic wood-decomposing fungi (FMH SAP wood) ($n = 7$; 36 data points with $n = 1$-10 per species), PMH Orchidaceae associated with ectomycorrhizal Ascomycota (PMH ECM Ascomycota) ($n = 4$), 28 data points with $n = 5$-9 per species), PMH Orchidaceae associated with ectomycorrhizal fungi (PMH ECM) ($n = 22$, 421 data points with $n = 2$-72 per species) and autotrophic reference species ($n = 240$, 1882 data points).
b) Green-leaved Orchidaceae species from the subfamilies Cypripedioideae ($n = 1$; *Cypripedium calceolus* with $n = 25$), Epidendroideae ($n = 9$, 106 data points with $n = 3$-45 per species), and Orchidoideae ($n = 53$; 535 data points with $n = 1$-49 per species) and autotrophic reference species ($n = 224$, 2033 data points).
Data extracted from Yagame *et al.* 2012; Hynson *et al.* 2013; Roy *et al.* 2013; Stöckel *et al.* 2014; Hynson *et al.* 2015; Ercole *et al.* 2015; Lee *et al.* 2015; Johansson *et al.* 2015; Liebel *et al.* 2015; Gebauer *et al.* 2016; Hynson 2016; Hynson *et al.* 2016; Sakamoto *et al.* 2016; Schiebold *et al.* 2017, 2018; Suetsugu *et al.* 2017; Jacquemyn *et al.* 2017b; Lallemand *et al.* 2017; Fay *et al.* 2018; Ogura-Tsujita *et al.* 2018; Schweiger *et al.* 2018 and Schweiger *et al.* 2019.

that sporocarps of ECM fungi are also more enriched in ^{15}N than fruiting bodies of saprotrophic litter- and wood-decomposing fungi occurring on the same sites (Gebauer & Taylor 1999; Gebauer *et al.* 2016). PMH orchids of the subfamilies Epidendroideae and Orchidoideae associated with Ascomycota and Basidiomycota usually forming ECMs are less enriched in ^{13}C than all last-named groups of FMH orchids (Fig. 7a). PMH orchids supplement their photosynthetic carbon nutrition that is ^{13}C-depleted with ^{13}C-enriched organic nutrients from fungi and are thus generally depleted in ^{13}C in comparison to FMH orchids solely exploiting their mycorrhizal fungi. As already mentioned, the ^{15}N enrichment was described to be not linearly related to the degree of heterotrophic C gain (Leake & Cameron 2010; Merckx 2013). However, the explanation of variations in ^{15}N enrichment found for fully, partially and initially mycoheterotrophic plants, but also for putatively autotrophic species is far less clear and remains to be investigated.

The overwhelming majority of green-leaved orchid species in the subfamilies Epidendroideae, Orchidoideae and Cypripedioideae is associated with rhizoctonia fungi (Dearnaley *et al.* 2012) and was thus assumed to be putatively autotrophic. However, the stable isotope profiles of rhizoctonia-associated orchids are most often characterised by conspicuously significant ^{15}N enrichment and either a lacking or modest but insignificant ^{13}C enrichment or even depletion relative to autotrophic references (Fig. 7b; Liebel *et al.* 2010; Girlanda *et al.* 2011; Johansson *et al.* 2015). The total leaf nitrogen concentrations of rhizoctonia-associated orchids are higher compared to autotrophic plants (Table S1). Thus, the assumed autotrophy of rhizoctonia-associated orchids has

been challenged and they have been called "cryptic mycoheterotrophs" (Hynson *et al.* 2013; Hynson 2016). However, N stable isotope abundances and higher N concentrations might indicate carbon gain from fungal sources additionally to photosynthesis and thus, partial mycoheterotrophy.

In a comparison between ^{13}C and ^{15}N enrichment of fully mycoheterotrophic protocorms and mature individuals of the same species, Stöckel *et al.* (2014) found that achlorophyllous and thus fully mycoheterotrophic seedlings of rhizoctonia-associated orchids were by far less enriched in ^{13}C and ^{15}N than protocorms of orchids that associate with ectomycorrhizal fungi. They pronounced that especially the ^{13}C enrichment measured in mature chlorophyllous orchids associated with saprotrophic rhizoctonia fungi might be too small to enable the detection of partial mycoheterotrophy, in contrast to a usually clear ^{13}C enrichment of mature chlorophyllous orchids associated with ectomycorrhizal fungi. In other words, the isotopic signature of the carbon source of rhizoctonia-associated orchid species, namely rhizoctonia fungi, is too close to the isotope abundance of autotrophic plants to be distinguished. Stöckel *et al.* (2014) concluded that the routinely used δ^{13}C ratios might not be sufficient to unequivocally identify partial mycoheterotrophy in the majority of orchid species associated with rhizoctonia fungi. Recently, Gebauer *et al.* (2016) provided clear evidence that the trophic strategy of partial mycoheterotrophy is far more widespread in orchids than previously assumed. They used δ^{2}H in addition to the routinely employed δ^{13}C and δ^{15}N measurements following the reasoning that not only carbon and nitrogen but also hydrogen atoms are present in organic molecules that move from fungus to orchid during peloton digestion and / or transfer across intact membranes. Consequently, δ^{2}H values could thus also be used as indicators for mycoheterotrophic nutrition. The δ^{2}H approach is based on the finding that secondary heterotrophic organic compounds (i.e. in our case compounds of fungal origin) are enriched in ^{2}H compared to primary photosynthetic organic compounds (Yakir 1992; Cormier *et al.* 2018). Consequently, a significant ^{2}H enrichment in plant tissue can serve as an indicator for flow of heterotrophic organic matter, i.e. carbon, from mycorrhizal fungi to plant and thus for partial mycoheterotrophy.

In Gebauer *et al.*'s (2016) study not only the FMH orchid species *Neottia nidus-avis* and the PMH *Cephalanthera damasonium*, *C. rubra* and *Epipactis helleborine*, all associated with ECM fungi, were enriched in ^{2}H simultaneously to ^{13}C and ^{15}N, serving as a proof of concept, but also four rhizoctonia-associated species (*Cypripedium calceolus*, *Neottia ovata*, *Ophrys insectifera* and *Platanthera bifolia*) growing in forests were positioned in their ^{2}H enrichment between autotrophic plants sampled as references and the FMH *N. nidus-avis* (Fig. 8). Thus, good evidence was provided to substitute H for C stable isotope abundance analysis in cases where C stable isotope abundances of C sources are poorly distinguished and mask the C flow between mycorrhizal fungi and orchids (Gebauer *et al.* 2016). However, whether this pattern also holds true for orchid species forming orchid mycorrhizae with rhizoctonia fungi growing in habitats with high irradiance level is still open.
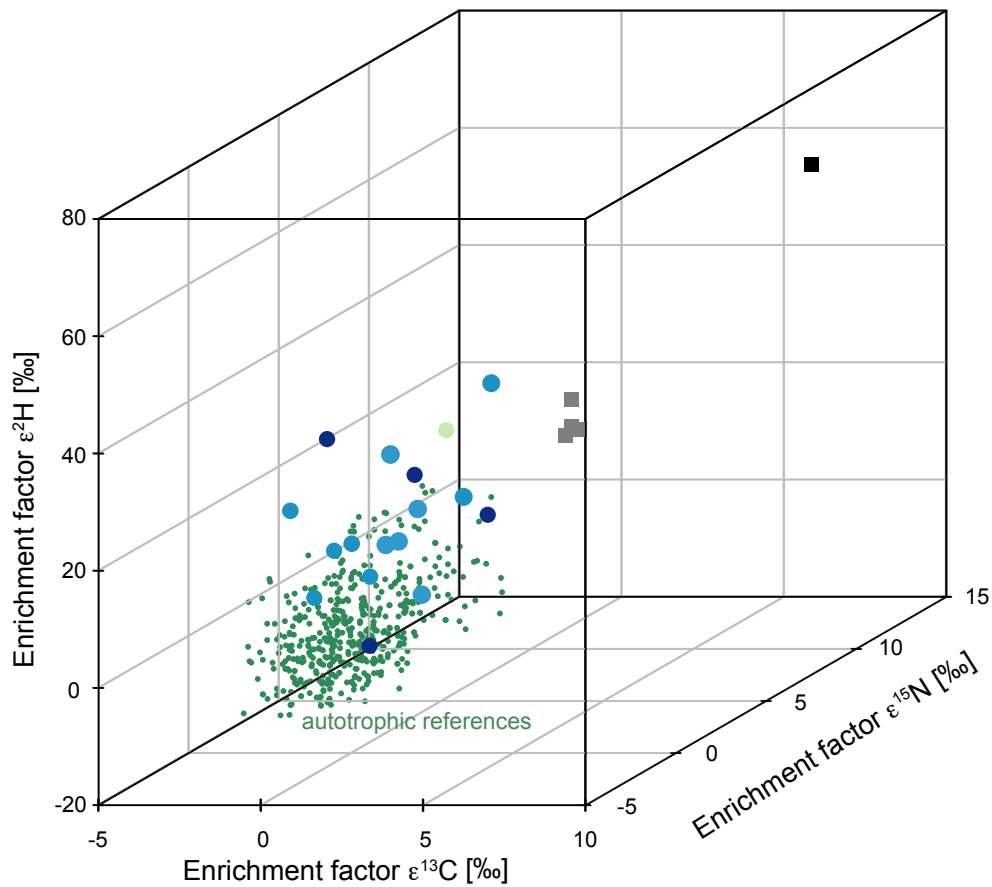
Fig. 8 Mean enrichment factors ε¹³C, ε¹⁵N and ε²H of fully mycoheterotrophic Orchidaceae species associated with ectomycorrhizal fungi (FMH ECM) (n = 1; *Neottia nidus-avis* with n = 10), partially mycoheterotrophic Orchidaceae species associated with ectomycorrhizal fungi (PMH ECM) (n = 4; 26 data points with n = 5-10 per species), green-leaved Orchidaceae species from the subfamilies Epidendroideae (n = 4; 30 data points with n = 5-15 per species), Cypripedioideae (n = 1; *Cypripedium calceolus* with n = 25) and Orchidoideae (n = 12; 81 data points with n = 5-16 per species) and autotrophic reference species (n = 48, 529 data points). The box represents ± 1 SD of the mean e values for the autotrophic references. See Fig. 7 for legend. For details on the 22 reviewed orchid species please refer to Table S2. This figure is an extension to the original figure published as a case study in the chapter "Orchid Mycorrhizal Associations" in the book "Conservation Methods of Terrestrial Orchids" (Fig. 3.3 in Gebauer & Schiebold 2017; Appendix I).
Data extracted from Gebauer *et al.* 2016; Fay *et al.* 2018; Schiebold *et al.* 2018; Schweiger *et al.* 2018 and Schweiger *et al.* 2019.

Objectives of this thesis

The complex ecology of partial mycoheterotrophy in the orchid family raises many questions, some of which were aimed at answering in this thesis.

Analyses of stable isotope abundances and molecular identification of mycorrhizal fungi are state-of-the-art techniques and the results of this thesis are mainly based on investigations using these two methods. A literature review was carried out to also synthesise published and previously unpublished stable isotope data to identify general patterns characterising partially and fully mycoheterotrophic species. Additionally, *in situ* micro-scale light climate measurements were conducted.

As presented in the following section, the five publications contributing to this thesis aimed to investigate the dependency of ecophysiological traits on the plant familial identity in Ericaceae and Orchidaceae [manuscript 1]; the drivers of isotopic enrichment in the orchid genus *Epipactis* [manuscript 2]; the presence of partial mycoheterotrophy in meadow orchids associated with rhizoctonia fungi [manuscript 3]; the percentage of organic matter gain in rhizoctonia-associated orchids using an improved methodological approach [manuscript 4] and the light-dependency of partial mycoheterotrophy in rhizoctonia-associated orchids [manuscript 5].

In detail, studies were conducted to achieve the following objectives:

[1] Identifying family-specific patterns in stable isotope abundances and nitrogen concentrations in partially and fully mycoheterotrophic Ericaceae and Orchidaceae associated with ectomycorrhizal fungi.

[2] Investigating the observed gradient in ^{15}N enrichment in initially and partially mycoheterotrophic plants species with the orchid genus *Epipactis* as case study.

[3] Testing whether partial mycoheterotrophy is a common nutritional strategy also in rhizoctonia-associated meadow orchids with a new analytical method.

[4] Elucidating the percental organic matter gained from mycorrhizal fungi in rhizoctonia-associated orchids using orchid seedlings in an improved methodological approach.

[5] Assessing the light-dependency of partial mycoheterotrophy in the rhizoctonia-associated orchid species *Neottia ovata* and *Ophrys insectifera.*

Synthesis

My thesis contributes to improve the understanding of the complex ecology of partial mycoheterotrophy especially in the orchid family. This was also possible by synthesising data published on mycoheterotrophic plants that were sampled in a reference system allowing enrichment factor calculations to gather site-independent and thus comparable stable isotope data (Gebauer & Meyer 2003). Data of publications following these criteria are collected in a database since, allowing studies synthesising isotopic signatures of plants with varying degrees of mycoheterotrophy, plant family and type of associated fungi (Hynson *et al.* 2013, 2016; Gebauer & Schiebold 2017; Appendix I). Besides data on Orchidaceae, the data bank also features all available stable isotope data on fully and partially mycoheterotrophic Burmanniaceae, Ericaceae, Gentianaceae and Triurdiaceae that were sampled in a reference system (Gebauer & Meyer 2003; Preiss & Gebauer 2008). With the begin of my thesis I took over the maintenance of the database (data acquisition and / or data entering into the database) and the number of entries for fully mycoheterotrophic (FMH) species have since almost doubled to 392 entries, more than tripled for initially (IMH) and partially mycoheterotrophic (PMH) plant species (2,080 entries) and more than doubled for autotrophic references (4,634 entries) until the end of January 2018. Among these contributions are also stable isotope abundance data for 32 orchid species that I sampled during this thesis (Fig. 4).

In the following I will shortly summarise my key findings and elaborate on how they contribute to the current state of the art by having identified and addressed essential knowledge gaps. The Orchidaceae and the Ericaceae clearly have received the most attention from researchers concerning prevalent fungi-mediated trophic strategies. It was thus apparent to investigate family-specific patterns in stable isotope abundances and nitrogen concentrations in PMH and FMH species in these two plant families by synthesising the largest available dataset on the ecophysiologies of those species associated with ectomycorrhizal fungi (Hynson *et al.* 2016, manuscript 1). We detected significant enrichments in ^{13}C and ^{15}N of PMH and FMH plants relative to autotrophic reference species, and confirmed that FMH ericaceous and orchidaceous species were more enriched in ^{13}C than their FMH counterparts. Additionally, we were able to distinguish mycoheterotrophic Ericaceae from mycoheterotrophic Orchidaceae according to their ^{13}C and ^{15}N enrichments and N concentrations. Most importantly, PMH and FMH Orchidaceae exhibited significantly higher nitrogen concentrations than those being present in the tissues of Ericaceae of any mycoheterotrophic degree or autotrophic reference species (Hynson *et al.* 2016, manuscript 1).

To summarise the state of the art in stable isotope natural abundance data of Orchidaceae of different degrees of mycoheterotrophy as target species and autotrophic plant species as reference for site conditions, I have compiled Figs. 7, 8 and 9 of published data originating from the database. Figs. 7 and 8 are extensions to the original figures published as a case study in the chapter "Orchid Mycorrhizal Associations" in the

book "Conservation Methods of Terrestrial Orchids" (Gebauer & Schiebold 2017, Appendix I). Details on data origin (publications) can be found in the individual figure captions; details on the species identities, categories assigned due to their degree of mycoheterotrophy, mean enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and mean nitrogen concentrations are summarised in Tables S1 and S2 (s. *Appendix to the Synopsis*).

The second study investigating the observed gradient in ^{15}N enrichment in species within the orchid genus *Epipactis* contributed an intriguing facet to the overall picture of stable isotope abundances of IMH and PMH plants. Interestingly, PMH orchids of the Epidendroideae genus *Epipactis* Zinn are more enriched in ^{15}N than other PMH orchids and even exceed the isotopic enrichment in ^{15}N of FMH orchid species (Fig. 7a; Schiebold *et al.* 2017, manuscript 2). Some *Epipactis* species are mycorrhizal with ECM Ascomycota such as several true truffle species of the genus *Tuber* while most other PMH orchid species are mycorrhizal dominantly with Basidiomycota and occasionally Ascomycota forming ECM (Schiebold *et al.* 2017, manuscript 2). Sporocarps of the ECM Ascomycota *Tuber* are significantly more enriched in ^{15}N than fruiting bodies of ECM Basidiomycota probably due to a unique set of exoenzymes enabling *Tuber* species to access ^{15}N-enriched recalcitrant organic matter as nitrogen source (Gebauer *et al.* 2016; Schiebold *et al.* 2017, manuscript 2). This pattern of ^{15}N enrichment is mirrored in the orchid species (*Epipactis*) that obtain nutrients from the mycorrhizal truffle species and is also evidence that orchids not only exploit their associated fungi for carbohydrates but also for organic molecules containing nitrogen (Schiebold *et al.* 2017, manuscript 2).

All groups displayed in Fig. 7a (FMH ECM, FMH SAP litter, FMH SAP wood, PMH ECM Ascomycota and PMH ECM; s. legend) and all green rhizoctonia-associated orchid species (Epidendroideae, Orchidoideae and Cypripedioideae; Fig 7b) summarised into one group could be significantly differentiated from each other using a Bray-Curtis dissimilarity matrix calculated from enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and nitrogen concentration data (Fig. 9). The groups are well segregated according to their individual pattern of ^{13}C and ^{15}N enrichments. The nitrogen concentration data also went into the calculation of the NMDS but it had no significant effect on the ordination (Fig. 9).

Elaborating further on the $\delta^{2}H$ approach to test whether partial mycoheterotrophy is a common nutritional strategy also in rhizoctonia-associated meadow orchids, we extended the dataset upon 13 further orchid species in the subfamilies Epidendroideae and Orchidoideae forming orchid mycorrhizae with rhizoctonia fungi growing in montane meadow habitats with high irradiance (Fig. 8; Schiebold *et al.* 2018, manuscript 3). We inferred partial mycoheterotrophy as nutritional mode for the majority of these meadow orchid species due to both significant ^{2}H and ^{15}N enrichment (Fig. 8) and high nitrogen concentrations (Table S2). The Small White Orchid *Pseudorchis albida* remained as the sole autotrophic species in this study.
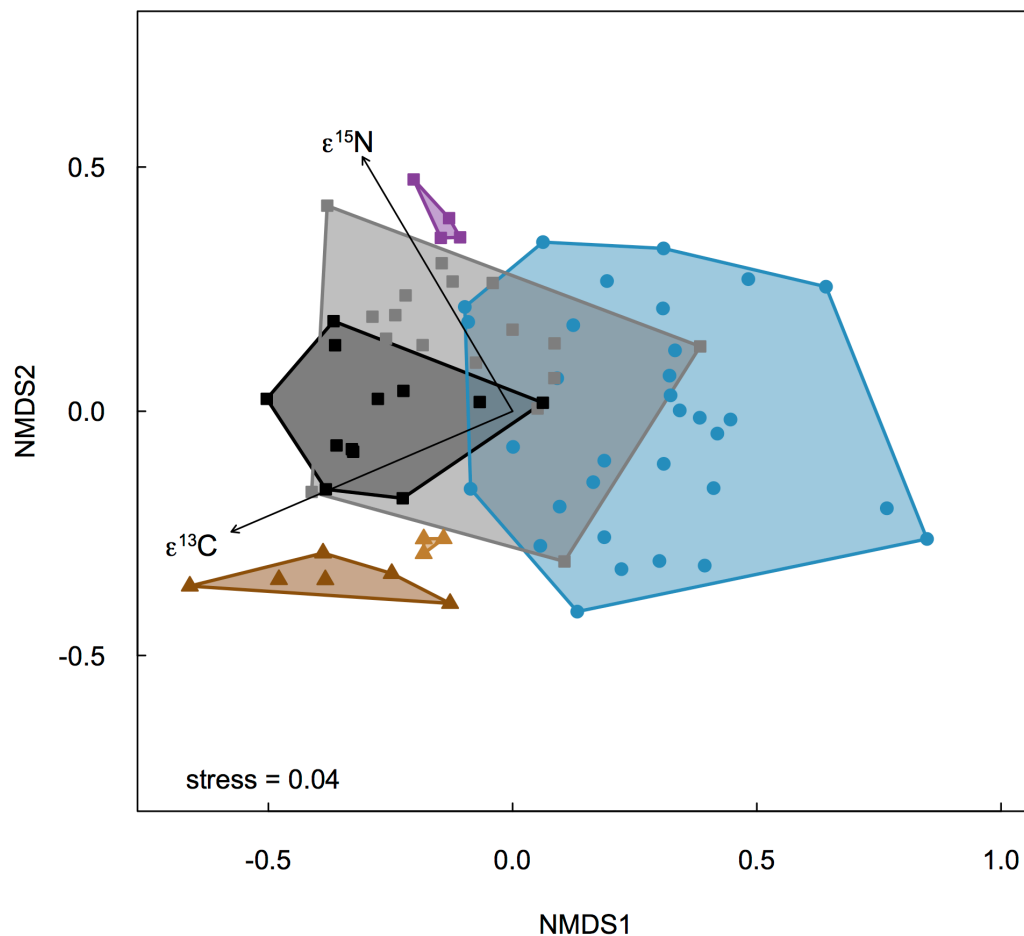
Fig. 9 NMDS plot visualises a Bray–Curtis dissimilarity matrix calculated from enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and nitrogen concentration data in a two-dimensional space. Fitted vectors display the response variables $\varepsilon^{13}C$ and $\varepsilon^{15}N$ in the ordination space and indicate the differences between the groups in association with these variables. All groups displayed in Fig. 7a (FMH ECM, FMH SAP litter, FMH SAP wood, PMH ECM Ascomycota and PMH ECM) and all green rhizoctonia-associated orchid species (Epidendroideae, Orchidoideae and Cypripedioideae; Fig 7b) summarised into one group (blue dots) could be significantly differentiated from each other; stress = 0.04, 1000 permutations; R^2 = 0.664; $P < 0.001$; pairwise tests between the groups: MANOVA $R^2 < 0.932$, $P < 0.03$; Generally, a stress value <0.05 provides an excellent representation in reduced dimensions. See Fig. 7 for legend.

We conclude that partial mycoheterotrophy in rhizoctonia-associated orchid species, as elucidated here by pronounced ^2H enrichment, plays a far greater role in orchids of open habitats than expected (Schiebold *et al*. 2018, manuscript 3).

In a next step we aimed to quantify the percental organic matter gained from mycorrhizal fungi by rhizoctonia-associated orchids using orchid seedlings in an improved methodological approach (Schweiger *et al*. 2018, manuscript 4). By inserting the mean enrichment factors $\varepsilon^{13}C$ and ε^2H of *in situ* grown orchid protocorms as FMH endpoint in a linear two-source mixing model, we calculated the organic matter gain derived by mature orchids from mycorrhizae to reach approximately 20 %. The PMH

exploitation of mycorrhizal fungi in rhizoctonia-associated orchids is thus clearly a supplement to the carbon nutrition from photosynthesis. That there is still down-flow of organic nutrients from plant to mycorrhizal fungi cannot be excluded (Schweiger *et al.* 2018, manuscript 4).

In the last step pursued during this thesis we assessed the degree of partial mycoheterotrophy in dependency on the light availability at the microhabitat in rhizoctonia-associated orchid species occurring in a variety of habitats (Schweiger *et al.* 2019, manuscript 5). We chose *Neottia ovata* and *Ophrys insectifera* for our case study and monitored the irradiance levels at their growing sites, an open grassland site and a closed-canopy forest. We found a significant enrichment in ^{13}C of both orchids relative to the autotrophic references at the forest but not at the grassland site. ^{13}C enrichment in *O. insectifera* but not in *N. ovata* was linearly correlated with the irradiance levels at its habitats. Concluding, both species can be considered as partially mycoheterotrophic and at least in *O. insectifera* the degree of partial mycoheterotrophy can be fine-tuned according to the light availability at the growing site (Schweiger *et al.* 2019, manuscript 5).

Outlook

Although my thesis contributes to improve the understanding of the complex ecology of partial mycoheterotrophy especially in the orchid family, a number of questions remain to be addressed.

A central question would be to investigate the bidirectional nutrient flow in the orchid mycorrhiza and especially the 'down-flow' of carbohydrates from plant to fungus to better integrate orchid mycorrhiza along the *mutualism-parasitism continuum*. An approach on how to realise Cameron *et al.*'s (2008) laboratory experiment showing an 'up-flow' of carbon from fungus to plant in the photosynthesising orchid *Goodyera repens* with a simultaneous 'down-flow' vice versa *in situ* under field conditions is thus required.

However the costs for the mycorrhizal fungi being exploited by partially mycoheterotrophic plants also remain unknown. Also the possible benefits for orchid mycorrhizal fungi remain to be explored, that could encompass providing a habitat for the fungus in the orchid roots or the provisioning with vitamins or other secondary substances (Selosse & Roy 2009).

It would also be fascinating to focus on the trade-offs between different mutualisms such as mycorrhiza vs. anti-herbivory defenses or reproduction (Hoeksema & Bruna 2000 and references herein). Belowground organisms such as mycorrhizal fungi can generally influence a wide variety of floral traits (Becklin *et al.* 2011; Barber & Gorden 2013). The effect of mycorrhization, the composition and diversity of a mycorrhizal community in orchids roots on the amount and the composition and thus attractiveness of floral volatiles and, in rewarding species, also the amount and composition of nectar can strongly affect plant fitness. These studies could be employed in nature conservation and help facilitate a better management of dwindling orchid populations. Especially orchids are known to not only engage in intricate mycorrhizal associations but also in complex plant-pollinator interactions such as food- and sexual-deception as briefly illustrated by the example of the pollination syndrome in bee orchids (genus *Ophrys*). In comparison to autotrophic plants, partially mycoheterotrophic orchids have access to greater carbon pools through their mycorrhizal fungi and could allocate this carbon into pollen dispersal mutualisms. On the contrary, many orchid species do not produce nectar to reward their pollinators and are possibly deceptive both above- and belowground. Few studies link the above- and belowground deceptions (Waterman & Bidartondo 2008; Waterman *et al.* 2011) and it would be interesting to further elaborate on this subject to find analogies among these mutualisms.

The absolute majority of orchids (> 80 %) have an epiphytic lifestyle but besides studies on their initially mycoheterotrophic seedling stages not much is known about trophic strategies in adult epiphytic orchids. This might be challenged and further complicated by the CAM-photosynthesis overlaying the signals of $\delta^{13}C$ ($^{13}CO_2$ discrimination; Farquhar, Ehleringer & Hubick 1989) and δ^2H (cycling of malic acid and pyruvate; Cormier *et al.* 2018) that are usually employed to identify and even quantify

the partial mycoheterotrophic nutrition in C$_3$ plants or fully mycoheterotrophic species. However, investigating trophic strategies in epiphytic orchids would open up an important new research area.

One the other hand surely more partially mycoheterotrophic plants in other families besides the Orchidaceae wait to be identified. Promising candidates are families with dust seeds featuring initial mycoheterotrophy as trophic mode and those families with already known fully mycoheterotrophic species. Heterotrophy in a family might serve as a strong predictor for further partially mycoheterotrophic species (Selosse & Roy 2009). Especially the dominant mycorrhizal type of arbuscular mycorrhiza (AM) with an ancient evolutionary origin might prove promising in discovering new partially mycoheterotrophic species. Many fully mycoheterotrophic species (e.g. in Burmanniaceae, Gentianaceae and Polygalaceae) and even entire families (e.g. Corsiaceae, Petrosaviaceae and Triurdiaceae) exploiting AM have been identified but comparably only relatively few partially mycoheterotrophic species (Merckx *et al.* 2013a).

Extended summaries

Manuscript 1 synthesises the largest available dataset on the ecophysiologies of partially and fully mycoheterotrophic species in the families Ericaceae and Orchidaceae that form mycorrhizae with ectomycorrhizal (ECM) fungi. Most research on the trophic strategies of partial and full mycoheterotrophy using stable isotope natural abundance analysis has been conducted on species belonging to the plant families Ericaceae and Orchidaceae. Hence, the critical mass of data now available is ideally suited to investigate the relationship between plant evolutionary history and mycoheterotrophic plant ecophysiology. We compiled published C and N stable isotope natural abundances ($\delta^{13}C$ and $\delta^{15}N$) and nitrogen concentration data of fully ($n = 18$) and partially ($n = 22$) mycoheterotrophic species in the Ericaceae and Orchidaceae and autotrophic reference plant species ($n = 156$) that we retrieved from 22 original studies using a traceable literature search using the web-based search engine *Web of Science*. Data was also contributed from further unpublished studies. The exact partially and fully mycoheterotrophic species were chosen depending on the type of mycorrhizal fungi that they form an mycorrhizal association with, namely ECM fungi, to avoid the effects of fungal functional guild on mycoheterotrophic plants' stable isotope values and nitrogen concentrations. All stable isotope abundances (δ values) were normalised to gain site-independent data and thus converted into enrichment factors ($\epsilon^{13}C$ and $\epsilon^{15}N$). We then tested for differences in ^{13}C and ^{15}N enrichment and N concentration among plant families and trophic strategies. As expected, we could detect significant enrichments in ^{13}C and ^{15}N of partially and fully mycoheterotrophic plants relative to autotrophic reference species, and confirmed that fully mycoheterotrophic ericaceous and orchidaceous species were more enriched in ^{13}C than their partially mycoheterotrophic counterparts. Additionally, we were able to distinguish mycoheterotrophic Ericaceae from mycoheterotrophic Orchidaceae according to their ^{13}C and ^{15}N enrichments and N concentrations. However, the nitrogen concentration in tissues of Orchidaceae and Ericaceae turned out to be an additional and hitherto insufficiently considered factor differentiating these two plant families: Partially and fully mycoheterotrophic Orchidaceae exhibited significantly higher nitrogen concentrations than those being present in the tissues of Ericaceae of any mycoheterotrophic degree or autotrophic reference species. We conclude that family- or species-specific characteristics in the physiology of matter exchange between fungi and plants are controlled by the plant over the assimilation and processing of C and N can be considered as the most likely reasons underlying the observed differences.

Manuscript 2 focuses on the occasionally observed ^{15}N enrichment in partially mycoheterotrophic orchids that can even exceed the ^{15}N enrichment in fully mycoheterotrophic orchid species. The ^{15}N enrichment is not linearly related to the ^{13}C enrichment, and thus to the degree of heterotrophic carbon gain (Leake & Cameron

2010; Merckx 2013). We hypothesised that the type of mycorrhizal fungi (rhizoctonia; ECM–forming Basidiomycota, ECM B; ECM–forming Ascomycota, ECM A) present in the roots of orchid species might be responsible for the differences in ^{15}N enrichment measured in their bulk leaf tissue. We used the genus *Epipactis* Zinn as a case study to analyse the relationship between the identities of mycorrhizal fungi extracted from the orchid roots and ^{15}N enrichment present in the leaf tissue. For this study we compiled stable isotope abundance data of seven *Epipactis* species and their mycorrhizal fungi from published studies and investigated five further taxa. We observed a pronounced gradient in ^{15}N enrichment in the studied *Epipactis* species ranging from 3.2 ± 0.8 ‰ in initially mycoheterotrophic *E. gigantea* to 24.6 ± 1.6 ‰ in partially mycoheterotrophic *E. neglecta*. The gradient in ^{15}N enrichment is strongly driven by the type and by the ^{15}N abundance of the orchids' mycorrhizal fungi. We detected Ascomycota of the true truffle genus *Tuber* as being the exclusively associated fungi in the roots of the *Epipactis* species *E. muelleri*, *E. leptochila* and *E. neglecta* and simultaneously, the highest ^{15}N enrichments in leaf tissue of the exact *Epipactis* species. Truffle ascocarps that we sampled subsequently from the same sites as the sampled *Epipactis* populations with the help of a truffle–hunting dog, were also highly enriched in ^{15}N in comparison to sporocarps of ECM–forming Basidiomycota or saprotrophic basidiomycetes (Gebauer *et al*. 2016). The truffles' N stable isotope abundances are mirrored in the ^{15}N enrichment of their hosting orchid species. Generally, we find the follwing gradient: ε^{15}N in *Epipactis* spp. exclusively associated with ECM–A > ε^{15}N in *Epipactis* spp. associated with ECM A and B > in *Epipactis* spp. associated with ECM B > ε^{15}N in *Epipactis* spp. associated with orchid mycorrhizal rhizoctonias. This manuscript thus also enhances the understanding of the role of Ascomycota (genus *Tuber*) as mycorrhizal fungi being able to form functional orchid mycorrhizae, a point that was challenged until recently (Smith & Read 2008). We furthermore conclude that orchids generally cover all their nitrogen demands via mycorrhizal fungi as their ^{15}N enrichment results from the nitrogen mobilised and assimilated by their associated fungi.

Manuscript 3 investigates the nutritional mode of partial mycoheterotrophy in chlorophyllous orchid species associated with rhizoctonia fungi that grow in habitats with high irradiance levels such as montane meadow habitats. This group of orchids belongs to the overwhelming majority of terrestrial orchid species that are known to be initially mycoheterotrophic during germination and seedling establishment. However, at adulthood they were assigned an unclear trophic status somewhere between *putatively autotrophic* and *cryptic mycoheterotrophic* (Hynson *et al*. 2013; Hynson 2016). From a stable isotope perspective, these orchids are characterised by a significant ^{15}N enrichment and higher leaf total nitrogen concentrations, but a lack of ^{13}C enrichment, or even ^{13}C depletion relative to autotrophic reference plant species. Here, we used H stable isotope abundances (δ^{2}H) in addition to the routinely employed δ^{13}C and δ^{15}N

measurements to test our hypothesis that also rhizoctonia-associated orchids of open habitats feature partial mycoheterotrophy as nutritional mode. A recent study (Gebauer *et al.* 2016) provided valuable evidence that H isotopes can be used as an additional explanatory variable to elucidate a mycoheterotrophic nutrition in orchids. We sampled 13 orchid species mainly growing in montane meadows in the Northern Limestone Alps of Europe, four forest orchid species and 34 autotrophic reference species to test for a general pattern of partial mycoheterotrophy. Most orchid species inhabiting sunny meadows lacked ^{13}C enrichment or were significantly depleted in ^{13}C relative to autotrophic references. However, we detected significant ^{2}H and ^{15}N enrichments, and high leaf total nitrogen concentrations in the tissue of the majority of rhizoctonia-associated orchid species growing under light-saturated conditions and we thus conclude a partially mycoheterotrophic nutritional mode for these orchids. *Pseudorchis albida* remained as the sole autotrophic orchid species in this study as this species exhibited neither enrichment in any isotope nor a distinctive leaf nitrogen concentration. In addition, we find δ^{2}H well suited to complement the isotopic toolbox for investigating trophic strategies in orchids. Our findings demonstrate that partial mycoheterotrophy is a trophic continuum between the extreme endpoints of autotrophy and full mycoheterotrophy, ranging from marginal to pronounced. In rhizoctonia-associated orchids, partial mycoheterotrophy plays a far greater role than previously assumed, even in full light conditions. We suggest that partial mycoheterotrophy in orchid species populating open habitats is a useful trait to improve competitive success and to facilitate the survival of unfavourable conditions during periods of dormancy.

Manuscript 4 tackles the question how much organic matter rhizoctonia-associated orchids gain exactly form their mycorrhizal fungi. In the previous study we already elucidated that also green-leaved orchids forming orchid mycorrhiza with rhizoctonia fungi are partially mycoheterotrophic (Schiebold *et al.*, 2018). However, the exact fraction of organic matter exploited from mycorrhizal fungi to supplement the orchids´ carbon nutrition remains unknown. This question has already been resolved for orchids even thriving under light-limited conditions such as the understorey of temperate beech forests that partner with ectomycorrhizal fungi forming a mutualistic relationship with forest trees at the same time. In these habitats, the proportion of carbon derived from ectomycorrhizal fungi was found to vary between 14 and 84 %, depending on the irradiance level of an orchid's habitat (Bidartondo *et al.* 2004; Hynson *et al.* 2013). These calculations have been conducted employing a linear two-source mixing model with the isotopic signature of the achlorophyllous bird´s nest-orchid (*Neottia nidus-avis*) or the mean value of up to 11 fully mycoheterotrophic orchid species as fully mycoheterotrophic endpoint (Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Preiss & Gebauer 2008; Hynson *et al.* 2013). However, employing these exact model settings to calculate the proportion of organic matter derived from mycorrhizal fungi in rhizoctonia-associated orchids might result in an underestimation as *N. nidus-avis* and

also the other fully mycoheterotrophic orchid species previously employed in the mixing model approach all form orchid mycorrhiza with ectomycorrhizal fungi. No fully mycoheterotrophic orchid species mycorrhizal with rhizoctonia fungi are known so far. We thus adjusted the linear two-source mixing model in the way that we used fully mycoheterotrophic underground seedlings (protocorms) of the exact rhizoctonia-associated partially mycoheterotrophic orchid species we aimed to investigate at adulthood, as fully mycoheterotrophic endpoints. We obtained the protocorms of seven orchid species from a field germination experiment using the seed packet technique by Rasmussen & Whigham (1993). The seeds remained buried for 19 – 30 months *in situ* at the sites of adult orchids and we could successfully retrieve protocorms for five orchid species that were also associated with rhizoctonia fungi as germination mycobionts. On average, the orchid protocorms were enriched in ^{13}C and ^{15}N relative to mature orchids and had higher mean nitrogen concentrations. By inserting the mean enrichment factors ε^{13}C and ε^{2}H of the orchid protocorms as fully mycoheterotrophic endpoint, the organic matter gain derived by mature orchids from mycorrhizae was estimated to reach approximately 20 % and thus a supplement to the carbon nutrition from photosynthesis. We conclude that green-leaved orchid species mycorrhizal with rhizoctonia fungi are predisposed to a nutrition complemented with organic matter originating from mycorrhizal fungi, due to their underground seedling stage where they rely completely on nutrient provisioning by fungi.

Manuscript 5 concentrates on the degree of mycoheterotrophic carbon gain in partially mycoheterotrophic Orchidaceae in dependence on the light availability at the habitat. Some partially mycoheterotrophic orchids forming orchid mycorrhiza with ectomycorrhizal fungi have been shown to flexibly increase the degree of organic matter exploitation from their mycorrhizal fungi with decreasing light levels. Whether orchid species forming orchid mycorrhizae with rhizoctonia-fungi can also dynamically adjust the intensity of fungal exploitation according to the light availability is a debated question. A few studies following up on the above-mentioned issue monitored rhizoctonia-associated orchid species under natural light gradients that were rather small due to the narrow habitat preference of the respective orchid species (Preiss *et al.* 2010; Liebel *et al.* 2015; Lallemand *et al.* 2018). They observed decreased δ^{13}C abundances in their study species (*Cypripedium calceolus*, *Goodyera repens*, *Epipactis palustris*) with decreasing irradiance levels such as in autotrophic plants. We thought that species occurring under larger natural light gradients such as open meadow habitats and closed-canopy forests might be more suitable to study their ability to adjust the degree of mycoheterotrophic nutrition with the light level at the microhabitat. We chose *Neottia ovata* and *Ophrys insectifera* for our study and hypothesised that these two orchid species associated with rhizoctonia fungi and distributed in a variety of habitats, are able to increase the exploitation of organic matter from their mycorrhizal fungi with

decreasing irradiance levels. We continuously measured the light availability next to 12 individuals of *N. ovata* and *O. insectifera* each at a grassland site and in a forest during their flowering and fruiting period and repeatedly sampled the leaves of *N. ovata*, *O. insectifera* and autotrophic reference species for stable isotope natural abundances and C and N concentrations. We found a significant enrichment in ^{13}C of both orchids relative to the autotrophic references at the forest but not at the grassland site. *N. ovata* and *O. insectifera* were significantly enriched in ^{2}H at both sites. ^{13}C enrichment in *O. insectifera* but not in *N. ovata* was linearly correlated with the irradiance levels at its habitats. Concluding, both species can be considered as partially mycoheterotrophic and at least in *O. insectifera* the degree of partial mycoheterotrophy can be fine-tuned according to the light availability at the growing site. However, the exploitation of mycorrhizal fungi appears to be not as flexible in rhizoctonia-associated orchids as in species forming orchid mycorrhiza with ectomycorrhizal fungi.

Declaration of own contribution to each manuscript

Manuscript 1

Title: **Plant family identity distinguishes patterns of carbon and nitrogen stable isotope abundance and nitrogen concentration in mycoheterotrophic plants associated with ectomycorrhizal fungi**

Authors: N. A. Hynson[*], **J. M.-I. Schiebold**[*] & G. Gebauer (2016)
[*]*authors contributed equally to this manuscript*

Journal: **Annals of Botany** 118 (2016): 467−479, doi: 10.1093/aob/ mcw119

Impact Factor: 4.41 (2016)

Own contribution: idea & concept: 40%, data analysis & figures: 100%, writing: 40%, literature survey: 100%

N.A.H. and G.G. had the idea for this investigation; J.M.-I.S. conducted the literature review, performed the data analysis and created the figures and tables; N.A.H. and J.M.-I.S. drafted the manuscript. Revision and rewriting of the manuscript was done by N.A.H., J.M.-I.S. and G.G. N.A.H. is the corresponding author.

Manuscript 2

Title: **You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species**

Authors: **J. M.-I. Schiebold**, M. I. Bidartondo, P. Karasch, B. Gravendeel & G. Gebauer

Journal: **Annals of Botany** 119 (2017): 1085−1095, doi: 10.1093/aob/mcw265

Impact Factor: 4.41 (2016)

Own contribution: idea & concept: 90%, field work: 100%, analysis of plant and fungal samples: 80%, data analysis & figures: 100%, writing: 90%

J.M.-I.S. and G.G. had the idea for this investigation; J.M.-I.S. performed the literature review, collected the plant samples and conducted the molecular analysis of mycorrhizal fungi at the Jodrell Laboratory at Royal Botanic Gardens, Kew; J.M.-I.S. collected the truffle samples together with P.K. and his truffle-hunting dog "Snoopy"; J.M.-I.S. prepared all samples for stable isotope analysis at the BayCEER−Laboratory of Isotope Biogeochemistry (see acknowledgements); M.I.B. conducted the molecular analysis of the truffle samples and supervised the molecular analysis of the mycorrhizal fungi from the

root samples; G.G. supervised the sample isotope abundance analysis; J.M.-I.S. performed the data analysis, drafted the manuscript and created the figures and tables. Revision and rewriting of the manuscript was done by J.M.-I.S., M.I.B. and G.G. All co-authors contributed critically to the drafts of the manuscript and gave final approval for publication. G.G. is the corresponding author.

Manuscript 3

Title: **Exploiting mycorrhizas in broad daylight: Partial mycoheterotrophy is a common nutritional strategy in meadow orchids**

Authors: **J. M.-I. Schiebold**, M. I. Bidartondo, F. Lenhard, A. Makiola & G. Gebauer

Journal: **Journal of Ecology** 106 (2018), 168−178, doi: 10.1111/1365-2745.12831

Impact Factor: 5.81 (2016)

Own contribution: idea & concept: 50%, field work: 90%, analysis of plant and fungal samples: 70%, data analysis & figures: 100%, writing: 90%

J.M.-I.S. and G.G. had the idea for this investigation; J.M.-I.S. collected the plant samples during students' field courses and with the help of student field assistants (see acknowledgements); J.M.-I.S., A.M. and F.L. prepared the samples for stable isotope analysis at the BayCEER−Laboratory of Isotope Biogeochemistry (see acknowledgements); J.M.-I.S.conducted the molecular analysis of mycorrhizal fungi at the Jodrell Laboratory at Royal Botanic Gardens, Kew; M.I.B. supervised the molecular analysis of mycorrhizal fungi; G.G. supervised the sample isotope abundance analysis; J.M.-I.S. performed the data analysis, created the figures and tables and drafted the manuscript. Revision and rewriting of the manuscript was done by J.M.-I.S., M.I.B. and G.G. All co- authors contributed critically to the drafts of the manuscript and gave final approval for publication. G.G. is the corresponding author.

Manuscript 4

Title: **Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi**

Authors: **J. M.-I. Schweiger**, M. I. Bidartondo & G. Gebauer

Journal: **Functional Ecology** 32 (2018), 870–881, doi: 10.1111/1365-2435.13042

Impact Factor: 5.63 (2016)

Own contribution: idea & concept: 30%, field work: 100%, analysis of plant and fungal samples: 100%, data analysis & figures: 100%, writing: 90%

G.G. had the idea for this investigation; J.M.-I.S. conducted the seed baiting experiment with support from 5 Hiwis (see acknowledgements), collected the plant samples, prepared all samples for stable isotope analysis at the BayCEER−Laboratory of Isotope Biogeochemistry (see acknowledgements) and conducted the molecular analysis of mycorrhizal fungi at the Jodrell Laboratory at Royal Botanic Gardens, Kew; M.I.B. supervised the molecular analysis of mycorrhizal fungi; G.G. supervised the sample isotope abundance analysis. J.M.-I.S. performed the data analysis, created the figures and tables and drafted the manuscript. Revision and rewriting of the manuscript was done by J.M.-I.S., M.I.B. and G.G. All co-authors contributed critically to the drafts of the manuscript and gave final approval for publication. G.G. is the corresponding author.

Manuscript 5

Title: **Light limitation and partial mycoheterotrophy in rhizoctonia-associated orchids**

Authors: **J. M.-I. Schweiger**, C. Kemnade, M. I. Bidartondo & G. Gebauer

Journal: **Oecologia** (accepted: 11 January 2019), Ms. No. OECO-D-18-00462R3

Own contribution: idea & concept: 20%, field work: 20%, analysis of plant samples: 25%, analysis of fungal samples 50%, data analysis & figures: 100%, writing: 90%

G.G. had the idea for this investigation; C.K. set up the field experiment and conducted the sampling during the light climate experiment and prepared all samples for stable isotope analysis at the BayCEER−Laboratory of Isotope Biogeochemistry (see acknowledgements); J.M.-I.S. collected additional plant samples for stable isotope analysis (δ^2H & δ^{18}O) and molecular analysis of mycorrhizal fungi; J.M.-I.S. conducted the molecular analysis of mycorrhizal fungi at the Jodrell Laboratory at Royal Botanic Gardens, Kew; M.I.B. supervised the molecular analysis of mycorrhizal fungi; G.G. supervised the sample isotope abundance analysis. J.M.-I.S. performed the data analysis, created the figures and tables and drafted the manuscript.

Table 1 Conference presentations of research related to this thesis. Contributions that were awarded with a prize as the best oral or poster presentation of the respective conference are marked with an asterisk (*).

Date	Title	Conference	Location	Type
2013	Elucidating the carbon and nitrogen gains of orchids in mountainous habitats – A stable isotope approach	31st New Phytologist Symposium	Università della Calabria, Cosenza, Italy	Poster
2013	Multielement-Stabilisotopenanalyse offenbart partielle Mykoheterotrophie von Orchideen auf Almwiesen der Alpen	Jahrestagung der Arbeitsgemeinschaft Stabile Isotope e.V.	Thünen Institut, Braunschweig, Germany	Talk
2013	Nutrient gains from fungi by adult orchids in temperate montane habitats – A stable isotope approach	5th BayCEER Workshop	University of Bayreuth, Bayreuth, Germany	Poster*
2013	Nutrient gains from fungi by adult orchids in temperate montane habitats	5th International Orchid Conservation Congress	Ile de la Réunion, France	Talk
2014	Nutrient gains from mycorrhizal fungi by adult orchids in temperate montane habitats	33rd New Phytologist Symposium	Agroscope, Zürich, Schweiz	Poster
2014	Partielle Mykoheterotrophie bei Orchideen	Treffen des Arbeitskreis Heimische Orchideen e.V.	Kersbach, Germany	Invited Talk
2014	Multi-element stable isotope analysis elucidates partial mycoheterotrophy of orchids in temperate montane meadow habitats	BIOGEOMON – 8th International Symposium on Ecosystem Behavior	University of Bayreuth, Bayreuth, Germany	Poster
2014	Partielle Mykoheterotrophie bei Orchideen	Jahrestagung der Deutschen Gesellschaft für Mykologie (DGfM e.V.)	Mettlach-Orscholz, Deutschland	Invited Talk
2014	Light limitation stimulates partial mycoheterotrophy in Rhizoctonia-associated orchids	Jahrestagung der Arbeitsgemeinschaft Stabile Isotope e.V.	Helmholtz Zentrum München, Munich, Germany	Talk

Table 1 (continued)

Date	Title	Conference	Location	Type
2015	From rags to riches: Partial mycoheterotrophy in the genus *Epipactis* ZINN	Terrestrial Orchid Research Conference	Samos, Greece	Talk
2015	Is a switch of carbon sources under light-limitation a general feature of partially mycoheterotrophic orchids?	Terrestrial Orchid Research Conference	Samos, Greece	Poster*
2015	Clades of mycorrhizal fungi differentiate isotope abundance patterns of partially mycoheterotrophic species in the orchid genus *Epipactis*	8th International Conference on Mycorrhizas (ICOM8)	Northern Arizona University (NAU), Flagstaff, Arizona, USA	Poster & Lightning Talk
2015	Does 'you are what you eat (plus a few permil)' also hold true for orchids?	Jahrestagung der Arbeitsgemeinschaft Stabile Isotope e.V.	University of Heidelberg, Germany	Talk
2015	An orchid´s exquisite taste for truffles – partial mycoheterotrophy in the genus *Epipactis*	7th BayCEER Workshop	University of Bayreuth, Bayreuth, Germany	Poster
2016	New insights into partial mycoheterotrophy: isotope signatures and fungal partners of orchid protocorms	6th International Orchid Conservation Congress	Hong Kong, China	Talk*
2016	Partielle Mykoheterotrophie bei Orchideen	34. Tagung der Vorstände der Arbeitskreise Heimische Orchideen Deutschlands	Arnstadt, Germany	Invited Talk
2017	Many ways to exploit mycorrhizas: the mycoheterotrophy continuum in orchids	8th International Conference on Mycorrhizas (ICOM8)	Prague, Czech Republic	Poster & Lightning Talk
2017	Many ways to exploit mycorrhizas: the mycoheterotrophy continuum in orchids	9th BayCEER Workshop	University of Bayreuth, Bayreuth, Germany	Poster

List of further publications

On this topic

G. Gebauer & **J. M.-I. Schiebold (Schweiger)**: Orchid Mycorrhizal Associations −
Stable isotope natural abundance analysis as a tool for understanding orchid
mycorrhizal nutrition *in* **Conservation Methods for Terrestrial Orchids** (2017), eds.
Nigel Swarts & Kingsley Dixon, J. Ross Publishing, USA.

added to this thesis as Appendix I

Y. Ogura-Tsujita, G. Gebauer, H. Xu, Y. Fukasawa, H. Umata, K. Tetsuka, M. Kubota,
J. M.-I. Schweiger, S. Yamashita, N. Maekawa, M. Maki, S. Isshiki & T. Yukawa: The
giant mycoheterotrophic orchid *Erythrorchis altissima* is associated mainly with a
divergent set of wood-decaying fungi, **Molecular Ecology** (2018) 27, 1324–1337,
doi:10.1111/ mec.14524; *Impact Factor:* 6.09 (2016).

added to this thesis as Appendix II

J. M.-I. Schiebold (Schweiger) & G. Gebauer: Orchideen als Feinschmecker -
Unterirdische Dreiecksbeziehungen sichern die Ernährung mit Trüffeln. **Spektrum
Universität Bayreuth** (2016) 2, 76−79.

On another topic

J. M.-I. Schiebold (Schweiger), S. Dötterl, M. Feulner & M. Lauerer: High congruence
of intra-specific variability in floral scent and genetic patterns in *Gentianella bohemica*
Skalický (Gentianaceae), **Biochemical Systematics and Ecology** (2017) 71, 50−58,
doi:10.1016/j.bse. 2017.01.004; *Impact Factor:* 0.93 (2016).

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Appendix to the Synopsis

Table S1 Details on the identity of the 109 reviewed orchid species displayed in Fig. 7. Stable isotope data originating from manuscripts 1 to 5 of this thesis are marked with "Y" in the column "Own Data".

Category	Subfamily	Species	n	mean N conc.	mean ε^{15}N	mean ε^{13}C	Own data
FMH ECM	**Epidendroideae**	Aphyllorchis caudata	4	1,93	7,20	8,37	N
		Aphyllorchis montana	3	2,63	10,88	10,33	N
		Cephalanthera damasonium (albino)	14	5,79	14,28	7,81	N
		Cephalanthera exigua	5	3,12	13,62	8,68	N
		Cephalanthera longifolia (albino)	9	3,77	11,77	6,59	N
		Corallorhiza maculata	25	1,82	13,60	7,05	N
		Corallorhiza striata	3	2,27	10,37	9,57	N
		Cymbidium macrorhizon (syn. Cymbidium aberrans)	9	1,43	12,78	9,96	N
		Epipogium aphyllum	8	3,04	7,45	8,98	N
		Neottia nidus-avis	62	2,42	11,34	7,43	Y
	Vanilloideae	Lecanorchis nigricans	3	1,97	16,43	7,90	N
		Lecanorchis thalassica	5	2,18	8,84	8,04	N
FMH SAP litter	**Epidendroideae**	Gastrodia appendiculata	5	3,00	5,04	7,88	N
		Gastrodia fontinalis	5	3,22	4,78	8,78	N
		Gastrodia nantoensis	5	3,32	5,16	8,04	N
FMH SAP wood	**Epidendroideae**	Gastrodia confusa	5	3,90	2,92	10,22	N
		Gastrodia flavilabella	5	2,56	4,72	12,04	N
		Gastrodia similis	10	NA	2,34	9,37	N
	Vanilloideae	Cyrtosia javanica	5	3,34	4,74	11,20	N
		Cyrtosia septentrionalis	1	0,80	5,10	10,20	N
		Erythrorchis altissima	5	1,72	4,50	10,30	Y
		Galeola falconeri	5	2,50	5,94	11,86	N
PMH ECM Ascomycota	**Epidendroideae**	Epipactis helleborine subsp. orbicularis (syn. Epipactis distans)	9	2,34	16,14	3,03	Y
		Epipactis leptochila	9	3,56	22,70	5,10	Y

Table S1 (continued)

Category	Subfamily	Species	n	mean N conc.	mean ϵ^{15}N	mean ϵ^{13}C	Own data
PMH ECM Ascomycota	**Epidendroideae** (continued)	*Epipactis leptochila* subsp. *neglecta* (syn. *Epipactis neglecta*)	5	2,58	24,58	4,22	Y
		Epipactis muelleri	5	2,92	20,74	3,84	Y
PMH ECM	**Epidendroideae**	*Cephalanthera damasonium*	72	2,67	10,03	3,72	N
		Cephalanthera erecta	20	3,80	11,71	3,18	N
		Cephalanthera erecta var. *subaphylla* (syn. *Cephalanthera subaphylla*)	19	NA	12,12	5,92	N
		Cephalanthera falcata	24	NA	6,75	2,11	N
		Cephalanthera longibracteata	8	NA	6,89	1,30	N
		Cephalanthera longifolia	65	2,62	6,43	2,18	N
		Cephalanthera rubra	35	2,61	6,89	1,57	Y
		Corallorhiza trifida	9	1,55	6,12	6,92	N
		Cymbidium goeringii	12	1,51	4,65	2,52	N
		Cymbidium lancifolium	6	1,88	11,23	4,53	N
		Epipactis albensis var. *fibri* (syn. *Epipactis fibri*)	29	2,72	17,12	4,24	N
		Epipactis atrorubens	26	2,49	9,37	2,00	N
		Epipactis helleborine	36	2,56	12,27	2,26	Y
		Epipactis helleborine subsp. *neerlandica*	15	2,09	11,77	2,42	Y
		Epipactis microphylla	5	1,50	21,52	5,70	Y
		Epipactis papillosa (syn. *Epipactis helleborine* var. *sayekiana*)	6	NA	12,86	3,52	N
		Epipactis purpurata	5	2,68	17,40	6,10	Y
		Limodorum abortivum	14	2,17	13,71	5,05	N
		Limodorum trabutianum	5	2,32	11,50	4,64	N
	Orchidoideae	*Cheirostylis griffithii*	2	3,70	2,70	4,75	N

Table S1 (continued)

Category	Subfamily	Species	n	mean N conc.	mean $\varepsilon^{15}N$	mean $\varepsilon^{13}C$	Own data
PMH ECM	Orchidoideae (continued)	*Ludisia discolor*	5	1,62	3,40	-1,72	N
		Platanthera minor	3	1,57	10,90	3,73	N
green Cypripedioideae rhizoctonia	Cypripedioideae	*Cypripedium calceolus*	25	1,66	3,64	0,91	N
green Epidendroideae rhizoctonia	Epidendroideae	*Calanthe discolor*	5	NA	1,66	-1,70	N
		Epipactis gigantea	5	2,32	3,16	-1,18	N
		Epipactis palustris	19	1,73	4,52	0,06	Y
		Liparis hawaiensis	3	2,23	1,20	-2,20	N
		Liparis loeselii	10	1,92	4,63	-2,31	Y
		Liparis nervosa	3	2,67	2,10	1,70	N
		Malaxis monophyllos	5	1,86	2,22	-0,68	Y
		Neottia cordata	11	2,06	2,01	1,74	Y
		Neottia ovata	45	2,13	2,83	0,67	Y
green Orchidoideae rhizoctonia	Orchidoideae	*Anacamptis laxiflora* (syn. *Orchis laxiflora*)	10	NA	3,38	-0,92	N
		Anacamptis morio (syn. *Orchis morio*)	37	2,19	3,53	-0,77	N
		Anacamptis morio subsp. *longicornu* (syn. *Orchis longicornu*)	5	NA	2,64	0,14	N
		Anacamptis papilionacea (syn. *Orchis papilionacea*)	5	NA	2,64	-1,14	N
		Anoectochilus sandvicensis	18	1,82	3,27	-1,09	N
		Dactylorhiza fuchsii	5	1,60	5,46	-1,20	N
		Dactylorhiza incarnata	10	1,54	2,28	-1,49	Y
		Dactylorhiza maculata	27	1,41	0,35	0,16	N
		Dactylorhiza majalis	14	1,70	2,49	-0,25	Y

Table S1 (continued)

Category	Subfamily	Species	n	mean N conc.	mean ε^{15}N	mean ε^{13}C	Own data
green Orchidoideae rhizoctonia	**Orchidoideae** (continued)						
		Dactylorhiza sambucina	11	2,37	0,99	0,57	N
		Dactylorhiza viridis	5	1,50	1,94	-1,60	Y
		Dactylorhiza praetermissa	5	1,06	4,82	-0,22	N
		Gennaria diphylla	10	NA	1,61	3,01	N
		Goodyera schlechtendaliana	1	1,30	0,60	-0,60	N
		Goodyera oblongifolia	18	NA	1,22	-1,77	N
		Goodyera repens	49	1,30	0,35	-2,46	N
		Gymnadenia conopsea	18	1,39	6,62	-0,91	Y
		Gymnadenia nigra (syn. *Nigritella nigra*)	5	1,62	2,04	0,34	Y
		Habenaria tridactylites	5	NA	1,12	2,64	N
		Herminium monorchis	10	1,24	2,35	-2,76	Y
		Himantoglossum metlesicsianum (syn. *Barlia metlesicsiana*)	5	NA	2,16	-0,08	N
		Himantoglossum robertianum (syn. *Barlia robertiana*)	5	NA	3,28	2,24	N
		Neotinea maculata	5	NA	1,28	-0,80	N
		Neotinea tridentata (syn. *Orchis tridentata*)	5	NA	4,42	0,56	N
		Neotinea ustulata (syn. *Orchis ustulata*)	10	2,13	5,67	-1,22	Y
		Ophrys apifera	5	NA	3,62	-1,28	N
		Ophrys sphegodes	5	NA	2,04	-1,16	N
		Ophrys fuciflora	9	NA	3,66	-1,06	N
		Ophrys insectifera	32	2,00	6,30	-0,04	Y
		Ophrys lutea subsp. *galilea* (syn. *Ophrys sicula*)	5	NA	4,24	-1,98	N

Table S1 (continued)

Category	Subfamily	Species	n	mean N conc.	mean ε¹⁵N	mean ε¹³C	Own data
green Orchidoideae rhizoctonia	**Orchidoideae** (continued)	Ophrys sphegodes subsp. atrata (syn. Ophrys incubacea)	5	NA	1,00	-0,84	N
		Orchis anthropophora (syn. Aceras anthropophorum)	10	NA	1,93	-1,24	N
		Orchis branciforti	5	NA	3,52	-1,02	N
		Orchis mascula	18	1,76	5,74	-1,83	N
		Orchis mascula subsp. ichnusae (syn. Orchis ichnusae)	5	NA	3,38	-1,20	N
		Orchis militaris	5	1,22	5,96	0,16	Y
		Orchis patens subsp. canariensis (syn. Orchis canariensis)	5	NA	2,22	-1,04	N
		Orchis pauciflora	5	NA	1,98	-1,08	N
		Orchis provincialis	5	NA	3,64	-0,82	N
		Orchis purpurea	10	NA	5,84	1,30	N
		Platanthera bifolia	25	NA	4,42	0,56	Y
		Platanthera chlorantha	4	1,50	3,01	1,43	N
		Platanthera dilatata var. leucostachys (syn. Platanthera leucostachys)	13	NA	1,95	1,58	N
		Pseudorchis albida	10	1,44	0,42	-1,08	Y
		Serapias cordigera	5	NA	1,32	-1,64	N
		Serapias lingua	5	NA	2,78	-0,76	N
		Serapias nurrica	5	NA	3,22	0,30	N
		Serapias parviflora	15	NA	3,05	1,23	N
		Serapias vomeracea	10	NA	1,48	-0,10	N
		Spiranthes aestivalis	5	1,98	4,34	-1,22	Y
		Spiranthes spiralis	5	NA	4,18	-0,64	N
		Traunsteinera globosa	5	2,50	4,96	-0,58	Y
		Zeuxine agyokuana	1	4,00	3,50	0,90	N

Table S2 Details on the identity of the 22 reviewed orchid species displayed in Fig. 8. Stable isotope data originating from manuscripts 1 to 5 of this thesis are marked with "Y" in the column "Own Data".

Category	Subfamily	Species	n	mean N conc.	mean ε^{15}N	mean ε^{13}C	mean ε^{2}H	Own data
FMH ECM	**Epidendroideae**	*Neottia nidus-avis*	10	2,29	12,98	6,96	57,30	Y
PMH ECM	**Epidendroideae**	*Cephalanthera damasonium*	5	2,70	6,30	3,52	23,97	N
		Cephalanthera rubra	10	2,88	8,15	2,07	19,68	N
		Epipactis atrorubens	6	2,55	9,50	1,50	18,77	N
		Epipactis helleborine	5	2,57	10,31	1,05	22,07	Y
green Cypripedioideae rhizoctonia	**Cypripedioideae**	*Cypripedium calceolus*	25	1,65	3,64	0,90	28,61	N
green Epidendroideae rhizoctonia	**Epidendroideae**	*Liparis loeselii*	5	1,73	3,19	-2,52	27,91	Y
		Malaxis monophyllos	5	1,87	2,21	-0,67	-5,62	
		Neottia cordata	5	1,63	3,03	2,52	15,28	Y
		Neottia ovata	15	2,00	1,58	1,08	24,67	Y
green Orchidoideae rhizoctonia	**Orchidoideae**	*Dactylorhiza incarnata*	5	2,01	2,13	-1,17	11,95	Y
		Dactylorhiza majalis	5	1,48	2,11	-0,59	6,37	Y
		Dactylorhiza viridis	5	1,52	1,93	-1,60	11,10	Y
		Gymnadenia conopsea	5	1,66	6,58	-1,61	9,96	Y
		Gymnadenia nigra (syn. *Nigritella nigra*)	5	1,62	2,03	0,33	12,50	Y
		Herminium monorchis	5	1,45	1,75	-2,85	18,23	Y
		Neotinea ustulata (syn. *Orchis ustulata*)	5	1,97	4,15	-1,24	8,15	Y
		Ophrys insectifera	15	2,00	6,52	0,69	31,53	Y
		Platanthera bifolia	16	1,53	3,83	1,33	16,91	Y
		Pseudorchis albida	5	1,45	0,26	-1,28	6,02	Y
		Spiranthes aestivalis	5	1,98	4,35	-1,21	23,21	Y
		Traunsteinera globosa	5	2,49	4,96	-0,58	-1,79	Y

MANUSCRIPTS OF THIS THESIS

MANUSCRIPT 1

Plant family identity distinguishes patterns of carbon and nitrogen stable isotope abundance and nitrogen concentration in mycoheterotrophic plants associated with ectomycorrhizal fungi

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Plant family identity distinguishes patterns of carbon and nitrogen stable isotope abundance and nitrogen concentration in mycoheterotrophic plants associated with ectomycorrhizal fungi

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● **Background and Aims** Mycoheterotrophy entails plants meeting all or a portion of their carbon (C) demands via symbiotic interactions with root-inhabiting mycorrhizal fungi. Ecophysiological traits of mycoheterotrophs, such as their C stable isotope abundances, strongly correlate with the degree of species' dependency on fungal C gains relative to C gains via photosynthesis. Less explored is the relationship between plant evolutionary history and mycoheterotrophic plant ecophysiology. We hypothesized that the C and nitrogen (N) stable isotope compositions, and N concentrations of fully and partially mycoheterotrophic species differentiate them from autotrophs, and that plant family identity would be an additional and significant explanatory factor for differences in these traits among species. We focused on mycoheterotrophic species that associate with ectomycorrhizal fungi from plant families Ericaceae and Orchidaceae.
● **Methods** Published and unpublished data were compiled on the N concentrations, C and N stable isotope abundances (δ^{13}C and δ^{15}N) of fully ($n = 18$) and partially ($n = 22$) mycoheterotrophic species from each plant family as well as corresponding autotrophic reference species ($n = 156$). These data were used to calculate site-independent C and N stable isotope enrichment factors (ε). Then we tested for differences in N concentration, ^{13}C and ^{15}N enrichment among plant families and trophic strategies.
● **Key Results** We found that in addition to differentiating partially and fully mycoheterotrophic species from each other and from autotrophs, C and N stable isotope enrichment also differentiates plant species based on familial identity. Differences in N concentrations clustered at the plant family level rather than the degree of dependency on mycoheterotrophy.
● **Conclusions** We posit that differences in stable isotope composition and N concentrations are related to plant family-specific physiological interactions with fungi and their environments.

Key words: Carbon and nitrogen, Ericaceae, mycoheterotrophy, mixotrophy, mycorrhizal fungi, Orchidaceae, plant adaptations, stable isotopes.

INTRODUCTION

The ability of plants to fix atmospheric carbon (C) and convert it into sugars through photosynthesis (autotrophy) sets this kingdom of organisms apart from others and is the key to the earth's primary productivity. However, some plants have completely lost this ability and rely on alternative means of nutrition such as feeding off symbiotic relationships with mycorrhizal fungi. Some plants retain the ability to photosynthesize, but under certain conditions meet a portion of their C demands via associations with mycorrhizal fungi. Both of these intriguing plant adaptations fall under the category of mycoheterotrophy. Mycoheterotrophy entails plants meeting all or a portion of their C and nutrient demands via symbiotic mycorrhizal fungi (Merckx, 2013). The most striking examples of mycoheterotrophic plants are those that have completely lost the ability to photosynthesize, and their above-ground structures serve only for dispersal and reproduction (Merckx, 2013). Over approximately the last decade, the

marriage of methods from plant ecophysiology and molecular ecology has led to new revelations about the population ecology of mycoheterotrophs (reviewed in Merckx *et al*., 2009; Selosse and Roy, 2009). However, fundamental questions remain on the ecology and evolution of this unique plant adaptation. Here we address a set of these questions – can nitrogen (N) concentrations and C and N stable isotope abundances of mycoheterotrophs distinguish distantly related plants that are dependent upon the same functional guild of mycorrhizal fungi?

Mycoheterotrophy has arisen independently in at least 17 plant families (Merckx, 2013), but Orchidaceae and Ericaceae species have received by far the most attention from researchers (Bidartondo, 2005; Hynson and Bruns, 2010; Dearnaley *et al*., 2012). In a Tansley Review from 1994, Jonathan Leake coined the term 'myco-heterotrophy' for plants that met their C and nutrient demands exclusively via fungi (Leake, 1994). On the heels of his review came a slew of new research that engaged recently developed tools from molecular biology such as

Sanger DNA sequencing of environmental samples to identify the fungal partners of ericaceous and orchidaceous mycoheterotroph populations from temperate forests (Cullings *et al.*, 1996; Taylor and Bruns, 1997; Bidartondo *et al.*, 2000; McKendrick *et al.*, 2000). These studies (among numerous others) revealed that many mycoheterotrophic taxa partnered with specific lineages of fungi that simultaneously formed ectomycorrhizal (EM) associations with trees that provide C to both the fungi and mycoheterotrophs. Relative to other plants that can partner with multiple EM fungi simultaneously, the apparent extreme specificity of the mycorrhizal interactions in ericaceous and orchidaceous mycoheterotrophic species stood out as an anomaly, and was likened to specialized host–parasite interactions (Smith and Read, 2008). This pattern of fungal specificity held for other mycoheterotrophic species that associated with different functional guilds of fungi such as arbuscular mycorrhizal (AM) fungi (Bidartondo *et al.*, 2002) and saprotrophs (Ogura-Tsujita *et al.*, 2009), leading researchers to believe that fungal specificity must be a requisite for the mycoheterotrophic lifestyle. However, more recent studies have shown that not all fully mycoheterotrophic species specialize on particular lineages of fungi. Instead, fungal specificity tends to lie at the level of functional guild (EM, AM or saprotrophic fungi), rather than fungal species identity (Hynson and Bruns, 2009; Roy *et al.*, 2009).

In tandem with the research on mycoheterotrophs and their fungal 'hosts' was the work of Gebauer and Meyer (2003) and Trudell *et al.* (2003) on the ecophysiology of mycoheterotrophy. These research teams analysed the natural abundances of C and N stable isotopes from fully mycoheterotrophic species, leafy green orchids and other vegetation. Working on different sides of the globe, they independently came to the same conclusions that the stable isotope signatures of mycoheterotrophs were significantly enriched in the heavy isotopes of both carbon (^{13}C) and nitrogen (^{15}N) compared with surrounding autotrophic species and most similar to those of EM fruit bodies. The work of Gebauer and Meyer (2003) also detected a new isotopic pattern among some species of apparently autotrophic orchids. A selected number of green orchid species from their study sites in southern France and Bavaria had intermediate ^{13}C enrichment values relative to fully mycoheterotrophic and autotrophic species. This finding was the first line of evidence for what is now known as partial mycoheterotrophy – a form of mixotrophy where a plant meets its C demands through both fungi and photosynthesis (Selosse and Roy, 2009). Additional lines of support for the existence of partial mycoheterotrophy in orchids came from the work of Bidartondo *et al.* (2004), Selosse *et al.* (2004), Julou *et al.* (2005) and Abadie *et al.* (2006) who found similar patterns of ^{13}C enrichment among other species of green orchids and also found that these orchids partnered with a diversity of EM fungi shared with surrounding trees rather than orchid mycorrhizal fungi in the genera *Tulasnella*, *Ceratobasidium* or taxa in the order Sebacinales (grouped in the polyphyletic 'rhizoctonias'). These results were later corroborated in ericaceous species in studies led by Zimmer *et al.* (2007) and Tedersoo *et al.* (2007).

To date, the most well-investigated groups of both partially (PMH) and fully mycoheterotrophic (FMH) plants remain orchidaceous and ericaceous species that partner with EM fungi (Hynson and Bruns, 2010). However, there are a rising number of studies that have examined the stable isotope profiles of FMH species that partner with AM (Merckx *et al.*, 2010; Courty *et al.*, 2011) and saprotrophic fungi (Ogura-Tsujita *et al.*, 2009; Martos *et al.*, 2009; Dearnaley and Bougoure, 2010; Lee *et al.*, 2015), but evidence of partial mycoheterotrophy among species that partner with these guilds remains sparse (Cameron and Bolin, 2010; Bolin *et al.*, 2015). The combined results of these efforts provide evidence that the ^{13}C and ^{15}N enrichment of FMH species can be distinguished based on the guild of their fungal host (AM, EM or saprotroph; Hynson *et al.*, 2013). Also, among full mycoheterotrophs there are often interspecific differences in their C and N stable isotope profiles, but these values are relatively consistent within a species across its geographical range. The total N concentration of full mycoheterotrophs also varies significantly from species to species (Hynson *et al.*, 2013).

Authors have put forth numerous explanations for these patterns, but most agree that due to mycoheterotrophs' dependency on fungi to meet all or a portion of their C and N demands, the identity(ies) of their fungal symbionts should influence their C and N stable isotope profiles and N concentrations (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Zimmer *et al.*, 2007; Tedersoo *et al.*, 2007; Hynson *et al.*, 2009; Liebel *et al.*, 2010). This is because among genera (and sometimes species) of fungi there exists a wide range of soil nutrient mining and catabolic abilities (Gebauer and Taylor, 1999; Emmerton *et al.*, 2001; Taylor *et al.*, 2004; Pritsch and Garbaye, 2011). Differences among fungi in the processing of C from surrounding autotrophs and N from the soil should affect their stable isotope composition, and in turn mycoheterotroph stable isotope profiles closely mirror those of their host fungi (Taylor *et al.*, 2003; Hobbie *et al.*, 2005; Mayor *et al.*, 2009). For example, if an FMH species is relatively depleted in the heavy isotope of N (^{15}N) this could be due to this species associating with a specific fungus that is particularly adept at accessing ^{15}N-depleted mineral N (Gebauer and Taylor, 1999). However, this does not explain differences in ^{15}N enrichment between mycoheterotrophic taxa that specialize on closely related fungi from the same functional guild with putatively similar biochemistry. For instance, the two ericaceous FMH species *Sarcodes sanguinea* Torr. and *Pterospora andromedea* Nutt. often grow in sympatry and partner with the same or closely related EM fungi in the genus *Rhizopogon*, but have significantly different enrichment in ^{15}N (Fig. 1B; Bidartondo and Bruns, 2002; Hynson *et al.*, 2013). The opposite pattern can also be seen in the ericaceous FMH species *Hypopitys monotropa* Crantz and *Monotropa uniflora* L. that each specialize on distantly related lineages of EM fungi, but share overlapping C and N stable isotope profiles (Fig. 1A; Bidartondo and Bruns, 2002; Hynson *et al.*, 2013). These findings all indicate that among mycoheterotrophs that partner with the same functional guild of fungi, there exists some form of plant, rather than fungal, control over the assimilation and processing of C and N.

With a critical mass of data now accumulated on both the identity and diversity of fungi that host species of orchidaceous and ericaceous mycoheterotrophs, we set out to test whether plant family identity is a significant predictor for N concentration and stable isotope abundances. To avoid the effects of fungal functional guild on mycoheterotroph stable isotope values and N concentration, we selected just those species that form symbioses with EM fungi. We compiled data from 22 published
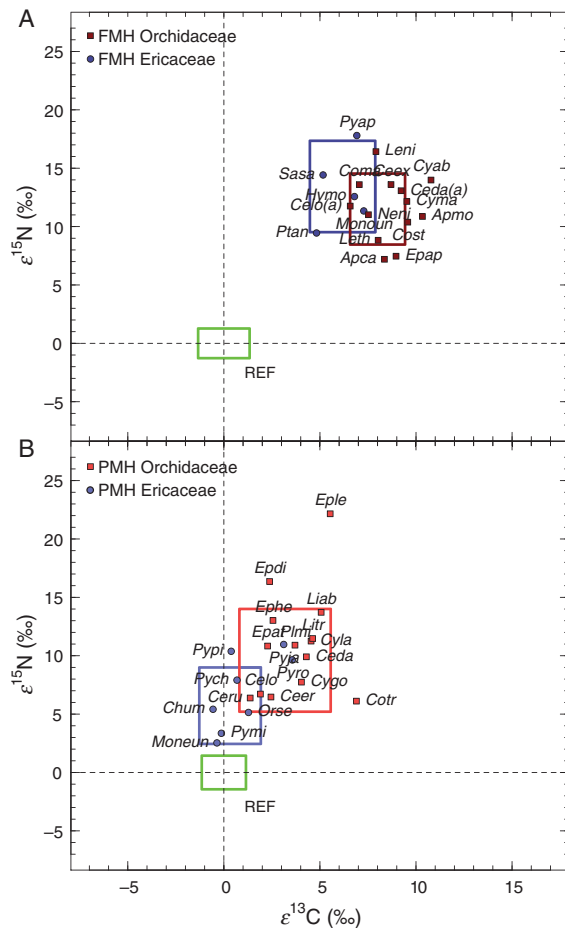
Fig. 1. Mean enrichment factors (ε) for ^{13}C and ^{15}N of (A) fully mycoheterotrophic (FMH) Orchidaceae and Ericaceae and (B) partially mycoheterotrophic (PMH) Orchidaceae and Ericaceae associated with fungi forming ectomycorrhizas. Boxes represent one standard deviation of the mean ε values for the four significantly distinguished groups of (partially) mycoheterotrophic Orchidaceae and Ericaceae and for their respective photosynthetic reference plants (REF, $n = 1433$. Abbreviations of the species and numbers of replicates (n) are as follows: FMH Orchidaceae ($n = 126$): *Apca, Aphyllorchis caudata*; *Apmo, A. montana*; *Ceda(a), Cephalanthera damasonium* albino; *Ceex, C. exigua*; *Celo(a), C. longifolia* albino; *Coma, Corallorhiza maculata*; *Cost, Corallorhiza striata*; *Cyab, Cymbidium aberrans*; *Cyma, C. macrorhizon*; *Epap, Epipogium aphyllum*; *Leni, Lecanorchis nigricans*; *Leth, Lecanorchis thalassica*; *Neni, Neottia nidus-avis*; (REF, $n = 628$). FMH Ericaceae ($n = 134$): *Hymo, Hypopitys monotropa*; *Monoun, Monotropa uniflora*; *Ptan, Pterospora andromedea*; *Pyap, Pyrola aphylla*; *Sasa, Sarcodes sanguinea*; (REF, $n = 403$). PMH Orchidaceae ($n = 189$): *Ceda, Cephalanthera damasonium*; *Ceer, C. erecta*; *Celo, C. longifolia*; *Ceru, C. rubra*; *Cotr, Corallorhiza trifida*; *Cygo, Cymbidium goeringii*; *Cyla, C. lancifolium*; *Epat, Epipactis atrorubens*; *Epdi, E. distans*; *Ephe, E. helleborine*; *Eple, E. leptochila*; *Liab, Limodorum abortivum*; *Litr, L. trabutianum*; *Plmi, Platanthera minor*; (REF, $n = 662$). PMH Ericaceae ($n = 606$): *Chum, Chimaphila umbellata*; *Moneun, Moneses uniflora*; *Orse, Orthilia secunda*; *Pych, Pyrola chlorantha*; *Pyja, P. japonica*; *Pymi, P. minor*; *Pypi, P. picta*; *Pyro, P. rotundifolia*; (REF, $n = 627$).

and unpublished studies on the stable isotope values and N concentration of FMH and PMH species in families Orchidaceae and Ericaceae. For our purposes, we considered full mycoheterotrophy to include achlorophyllous species known to share

EM fungi with trees and that are enriched in both ^{13}C and ^{15}N relative to neighbouring autotrophs. We considered partial mycoheterotrophy to include leafy green species that associate with EM fungi shared with trees and that are enriched in ^{13}C and ^{15}N, or those only significantly enriched in ^{15}N relative to surrounding autotrophs. Even though enrichment in both ^{13}C and ^{15}N provides the clearest indicator of partial mycoheterotrophy, we chose also to include those species only enriched in ^{15}N because there is substantial variation in the ^{13}C enrichment of EM fungi (Mayor *et al.*, 2009), ^{13}C enrichment in some partial mycoheterotrophs may turn out to be too small to be unequivocally identified (Selosse and Martos 2014; Stöckel *et al.*, 2014; Gebauer *et al.*, 2016). The ^{13}C enrichment in fungal tissue, as well as in FMH plants, is always accompanied by enrichment in ^{15}N. Thus, ^{15}N enrichment in plants associated with EM fungi that are not significantly enriched in ^{13}C serves as a substitute to identify organic matter (and thus C gain) from a fungal source. Hynson *et al.* (2013) called these plants 'cryptic mycoheterotrophs'.

To make comparisons across plant populations, we used an isotope enrichment factor approach to normalize the data (Preiss and Gebauer, 2008). With these data we tested the hypotheses that: (1) C and N stable isotope abundances and N concentration would distinguish FMH Orchidaceae from FMH Ericaceae; (2) C and N stable isotope abundances and N concentration would distinguish PMH Orchidaceae from PHM Ericaceae; and (3) similar to previous population-level studies, C and N stable isotopes abundances would differentiate FMH and PMH plants from each other and autotrophic species across multiple populations.

MATERIALS AND METHODS

Data compilation

To test our hypotheses with an exhaustive data set, we conducted a traceable literature search (Koricheva and Gurevitch, 2014) using the web-based search engine Web of Science (Thomson Reuters, 2015) and the key words 'mycoheterotroph*' OR 'myco-heterotroph*' AND 'stable isotope*' on 3 February 2016 that returned 252 hits. Document types were restricted to articles, and duplicates were removed from the retrieved results, limiting the number of hits to 238. Only publications focused on full or partial mycoheterotrophs in the plant families Ericaceae and Orchidaceae with species partnering with EM fungi were included in our study. We analysed the full text of the resulting papers and included only those studies that performed sampling of neighbouring autotrophic reference plant samples together with target plant samples (FMH or PMH) in a suitable manor for enrichment factor calculations, i.e. a replicated plot-wise sampling of FMH or PMH target plants together with closely neighbouring autotrophic reference plants (Preiss and Gebauer, 2008). We identified 21 publications suitable for our study published between 2003 and 2015 and added one further so far unpublished data set (Table 1). We explicitly excluded from our data set investigations for which the sampling design did not allow calculation of enrichment factors (Trudell *et al.*, 2003) or for which C and N isotope abundance was affected by experimental manipulations [shading and trenching (Hynson *et al.*, 2012); fungicide application (Bellino *et al.*, 2014); defoliation (Gonneau *et al.*, 2014)], by

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TABLE 1. *Fully (FMH) and partially mycoheterotrophic (PMH) species of the plant families Ericaceae and Orchidaceae included in this investigation, their numbers of replicates for C and N stable isotope natural abundance (n $\varepsilon^{13}C =$ and n $\varepsilon^{15}N =$) and total N concentration (n N conc. =) and the respective sources where the data were originally published*

Species	Type	$n\varepsilon^{13}$C=	$n\varepsilon^{15}$N=	nN conc.=	Publication
Family: Ericaceae					
Hypopitys monotropa	FMH	38	38	31	Tedersoo *et al.* (2007), Zimmer *et al.* (2007, 2008), Hynson *et al.* (2015), Johansson *et al.* (2015), B. Burghardt & G. Gebauer (unpubl. res.)
Monotropa uniflora	FMH	8	8	8	Ogura-Tsujita *et al.* (2009), Motomura *et al.* (2010)
Pterospora andromedea	FMH	34	34	32	Zimmer *et al.* (2007), Hynson *et al.* (2009)
Pyrola aphylla	FMH	39	39	37	Zimmer *et al.* (2007), Hynson *et al.* (2009)
Sarcodes sanguinea	FMH	15	15	15	Zimmer *et al.* (2007)
FMH Ericaceae		134	134	123	
Chimaphila umbellata	PMH	138	138	132	Tedersoo *et al.* (2007), Zimmer *et al.* (2007) Hynson *et al.* (2009), Johansson *et al.* (2015)
Moneses uniflora	PMH	99	99	99	Hynson *et al.* (2015), Johansson *et al.* (2015)
Orthilia secunda	PMH	140	140	134	Tedersoo *et al.* (2007), Zimmer *et al.* (2007), Liebel *et al.* (2009), Johansson *et al.* (2015)
Pyrola chlorantha	PMH	116	116	110	Tedersoo *et al.* (2007), Zimmer *et al.* (2007), Johansson *et al.* (2015)
Pyrola japonica	PMH	5	5	5	Matsuda *et al.* (2012)
Pyrola minor	PMH	48	48	48	Zimmer *et al.* (2007), Liebel *et al.* (2009), Johansson *et al.* (2015)
Pyrola picta	PMH	54	54	51	Zimmer *et al.* (2007), Hynson *et al.* (2009)
Pyrola rotundifolia	PMH	6	6	0	Tedersoo *et al.* (2007)
PMH Ericaceae		606	606	579	
Total Ericaceae		740	740	702	
Family: Orchidaceae					
Aphyllorchis caudata	FMH	3	3	3	Roy *et al.* (2009)
Aphyllorchis montana	FMH	4	4	4	Roy *et al.* (2009)
Cephalanthera damasonium albino	FMH	10	10	10	Julou *et al.* (2005)
Cephalanthera exigua	FMH	5	5	5	Roy *et al.* (2009)
Cephalanthera longifolia albino	FMH	9	9	9	Abadie *et al.* (2006)
Corallorhiza maculata	FMH	15	15	15	Zimmer *et al.* (2007), Hynson *et al.* (2009)
Corallorhiza striata	FMH	3	3	3	Hynson *et al.* (2015)
Cymbidium aberrans	FMH	3	3	3	Motomura *et al.* (2010)
Cymbidium macrorhizon	FMH	6	6	6	Motomura *et al.* (2010)
Epipogium aphyllum	FMH	8	8	8	Liebel and Gebauer (2011), Hynson *et al.* (2015)
Lecanorchis nigricans	FMH	3	3	3	Motomura *et al.* (2010)
Lecanorchis thalassica	FMH	5	5	5	Lee *et al.* (2015)
Neottia nidus-avis	FMH	52	52	38	Gebauer and Meyer (2003), Bidartondo *et al.* (2004), Zimmer *et al.* (2007), Zimmer *et al.* (2008), Liebel *et al.* (2010), Preiss *et al.* (2010), Stöckel *et al.* (2014)
FMH Orchidaceae		126	126	112	
Cephalanthera damasonium	PMH	39	43	39	Gebauer and Meyer (2003), Bidartondo *et al.* (2004) Julou *et al.* (2005), Liebel *et al.* (2010), Preiss *et al.* (2010)
Cephalanthera erecta	PMH	3	3	3	Motomura *et al.* (2010)
Cephalanthera longifolia	PMH	42	42	42	Abadie *et al.* (2006), Liebel *et al.* (2010), Johansson et al. (2015)
Cephalanthera rubra	PMH	25	25	25	Gebauer and Meyer (2003), Bidartondo *et al.* (2004), Preiss *et al.* (2010)
Corallorhiza trifida	PMH	9	9	4	Zimmer *et al.* (2008)
Cymbidium goeringii	PMH	7	7	7	Motomura *et al.* (2010)
Cymbidium lancifolium	PMH	6	6	6	Motomura *et al.* (2010)
Epipactis atrorubens	PMH	11	11	11	Gebauer and Meyer (2003), Bidartondo *et al.* (2004), Tedersoo *et al.* (2007)
Epipactis distans	PMH	4	4	4	Bidartondo *et al.* (2004)
Epipactis helleborine	PMH	21	21	21	Gebauer and Meyer (2003), Bidartondo *et al.* (2004), Abadie *et al.* (2006), Liebel *et al.* (2010), Johansson et al. (2015)
Epipactis leptochila	PMH	4	4	4	B. Burghardt and G. Gebauer (unpubl. res.)
Limodorum abortivum	PMH	10	14	14	Gebauer and Meyer (2003), Liebel *et al.* (2010)
Limodorum trabutianum	PMH	5	5	5	Liebel *et al.* (2010)
Platanthera minor	PMH	3	3	3	Yagame *et al.* (2012)
PMH Orchidaceae		189	197	188	
Total Orchidaceae		315	323	300	

investigation of chlorophyll concentration gradients (Stöckel *et al.*, 2011) or by investigation of different developmental stages (Roy *et al.*, 2013; Gonneau *et al.*, 2014). We did include data from mutant achlorophyllous (albino) orchids that are fully mycoheterotrophic. Data on C and N stable isotope natural abundances as well as total N concentrations in leaf or stem tissues of FMH and PMH species in the plant families Ericaceae

and Orchidaceae known to partner with EM fungi were either directly extracted from the original publications or were obtained by personal contact with the respective authors. Specifically, unpublished data on plant N concentrations from the investigations by Bidartondo *et al.* (2004), Zimmer *et al.* (2007, 2008), Hynson *et al.* (2009, 2015), Liebel *et al.* (2009), Motomura *et al.* (2010), Preiss *et al.* (2010), Yagame *et al.*

FIG. 2. Single values for enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and nitrogen concentrations (mmol g d. wt^{-1}) of (A) fully mycoheterotrophic (FMH) Orchidaceae and Ericaceae and the respective photosynthetic reference plants (REF, n = 804) and (B) partially mycoheterotrophic (PMH) Orchidaceaeand Ericaceae associated with fungi forming ectomycorrhizas and the respective photosynthetic reference plants (REF, n = 1191).

^{13}C and ^{15}N abundances for full mycoheterotrophs, 795 data points for ^{13}C and 803 data points for ^{15}N abundances for partial mycoheterotrophs and 1433 data points for ^{13}C and 1461 data points for ^{15}N abundances for neighbouring autotrophic references (Figs 2 and 3). Nitrogen concentration data were only available for a reduced data set of 235 data points for full mycoheterotrophs, 767 for partial mycoheterotrophs and 1355 for autotrophic references (Figs 2 and 3). For non-mycoheterotrophic Ericaceae within the autotrophic reference species, 118 and 126 data points were available for ^{13}C and ^{15}N abundances, respectively, and 111 data points for N concentration data.

Data treatment and statistical analysis

To enable comparisons of C and N stable isotope abundances across populations, between species and at the familiar level, we used an isotope enrichment factor approach to normalize the data. If isotope abundance data were published as δ values, normalized enrichment factors (ε) were calculated as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single value of an autotrophic, a PMH or FMH plant, and δ_{REF} is the mean value of all autotrophic reference plants by plot (Preiss and Gebauer, 2008). Some of the N concentration data were published as percentage nitrogen content (%N). To unify N concentration, these data were converted into millimoles of nitrogen per gram dry weight (mmol N g d.wt^{-1}).

We tested for differences between the groups FMH Orchidaceae, FMH Ericaceae, PMH Orchidaceae, PMH Ericaceae and corresponding autotrophic reference plants' isotopic enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) and N concentrations with non-parametric statistics due to non-normally distributed data using the Kruskal–Wallis H-test in combination with a post-hoc Mann–Whitney U-test for multiple comparisons. P-values were adjusted using the sequential Bonferroni correction (Holm, 1979). To account for different sample sizes in pairwise comparisons and to standardize for the magnitude of an observed effect we calculated Cohen's d effect size with:

$$d = (\bar{x}_{group1} - \bar{x}_{group2})/\sigma_{pooled}$$

and

$$\sigma_{pooled} = \left[\left(\sigma^2_{group1} + \sigma^2_{group2} \right)/2 \right] \times 0{\cdot}5^{-1}$$

where $\bar{x}$ is the group mean and σ the groups' standard deviations (Cohen, 1988). Effect sizes >0·8 are considered as large (Cohen, 1992). Variance, v_d, of the effect size d was calculated using:

$$v_d = \left(\sigma^2_{group1}/n_1 \right) + \left(\sigma^2_{group2}/n_2 \right)$$

where n is the groups' sample size (Borenstein et al., 2009). The same statistical tools were used to test for differences in enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) and N concentrations between the groups FMH, PMH and autotrophic Ericaceae and the remaining reference plants.

(2012) and Johansson et al. (2015) were kindly made available by the authors. Unpublished data on N stable isotope abundance were supplied by Preiss et al. (2010). Furthermore, B. Burghardt and G. Gebauer provided unpublished C and N stable isotope abundance and N concentration data on *Hypopitys monotropa* and *Epipactis leptochila* (Godfrey) Godfrey.

Thus, in total, we compiled C and N stable isotope abundance and N concentration data from 22 studies for a total of 18 FMH species, 22 PMH species and 156 of their neighbouring autotrophic reference species, of which 11 species were non-mycoheterotrophic Ericaceae (Table 1). We did not include any green orchids that partner with rhizoctonia fungi as references because all orchids are initially mycoheterotrophic in their germination stages. Data collection resulted in 260 data points for

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FIG. 3. Box-and-whisker plots and summary statistics for the compiled data sets on FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references in (A) enrichment factor $\varepsilon^{13}C$, (B) enrichment factor $\varepsilon^{15}N$ and (C) nitrogen concentration. The box spans the first and third quartile, while the horizontal line in the box represents the median; whiskers extend to data extremes. Different letters indicate significant differences between the groups.

We used non-metric multidimensional scaling (NMDS) to visualize the organization of samples in two-dimensional space graphically, whereas their spatial arrangement exactly represents the similarity between the objects. For this, the Bray–Curtis index was used to calculate distance matrices from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data using the function 'metaMDS' with two dimensions and 100 permutations in the R package 'vegan' (Oksanen *et al*., 2015). Stress values were calculated to

evaluate how well the configuration provides a representation of the distance matrices; generally, a stress value <0·05 provides an excellent representation in reduced dimensions. Fitted vectors were calculated to display the response variables $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration in the ordination space and to indicate the differences between the groups in association with these variables (Fig. 4). Each arrow shows the direction of the increasing response variable while its length is proportional to the correlation (R^2) between the variable and the ordination (Fig. 4, Oksanen *et al*., 2015).

The function 'adonis' in the R package 'vegan' was used to perform a permutational MANOVA (multivariate analysis of variance) to test for significance of differences between group means using the aforementioned calculated distance matrices (Anderson, 2001). *P*-values from multiple pairwise comparisons were adjusted (P_{adj}) using the sequential Bonferroni correction. For statistical analyses, we used the software environment R [version 3.1.2 (Pumpkin Helmet); R Development Core Team, 2014)] supported by the add-on packages 'coin' (version 1.0-24; Hothorn *et al*., 2006, 2008*a*), 'multcomp' (version 1.3-8; Hothorn *et al*., 2008*b*), 'scattergrid' (version 1.0; Gassem, 2015), 'scatterplot3d' (version 0.3-35; Ligges and Mächler, 2003) and 'vegan' (version 2.2-1; Oksanen *et al*., 2015) with a significance level of $\alpha = 0\cdot05$.

RESULTS

Based on comparisons of FMH and PMH Orchidaceae and Ericaceae, significant patterns have emerged (Fig. 3; Supplementary Data Table S2). Differences among our groups in ^{13}C enrichment (ε) found support for all three of our hypotheses (Fig. 3A; Table S2). In support of hypotheses (1) and (2), FMH and PMH Orchidaceae were on average significantly more enriched in ^{13}C than FMH and PMH Ericaceae (Fig. 3A; Table 2). In support of hypothesis (3) and as anticipated from previous plant population studies, autotrophic reference species were less enriched in ^{13}C relative to PMH species from both families, which were less enriched in ^{13}C than all FMH species (Fig. 3A; Table 2). A comparison of FMH and PMH Ericaceae with autotrophic Ericaceae from the reference plant group confirms these findings. Autotrophic Ericaceae were significantly depleted in ^{13}C compared with FMH and PMH Ericeaceae and even more depleted in ^{13}C than the remaining reference plants (Supplementary Data Fig. S1A; Table S1). Congruent with our non-parametric comparisons, effect sizes for $\varepsilon^{13}C$ among groups were high (Table 3), especially so for FMH Orchidaceae and Ericaceae relative to autotrophic references ($d = 5\cdot979$ and $4\cdot463$, respectively). Interestingly, $\varepsilon^{13}C$ values of PMH Orchidaceae had a higher scatter than all other groups (Figs 2B and 3A) despite this variation in the data, *P*-values and effect sizes between this group, autotrophic references, FMH species and PMH Ericaceae were significant and high. The more tightly clustered $\varepsilon^{13}C$ values of PMH Ericaceae varied little based on effect size from references ($d = 0\cdot229$), while being statistically distinguishable from references based on our non-parametric test ($P_{\mathrm{adj}} < 0\cdot001$).
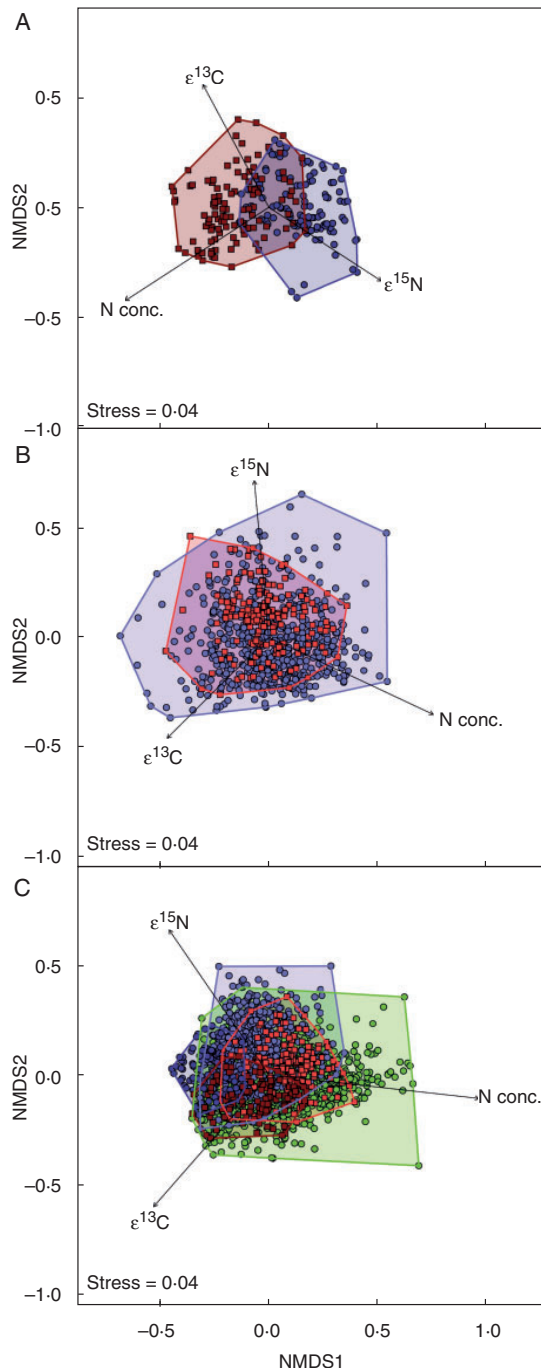
FIG. 4. NMDS plots visualize Bray–Curtis dissimilarity matrices calculated from enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and nitrogen concentration data in two-dimensional space. Fitted vectors display the response variables $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration in the ordination space and indicate the differences between the groups in association with these variables. (A) FMH Ericaceae and Orchidaceae, stress = 0·04, 100 permutations; MANOVA $R^2 = 0.185$, $P < 0.001$; (B) PMH Ericaceae and Orchidaceae, stress = 0·04, 100 permutations; MANOVA $R^2 = 0.22$, $P < 0.001$; and (C) FMH Ericaceae, FMH Orchidaceae, PMH Ericaceae, PMH Orchidaceae and respective autotrophic references, stress = 0·04, 100 permutations; MANOVA $R^2 = 0.678$, $P_{adj} < 0.001$.

From comparisons of ^{15}N enrichment among our study groups, a similar pattern emerges where we found support for all three of our hypotheses (Fig. 3B; Table S2). However, in contrast to patterns of ^{13}C enrichment, FMH Ericaceae were significantly more enriched in ^{15}N relative to FMH Orchidacae ($P_{adj} = 0.005$; Fig. 3B; Table 2). Similar to ^{13}C, PMH Orchidaceae were significantly more enriched in ^{15}N relative to PMH Ericaceae ($P_{adj} < 0.001$; Fig. 3B; Table 2). All groups were significantly more enriched in ^{15}N relative to references, and full mycoheterotrophs were significantly more enriched in ^{15}N than partial mycoheterotrophs (Figs 2 and 3B; Table 2). Effect sizes among all groups were also high (Table 3), especially so for FMH Orchidacae and Ericaceae vs. references ($d = 4.866$ and 4.711, respectively). However, based on our non-parametric test, FMH Orchidaceae were significantly more enriched in ^{15}N relative to PMH Orchidaceae ($P_{adj} < 0.001$), effect size between these two groups was medium ($d = 0.480$). Autotrophic Ericaceae were significantly depleted in ^{15}N compared with FMH and PMH Ericaceae and only slightly enriched in ^{15}N compared with the remaining reference plants (Supplementary Data Fig. S1B; Table S1).

While N concentration data werenot available for all species for which we had stable isotope profiles (Fig. 3C; Table S2), we were still able to detect significant differences among groups. We found support for our first hypothesis where FMH Ericaceae had, on average, lower N concentrations relative to FMH Orchidaceae ($P_{adj} < 0.001$; Fig. 3C; Table 2). We also found support for hypothesis (2) where PMH Orchidaceae had significantly higher N concentrations relative to PMH Ericaceae ($P_{adj} < 0.001$; Fig. 3C; Table 2). However, we did not find significant differences between the N concentrations of FMH and PMH Ericaceae ($P_{adj} = 1.0$) or FMH and PMH Orchidaceae ($P_{adj} = 1.0$) (Fig. 3C; Table 2). The effect sizes for differences in N concentration lend further support to hypotheses (1) and (2) where comparisons between FMH Ericaceae and Orchidaceae and partial mycoheterotrophs in both families were high (Table 3). In general, mean N concentrations in FMH and PMH Orchidaceae were about twice as high as in reference plants (Fig. 3C; Table 2) while mean N concentrations in FMH and PMH Ericaceae were, on average, lower than in reference plants (Fig. 3C; Table 2). Furthermore, N concentrations of autotrophic Ericaceae among the reference plants which included both arbutoid and ericoid mycorrhizal species were lower than those found for FMH and PMH Ericaceae (Supplementary Data Fig. S1C; Table S1).

Ordination of a Bray–Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data of FMH Orchidaceae and Ericaceae with NMDS supports hypothesis (1) as the groups are segregated in ordination space (Fig. 4A), and a MANOVA showed a significant effect of group on the ordination ($R^2 = 0.185$, $P = 0.001$). Fitted vectors in the ordination of FMH Orchidaceae and Ericaceae were maximally correlated with N concentration ($R^2 = 0.821$, $P < 0.001$), $\varepsilon^{15}N$ ($R^2 = 0.500$, $P < 0.001$) and $\varepsilon^{13}C$ ($R^2 = 0.493$, $P < 0.001$). NMDS for PMH Orchidaceae and Ericaceae (Fig. 4B) also supports hypothesis (2) as a MANOVA revealed a significant effect of group on the ordination ($R^2 = 0.220$, $P = 0.001$). Here, fitted vectors in the ordination of PMH

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TABLE 2. *Results from post-hoc pairwise comparisons between the groups FMH Orchidaceae, FMH Ericaceae, PMH Orchidaceae, PMH Ericaceae and autotrophic references with the non-parametric Mann–Whitney U-test after significant Kruskal–Wallis H-test* ($\varepsilon^{13}C$: H = 933·705, d.f. = 4, P < 0·001; $\varepsilon^{15}N$: H = 1793·556, d.f. = 4, P < 0·001; N concentration: H = 574·618, d.f. = 4, P < 0·001)

	FMH Ericaceae		FMH Orchidaceae		PMH Ericaceae		PMH Orchidaceae	
	U	P_{adj}	U	P_{adj}	U	P_{adj}	U	P_{adj}
$\varepsilon^{13}C$								
FMH Orchidaceae	3409	<0·001						
PMH Ericaceae	79 505	<0·001	75 590	<0·001				
PMH Orchidaceae	21 339	<0·001	22 553·5	<0·001	17 892·5	<0·001		
REF	191 188	<0·001	180 511	< 0·001	479 972·5	<0·001	242 162·5	<0·001
$\varepsilon^{15}N$								
FMH Orchidaceae	10 557·5	<0·001						
PMH Ericaceae	74 952	<0·001	67 571	<0·001				
PMH Orchidaceae	19 772	<0·001	16 479	<0·001	27 974	<0·001		
REF	195 774	<0·001	184 069	<0·001	849 267	<0·001	285 895	<0·001
N concentration								
FMH Orchidaceae	1245	<0·001						
PMH Ericaceae	36 479	1·0	59 398	<0·001				
PMH Orchidaceae	700	<0·001	10 458	1·0	3581	<0·001		
REF	72 306·5	0·1	131 120	<0·001	334 105	<0·001	234 909·5	<0·001

P-values were adjusted using the sequential Bonferroni-correction.

TABLE 3. *Results of Cohen's* d *effect size and variance* v_d *calculations for* $\varepsilon^{13}C$, $\varepsilon^{15}N$ *and N concentration in FMH Ericaceae, FMH Orchidaceae, PMH Ericaceae, PMH Orchidaceae and autotrophic references*

	FMH Ericaceae		FMH Orchidaceae		PMH Ericaceae		PMH Orchidaceae	
	d	v_d	d	v_d	d	v_d	d	v_d
$\varepsilon^{13}C$								
FMH Orchidaceae	1·246	0·023						
PMH Ericaceae	3·775	0·014	5·091	0·014				
PMH Orchidaceae	1·481	0·024	2·434	0·024	1·394	0·015		
REF	4·463	0·012	5·979	0·012	0·229	0·004	1·652	0·014
$\varepsilon^{15}N$								
FMH Orchidaceae	0·570	0·053						
PMH Ericaceae	2·173	0·034	1·817	0·030				
PMH Orchidaceae	0·911	0·052	0·480	0·047	0·976	0·029		
REF	4·711	0·029	4·866	0·025	2·281	0·006	2·851	0·024
N concentration								
FMH Orchidaceae	1·930	0·011						
PMH Ericaceae	0·040	0·004	1·953	0·009				
PMH Orchidaceae	2·314	0·007	0·023	0·012	2·364	0·004		
REF	0·321	0·004	1·598	0·092	0·304	0·001	1·875	0·004

Orchidaceae and Ericaceae were maximally correlated with $\varepsilon^{15}N$ ($R^2 = 0.424$, $P < 0.001$), N concentration ($R^2 = 0.313$, $P < 0.001$) and $\varepsilon^{13}C$ ($R^2 = 0.261$, $P < 0.001$). An ordination of a Bray–Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data of FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references with NMDS (Fig. 4C) supports hypothesis (3) through the distinct clustering of the groups (PMH, FMH and autotrophic species) in the ordination space, and a MANOVA showed a significant effect of group on the ordination ($R^2 = 0.678$, $P_{adj} = 0.001$). Here, fitted vectors in the ordination of FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references were maximally correlated with N concentration ($R^2 = 0.442$, $P < 0.001$), $\varepsilon^{15}N$ ($R^2 = 0.306$, $P < 0.001$) and $\varepsilon^{13}C$ ($R^2 = 0.301$, $P < 0.001$). Generally, the stress values of all ordinations provide an excellent representation in reduced dimensions (Fig. 4).

DISCUSSION

Overall patterns of nitrogen concentrations and stable isotope enrichment among partially and fully mycoheterotrophic plants and autotrophs

By assembling all available data sets of mycoheterotrophic species, we have confirmed that with an increasing dependency on fungal nutrition, there is a corresponding increase in N, and especially C isotope enrichment (Figs 1–3). Previous studies from plant populations have found similar patterns in isotope abundances where autotrophs, partial mycoheterotrophs and full mycoheterotrophs fit the theoretical principles of isotope enrichment along a food chain (Fry, 2006). However, we now find that this pattern holds across a much larger sample size assembled from study sites across the globe. Furthermore, by synthesizing the data from this extensive sampling, additional patterns have emerged. Despite depending upon the same

functional guild of fungi, FMH and PMH Orchidaceae and Ericaceae associated with EM fungi behave isotopically dissimilarly. Also, the N concentration turns out to be an additional and critical factor to consider when examining the ecophysiology of putatively mycoheterotrophic taxa within these two plant families.

Drivers of nitrogen concentrations among plant families and trophic groups

Why are N concentrations so much higher in FMH and PMH orchids than in corresponding Ericaceae? One prediction might be that the physiology or substrate use of the EM fungi that partner with orchids differs from those that partner with ericaceous species (*sensu* Gebauer and Taylor, 1999; Taylor *et al.*, 2003). This prediction is certainly worth investigating. However, the fact that there is substantial overlap in fungal partnerships among some species of mycoheterotrophic Orchidaceae and Ericaceae (e.g. Russulaceae spp. host both FMH orchids and ericaceous species), it seems to be the least likely explanatory factor. A potentially more important factor is differences in the anatomy and physiology of orchid vs. ericaceous mycorrhizae. When EM fungi colonize orchid protocorms or roots, they form intracellular pelotons (Burgeff, 1959). These pelotons are digested by the orchid, which probably uses the fungal biomass for its own growth (Bougoure *et al.*, 2014). However, the relative flux of compounds from fungi to orchids via peloton digestion vs. active fungus–plant membrane transport is currently an unresolved question [see contradictory findings by Bougoure *et al.* (2014) and Kuga *et al.* (2014)]. Conversely, EM fungi associating with PMH or FMH Ericaceae form either monotropoid or arbutoid mycorrhizal structures (Smith and Read, 2008). While the exact functions of these structures are unknown (Smith and Read, 2008; Imhof *et al.*, 2013, and references therein), ericaceous mycoheterotrophic species may rely more on active membrane transport of fungal compounds rather than mass flow, where the former probably represents a much more selective system. Because EM fungi have much higher N concentrations in their tissues than autotrophic plants from identical habitats (Gebauer and Dietrich, 1993; Gebauer and Taylor, 1999), high N concentration among mycoheterotrophic Orchidaceae may be largely due to differences in N transport mechanisms, where mass flow of N via digestion of fungal tissue would lead to an increased N concentration in orchids compared with other species (Tedersoo *et al.*, 2007; Stöckel *et al.*, 2014). However, to date, explicit tests of the relative contributions of fungal compounds by mass flow vs. active membrane transport to mycoheterotrophic Orchidaceae or Ericaceae are mostly lacking.

Differences in the life history strategies of orchids and ericaceous species may also contribute to explaining differences in their N concentrations. The majority of PMH EM-associated orchid species are deciduous, while PMH Ericaceae are evergreen and sclerophyllous. In general, evergreen sclerophyllous plant tissues tend to have lower N concentrations than deciduous tissues (Gebauer *et al.*, 1988). In our analyses, many more deciduous species than evergreen species served as reference plants, so mean N concentrations in PMH Ericaceae significantly lower than in reference plants may be explained by these morphological differences. Despite their perennial nature as geophytes that is more similar to orchids, the maintenance of low N concentrations in FMH Ericaceae points towards plant evolutionary history rather than trophic strategy as a determinant of N concentrations. However, N concentrations in FMH and PMH Orchidaceae twice as high as in reference plants cannot be explained exclusively by their perennial nature or evolutionary history.

Drivers of carbon and nitrogen stable isotope enrichment among plant families and trophic groups

Carbon isotope abundance in plant bulk tissues is mainly driven by three factors: the type of photosynthetic pathway (C_3, C_4 or CAM), stomatal regulation and origin of the carbon source. Since no CAM orchids are included in our data set, all investigated target plants (Orchidaceae and Ericaceae) are either C_3 or are non-photosynthetic full mycoheterotrophs. Thus, differences in ^{13}C discrimination among photosynthetic pathways can be ruled out as a driver for the differences in carbon isotope abundance patterns observed here. A decrease in stomatal conductance of C_3 plants shifts their carbon isotope abundances towards ^{13}C enrichment (Farquhar *et al.*, 1989). Thus, one might assume that low stomatal conductance is a factor contributing to the overall ^{13}C enrichment of PMH and FMH plants as well as the differences observed between the plant families Orchidaceae and Ericaceae. However, the patterns observed here of ^{13}C depletion in evergreen sclerophyllous PMH Ericaceae relative to deciduous PMH Orchidaceae do not fit with the general tendency of sclerophyllous plants towards lower stomatal conductance and therefore greater ^{13}C enrichment (Larcher, 2003). Furthermore, for non-photosynthetic FMH albino individuals of the orchid *Cephalanthera damasonium*, a significantly higher stomatal conductance and simultaneously higher ^{13}C enrichment than in PMH individuals has been found (Julou *et al.*, 2005; Roy *et al.*, 2013). Consequently, systematic differences in stomatal conductance are also unlikely to be responsible for the differences in ^{13}C enrichment found for FMH and PMH plants of the Orchidaceae and Ericaceae. Thus, the origin of the carbon source remains as the most likely factor responsible for the general ^{13}C enrichment of FMH and PHM orchids and ericaceous species in relation to their reference plants and each other.

For FMH plants, all of their carbon originates from the fungal source. Therefore, differences in the ^{13}C enrichment of fungi that serve as carbon sources for FMH Orchidaceae and Ericaceae are probably responsible for their relative ^{13}C enrichment. The greater ^{13}C enrichment found on average in FMH Orchidaceae relative to Ericaceae may be due to differences in the biochemical make-up of tissues (*sensu* Gebauer and Schulze, 1991; Badeck *et al.*, 2005; Cernusak *et al.*, 2009) or, again, possibly due to greater relative fungal C contributions from the digestion of pelotons entailing little ^{13}C discrimination, as opposed to active C transport which discriminates against ^{13}C.

For PMH plants, the situation is more complex, because they are composed of C from two different origins, atmospheric CO_2

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gained through C_3 photosynthesis and organic matter from the fungal source, and the ratios of these two sources can vary based on environmental factors. For example, light availability has been shown to be an important determinant for the ^{13}C enrichment of some PMH orchids and at least one PMH ericaceous species (Preiss *et al.*, 2010; Matsuda *et al.*, 2012). These studies found that as light becomes more limiting, some partial mycoheterotrophs increase their dependency on ^{13}C-enriched fungal C. So, if some of the PMH species included in this study were collected in different light environments, this could have led to significant differences in their $\varepsilon^{13}C$ values. Another example is the leafless, but still stem-chlorophyllous PMH orchid *Corallorhiza trifida*. This species is significantly more enriched in ^{13}C relative to other PMH orchids, while it is less enriched in ^{15}N (Fig. 1B). There has been some debate in the literature regarding the ability of *C. trifida* to gain significant amounts of carbon through photosynthesis (Zimmer *et al.*, 2008; Cameron *et al.*, 2009), so, while we include this species among the partial mycoheterotrophs, it may actually be more similar to FMH orchids.

Similar to FMH species, the identity of fungal symbionts associating with partial mycoheterotrophs and differences in their C substrate use cannot be completely ruled out as a possible additional factor affecting partial mycoheterotrophs' ^{13}C enrichment. However, partial mycoheterotrophs studied thus far tend to associate with a diversity of EM fungi and there is substantial overlap in the fungal taxa known to partner with PMH Orchidaceae and Ericaceae. Until future studies determine whether all or a sub-set of these partners are responsible for mycoheterotrophic C gains, there are no grounds to assume that differences in fungal partner identities are leading to differences in ^{13}C enrichment between partial mycoheterotrophs in these families.

Nitrogen isotope abundance in plant tissue integrates the isotopic composition of the various N sources utilized by a plant (Robinson, 2001). From our investigations ^{15}N enrichment of EM-associated FMH and PMH Orchidaceae and Ericaceae in comparison with reference plants indicates the utilization of different N sources by FMH and PMH plants relative to autotrophic plants. Ectomycorrhizal fungi can be highly variable in their ^{15}N enrichment (Taylor *et al.*, 2003; Hobbie *et al.*, 2005; Mayor *et al.*, 2009), because of a wide range of soil nutrient mining and catabolic abilities among genera (and sometimes species) of fungi (Gebauer and Taylor, 1999; Emmerton *et al.*, 2001; Taylor *et al.*, 2004; Pritsch and Garbaye, 2011). Similar to differences in ^{13}C enrichment, interactions with different fungal hosts that differ in their N acquisition strategies and differences in the physiology of the fungus–plant matter exchange may explain some, but not all, of the significant interfamilial and interspecific variations in ^{15}N enrichment among FMH and PMH Ericaceae and Orchidaceae. However, future investigations of two outlier species among the PMH Orchidaceae are needed; *Epipactis distans* and *E. leptochila* are significantly more enriched in ^{15}N than all other PMH species (Fig. 1B). Interestingly, these two species are exclusively associated with EM Ascomycetes (J.M.-I. Schiebold, unpubl. data). Future investigation should test whether above-average ^{15}N enrichment among PMH Orchidaceae is related to the ^{15}N enrichment of EM Ascomycetes. Furthermore, it remains unknown why species that partner with closely related fungi (e.g. *Sarcodes*

sanguinea and *Pterospora andromedea*) and grow in sympatry exhibit such significant differences in ^{15}N enrichment (Fig. 1B). These species provide a potentially fruitful study system for examining the ecology of mycoheterotrophy; specifically niche partitioning through differences in ecophysiological traits.

Future directions

Given that we have confirmed a general isotope food chain model for FMH and PMH species across a large data set and geographic sampling area, while also finding that there are significant differences among plant families that occupy the same trophic position, how should future studies progress? We suggest that future studies should focus on identifying the physiological mechanisms leading to differences between mycoheterotrophic orchids and ericaceous species that associate with similar guilds of fungi. Similarly, future investigations should attempt to identify mechanisms leading to interspecific differences in isotope enrichment within the same plant family and trophic groups. Mycoheterotrophic species that partner with similar fungi and grow in sympatry, but have disparate $\varepsilon^{13}C$ and $\varepsilon^{15}N$ values, provide ideal study systems. Furthermore, until we have a better understanding of why these familial differences exist, future population studies of putative partial mycoheterotrophs that use a mixing model approach to identify the degree of partial mycoheterotrophy should only use FMH species from the same family as the FMH end-member (e.g. Tedersoo *et al.*, 2007).

Adding N concentration as an additional explanatory variable will aid future research in distinguishing differences among full and partial mycoheterotrophs and autotrophs. When N concentrations along with $\varepsilon^{15}N$ and $\varepsilon^{13}C$ values were incorporated into an ordination of a Bray–Curtis dissimilarity matrix with NMDS, MANOVA showed a significant effect of group on the ordination ($P_{adj} = 0.001$) and these three factors combined explain approx. 68 % of the variation in the data set (Fig. 4). Similar models that segregated FMH Orchidaceae from Ericaceae and did the same for partial mycoheterotrophs for each family found significant differences between plant families, but the three factors only explained about 19 and 22 % of the variation in the data sets, respectively (Fig. 4A, B). Therefore, future investigations should consider measurements of additional explanatory response variables. For instance, analysis of concentrations and stable isotope abundances of additional elements involved in organic matter exchange such as hydrogen, oxygen or sulphur, may prove informative for teasing apart the dependency of mycoheterotrophic plants on fungal-derived organic matter (Gebauer *et al.*, 2016). Also, studies that identify the C and N compounds and transfer pathways among different types of mycoheterotrophic plants are urgently needed.

Finally, much of the intra- and interspecific variation in N concentrations, and ^{13}C and ^{15}N enrichment of partial mycoheterotrophs is probably due to the environment in which these plants are subsisting (Preiss *et al.*, 2010; Hynson *et al.*, 2012; Matsuda *et al.*, 2012). So, measurements of stable isotope composition throughout the life cycle of individual plants and over time within adult plants could add valuable explanatory power

to these models, as would data on light environment, leaf chlorophyll concentrations, and plant–water and plant–nutrient relations (Preiss *et al.*, 2010; Stöckel *et al.*, 2011; Hynson *et al.*, 2012; Matsuda *et al.*, 2012).

Conclusions

In summary, we have found that measurements of C and N stable isotope abundances are able to distinguish mycoheterotrophic Ericaceae from mycoheterotrophic Orchidaceae and confirmed that isotopic differences among partial and full mycoheterotrophs and autotrophs hold across plant populations and are geographically widespread. Furthermore, N concentration in tissues of Orchidaceae and Ericaceae turned out to be an additional and hitherto insufficiently considered factor differentiating these two plant families. Though different identities of fungal hosts cannot be ruled out as factors contributing to the differences in C and N isotopic composition and N concentration between FMH and PMH Orchidaceae and Ericaceae, family- or species-specific characteristics in the physiology of matter exchange between fungi and plants are considered as the most likely reasons underlying the observed differences.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: box-and-whisker plots and summary statistics for the compiled data sets on FMH Ericaceae, PMH Ericaceae, autotrophic Ericaceae among the references and the remaining autotrophic references in enrichment factor ε^{13}C, enrichment factor ε^{15}N and nitrogen concentration. Table S1: (A) Results from post-hoc pairwise comparisons between the groups of FMH Ericaceae, PMH Ericaceae, autotrophic Ericaceae among the references and the remaining autotrophic references. (B) Results from effect-size calculations. Table S2: mean enrichment factors ε^{13}C and ε^{15}N, mean nitrogen concentration data, standard deviation and number of samples for each fully mycoheterotrophic and partially mycoheterotrophic Ericaceae and Orchidaceae species.

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Suppl. Table 1: A) Results from *post hoc* pairwise comparisons between the groups FMH Ericaceae, PMH Ericaceae, autotrophic Ericaceae among the references and remaining autotrophic references (REF) with the non-parametric Mann-Whitney U-test after significant Kruskal-Wallis *H*-test (ε^{13}C: H = 396.900, df = 3, P < 0.001; ε^{15}N: H = 1365.375, df = 3, P < 0.001; N concentration: H = 70.596, df = 3, P < 0.001). P-values were adjusted using the sequential Bonferroni-correction. B) Results from effect-size calculations.

A)

ε^{13}C	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	< 0.001	-	-
autotrophic Ericaceae	< 0.001	< 0.001	-
remaining REF	< 0.001	0.002	< 0.001

ε^{15}N	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	< 0.001	-	-
autotrophic Ericaceae	< 0.001	< 0.001	-
remaining REF	< 0.001	< 0.001	< 0.001

N concentration	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	1.000	-	-
autotrophic Ericaceae	0.027	< 0.001	-
remaining REF	0.010	< 0.001	< 0.001

B)

ε^{13}C	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	3.544	-	-
autotrophic Ericaceae	4.574	0.524	-
remaining REF	4.140	0.205	0.366

ε^{15}N	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	2.138	-	-
autotrophic Ericaceae	4.396	2.065	-
remaining REF	4.590	2.295	0.419

N concentration	FMH Ericaceae	PMH Ericaceae	autotrophic Ericaceae
PMH Ericaceae	0.040	-	-
autotrophic Ericaceae	0.303	0.352	-
remaining REF	0.381	0.358	0.622

Suppl. Table 2: Mean enrichment factors ε^{15}C and ε^{15}N , mean nitrogen concentration data [mmol g$_{dw}^{-1}$], standard deviation (SD) and number of samples (n) for each fully mycohetero-trophic (FMH) and partially myco-heterotrophic (PMH) Ericaceae and Orchidaceae species.

	Species		ε^{15}N [‰]	ε^{13}C [‰]	N conc. [mmol g$_{dw}^{-1}$]
Ericaceae					
FMH	*Hypopitys monotropa*	mean	12.57	6.80	1.07
		SD	2.67	1.71	0.23
		n	38	38	31
FMH	*Monotropa uniflora*	mean	11.34	7.28	1.08
		SD	1.73	0.67	0.18
		n	8	8	8
FMH	*Pterospora andromedea*	mean	9.45	4.82	1.37
		SD	1.24	1.62	0.27
		n	34	34	32
FMH	*Pyrola aphylla*	mean	17.80	6.92	1.47
		SD	2.74	0.86	0.39
		n	39	39	37
FMH	*Sarcodes sanguinea*	mean	14.42	5.16	0.68
		SD	1.98	1.59	0.15
		n	15	15	15
PMH	*Chimaphila umbellata*	mean	5.41	-0.56	1.08
		SD	2.51	1.41	0.31
		n	138	138	132
PMH	*Moneses uniflora*	mean	2.53	-0.36	1.60
		SD	1.33	1.58	0.42
		n	99	99	99
PMH	*Orthilia secunda*	mean	5.14	1.28	1.05
		SD	2.68	1.46	0.26
		n	140	140	134
PMH	*Pyrola chlorantha*	mean	7.91	0.68	1.25
		SD	2.76	1.21	0.26
		n	116	116	110
PMH	*Pyrola japonica*	mean	10.96	3.12	1.41
		SD	1.38	1.31	0.15
		n	5	5	5

Suppl. Table 2 (continued)

	Species		$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]	N conc. [mmol g_{dw}^{-1}]
PMH	*Pyrola minor*	mean	3.36	-0.13	1.24
		SD	1.25	0.74	0.25
		n	48	48	48
PMH	*Pyrola picta*	mean	10.38	0.38	1.31
		SD	1.91	1.52	0.28
		n	54	54	51
PMH	*Pyrola rotundifolia*	mean	9.64	3.56	NA
		SD	0.73	0.45	NA
		n	6	6	0

Orchidaceae

	Species		$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]	N conc. [mmol g_{dw}^{-1}]
FMH	*Aphyllorchis caudata*	mean	7.20	8.36	1.95
		SD	2.26	0.04	1.04
		n	3	3	3
FMH	*Aphyllorchis montana*	mean	10.86	10.34	2.63
		SD	0.64	0.50	0.52
		n	4	4	4
FMH	*Cephalanthera damasonium* albino	mean	13.27	9.26	4.03
		SD	1.49	0.87	0.23
		n	10	10	10
FMH	*Cephalanthera exigua*	mean	13.60	8.70	3.12
		SD	2.92	0.94	0.49
		n	5	5	5
FMH	*Cephalanthera longifolia* albino	mean	11.76	6.58	3.76
		SD	1.61	0.24	0.24
		n	9	9	9
FMH	*Corallorhiza maculata*	mean	13.60	7.05	1.81
		SD	2.09	1.25	0.49
		n	15	15	15
FMH	*Corallorhiza striata*	mean	10.38	9.57	2.26
		SD	0.66	1.02	0.43
		n	3	3	3
FMH	*Cymbidium aberrans*	mean	13.99	10.78	1.23
		SD	1.59	0.62	0.20
		n	3	3	3

Suppl. Table 2 (continued)

	Species		$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]	N conc. [mmol g_{dw}^{-1}]
FMH	*Cymbidium macrorhizon*	mean	12.15	9.52	1.52
		SD	1.88	1.09	0.48
		n	6	6	6
FMH	*Epipogium aphyllum*	mean	7.46	8.96	3.03
		SD	2.92	0.70	0.56
		n	8	8	8
FMH	*Lecanorchis nigricans*	mean	16.42	7.91	1.97
		SD	1.03	0.10	0.50
		n	3	3	3
FMH	*Lecanorchis thalassica*	mean	8.83	8.03	2.20
		SD	2.66	1.03	0.41
		n	5	5	5
FMH	*Neottia nidus-avis*	mean	11.02	7.53	2.45
		SD	2.98	1.18	0.64
		n	52	52	38
PMH	*Cephalanthera damasonium*	mean	9.91	4.29	2.91
		SD	3.38	2.28	0.40
		n	39	43	39
PMH	*Cephalanthera erecta*	mean	6.46	2.45	3.79
		SD	1.99	0.53	0.09
		n	3	3	3
PMH	*Cephalanthera longifolia*	mean	6.72	1.90	2.63
		SD	2.39	1.78	0.51
		n	42	42	42
PMH	*Cephalanthera rubra*	mean	6.38	1.37	2.49
		SD	1.81	1.30	0.43
		n	25	25	25
PMH	*Corallorhiza trifida*	mean	6.11	6.90	1.56
		SD	1.17	1.02	0.30
		n	9	9	4
PMH	*Cymbidium goeringii*	mean	7.74	4.04	1.50
		SD	3.86	2.59	0.35
		n	7	7	7

Suppl. Table 2 (continued)

	Species		$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]	N conc. [mmol g_{dw}^{-1}]
PMH	*Cymbidium lancifolium*	**mean**	11.25	4.54	1.90
		SD	1.01	2.15	0.48
		n	6	6	6
PMH	*Epipactis atrorubens*	**mean**	10.83	2.28	2.33
		SD	3.49	1.57	0.30
		n	11	11	11
PMH	*Epipactis distans*	**mean**	16.36	2.38	2.47
		SD	3.64	1.92	0.11
		n	4	4	4
PMH	*Epipactis helleborine*	**mean**	13.02	2.56	2.76
		SD	4.40	2.70	0.66
		n	21	21	21
PMH	*Epipactis leptochila*	**mean**	22.16	5.54	4.69
		SD	0.52	0.83	0.50
		n	4	4	4
PMH	*Limodorum abortivum*	**mean**	13.72	5.06	2.16
		SD	3.35	0.50	0.36
		n	10	14	14
PMH	*Limodorum trabutianum*	**mean**	11.48	4.62	2.35
		SD	3.46	1.03	0.20
		n	5	5	5
PMH	*Platanthera minor*	**mean**	10.91	3.70	1.57
		SD	2.94	0.84	0.12
		n	3	3	3

MANUSCRIPT 2

You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species

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You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species

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• **Background and Aims** Partially mycoheterotrophic plants are enriched in ^{13}C and ^{15}N compared to autotrophic plants. Here, it is hypothesized that the type of mycorrhizal fungi found in orchid roots is responsible for variation in ^{15}N enrichment of leaf tissue in partially mycoheterotrophic orchids.
• **Methods** The genus *Epipactis* was used as a case study and carbon and nitrogen isotope abundances of eight *Epipactis* species, fungal sporocarps of four *Tuber* species and autotrophic references were measured. Mycorrhizal fungi were identified using molecular methods. Stable isotope data of six additional *Epipactis* taxa and ectomycorrhizal and saprotrophic basidiomycetes were compiled from the literature.
• **Key Results** The ^{15}N enrichment of *Epipactis* species varied between $3{\cdot}2 \pm 0{\cdot}8\,‰$ (*E. gigantea*; rhizoctonia-associated) and $24{\cdot}6 \pm 1{\cdot}6\,‰$ (*E. neglecta*; associated with ectomycorrhizal ascomycetes). Sporocarps of ectomycorrhizal ascomycetes ($10{\cdot}7 \pm 2{\cdot}2\,‰$) were significantly more enriched in ^{15}N than ectomycorrhizal ($5{\cdot}2 \pm 4{\cdot}0\,‰$) and saprotrophic basidiomycetes ($3{\cdot}3 \pm 2{\cdot}1\,‰$).
• **Conclusions** As hypothesized, it is suggested that the observed gradient in ^{15}N enrichment of *Epipactis* species is strongly driven by ^{15}N abundance of their mycorrhizal fungi; i.e. $\varepsilon^{15}N$ in *Epipactis* spp. associated with rhizoctonias $< \varepsilon^{15}N$ in *Epipactis* spp. with ectomycorrhizal basidiomycetes $< \varepsilon^{15}N$ in *Epipactis* spp. with ectomycorrhizal ascomycetes and basidiomycetes $< \varepsilon^{15}N$ in *Epipactis* spp. with ectomycorrhizal ascomycetes.

Key words: Ascomycetes, basidiomycetes, carbon, *Epipactis*, mycorrhiza, nitrogen, Orchidaceae, partial mycoheterotrophy, stable isotopes, *Tuber*.

INTRODUCTION

Partial mycoheterotrophy (PMH) is a trophic strategy of plants defined as a plant's ability to obtain carbon (C) simultaneously through photosynthesis and mycoheterotrophy via a fungal source exhibiting all intermediate stages between the extreme trophic endpoints of autotrophy and mycoheterotrophy (Merckx, 2013). However, all so far known partially mycoheterotrophic plants feature a change of trophic strategies during their development. In addition to all fully mycoheterotrophic plants, all species in the Orchidaceae and the subfamily Pyroloideae in the Ericaceae produce minute seeds that are characterized by an undifferentiated embryo and a lack of endosperm. These 'dust seeds' are dependent on colonization by a mycorrhizal fungus and supply of carbohydrates to facilitate growth of non-photosynthetic protocorms in this development stage termed initial mycoheterotrophy (Alexander and Hadly, 1985; Leake, 1994; Rasmussen, 1995; Rasmussen and Whigham, 1998; Merckx *et al.*, 2013). At adulthood these initially mycoheterotrophic plants either stay fully mycoheterotrophic (e.g. *Neottia nidus-avis*) or they become (putatively) autotrophic or partially mycoheterotrophic. With approximately 28000 species in 736 genera the Orchidaceae is the largest

angiosperm family with worldwide distribution constituting almost a tenth of described vascular plant species (Chase *et al.*, 2015; Christenhusz and Byng, 2016) making initial mycoheterotrophy the most widespread fungi-mediated trophic strategy. Nevertheless, PMH has been detected not only in green Orchidaceae species, but also in Burmanniaceae, Ericaceae and Gentianaceae (Zimmer *et al.*, 2007; Hynson *et al.*, 2009; Cameron and Bolin, 2010; Merckx *et al.*, 2013; Bolin *et al.*, 2015).

Analysis of food webs and clarification of trophic strategies with $\delta^{13}N$ and $\delta^{15}N$ stable isotope abundance values have a long tradition in ecology (DeNiro and Epstein, 1978, 1981). DeNiro and Epstein coined the term 'you are what you eat – plus a few permil' (DeNiro and Epstein, 1976) to highlight the systematic increase in the relative abundance of ^{13}C and ^{15}N at each trophic level of a food chain. Gebauer and Meyer (2003) and Trudell *et al.* (2003) were the first to employ stable isotope natural abundance analyses of C and N to distinguish the trophic level of mycoheterotrophic orchids from surrounding autotrophic plants.

Today, stable isotope analysis together with the molecular identification of fungal partners have become the standard tools for research on trophic strategies in plants, especially orchids

(Leake and Cameron, 2010). Since the first discovery of partially mycoheterotrophic orchids (Gebauer and Meyer, 2003), the number of species identified as following a mixed type of trophic strategy has grown continuously (Hynson *et al*., 2013, 2016; Gebauer *et al*., 2016). One of the relatively well-studied orchid genera in terms of stable isotopes and molecular identification of mycorrhizal partners is the genus *Epipactis* Zinn (Bidartondo *et al*., 2004; Tedersoo *et al*., 2007; Hynson *et al*., 2016). *Epipactis* is a genus of terrestrial orchids comprising 70 taxa (91 including hybrids) (The Plant List, 2013) with a mainly Eurasian distribution. *Epipactis gigantea* is the only species in the genus native to North America, and *Epipactis helleborine* is naturalized there. All *Epipactis* species are rhizomatous and summergreen and they occur in various habitats ranging from open wet meadows to closed-canopy dry forests (Rasmussen, 1995). PMH of several *Epipactis* species associated with ectomycorrhizal fungi (*E. atrorubens*, *E. distans*, *E. fibri* and *E. helleborine*) has been elucidated using stable isotope natural abundances of C and N. They all turned out to be significantly enriched in both ^{13}C and ^{15}N (Hynson *et al*., 2016). Orchid mycorrhizal fungi of the *Epipactis* species in the above-mentioned studies were ascomycetes and basidiomycetes simultaneously ectomycorrhizal with neighbouring forest trees, and in some cases additionally basidiomycetes belonging to the polyphyletic rhizoctonia group well known as forming orchid mycorrhizas have also been detected (Gebauer and Meyer, 2003; Bidartondo *et al*., 2004; Abadie *et al*., 2006; Tedersoo *et al*., 2007; Selosse and Roy, 2009; Liebel *et al*., 2010; Gonneau *et al*., 2014). *Epipactis gigantea* and *E. palustris*, the only two *Epipactis* species colonizing open habitats and exhibiting exclusively an association with rhizoctonias, showed no ^{13}C and only minor ^{15}N enrichment (Bidartondo *et al*., 2004; Zimmer *et al*., 2007).

The definition of trophic strategies in vascular plants is restricted to an exploitation of C and places mycoheterotrophy into direct contrast to autotrophy. The proportions of C gained by partially mycoheterotrophic orchid species from fungi have been quantified by a linear two-source mixing-model approach (Gebauer and Meyer, 2003; Preiss and Gebauer, 2008; Hynson *et al*., 2013). Variations in percental C gain of partially mycoheterotrophic orchids from the fungal source are driven by plant species identity placing, for example, the leafless *Corallorhiza trifida* closely towards fully mycoheterotrophic orchids (Zimmer *et al*., 2008; Cameron *et al*., 2009) and by physiological and environmental variables such as leaf chlorophyll concentration (Stöckel *et al*., 2011) and light climate of their microhabitats (Preiss *et al*., 2010). Carbon gain in the orchid species *Cephalanthera damasonium*, for example, can range from 33 % in an open pine forest to about 85 % in a dark beech forest (Gebauer, 2005; Hynson *et al*., 2013).

Far less clear is the explanation of variations in ^{15}N enrichment found for fully, partially and initially mycoheterotrophic plants, but also for putatively autotrophic species (Gebauer and Meyer, 2003; Abadie *et al*., 2006; Tedersoo *et al*., 2007; Preiss and Gebauer, 2008; Selosse and Roy, 2009; Liebel *et al*., 2010, Hynson *et al*., 2013). This ^{15}N enrichment was found to be not linearly related to the degree of heterotrophic C gain (Leake and Cameron 2010; Merckx *et al*., 2013). Using the linear two-source mixing-model approach to obtain quantitative information of the proportions of N gained by partially mycoheterotrophic orchid species from the fungal source, some species

even exhibited an apparent N gain above 100 % (Hynson *et al*., 2013). Reasons for this pattern remained unresolved and could just be explained by lacking coverage of variability in ^{15}N signatures of the chosen fully mycoheterotrophic endpoint due to different fungal partners (Preiss and Gebauer, 2008; Hynson *et al*., 2013).

Here, we hypothesize that the type of mycorrhizal fungi in the roots of orchid species (i.e. ectomycorrhizal basidiomycetes, ectomycorrhizal ascomycetes or basidiomycetes of the rhizoctonia group) is responsible for the differences in ^{15}N enrichment measured in leaf bulk tissue. We used the genus *Epipactis* as case study due to already existing literature on their mycorrhizal partners and natural abundance stable isotope values and extended the data to six additional *Epipactis* taxa.

MATERIALS AND METHODS

Study locations and sampling scheme

Eight *Epipactis* taxa were sampled at nine sites in the Netherlands and Germany in July 2014 following the plot-wise sampling scheme proposed by Gebauer and Meyer (2003). Leaf samples from flowering individuals of all *Epipactis* species in this survey were taken in five replicates (resembling five 1-m^2 plots) together with three autotrophic non-orchid, non-leguminous reference plant species each (listed in Supplementary Data Table S1). *Epipactis helleborine* (L.) Crantz and *E. helleborine* subsp. *neerlandica* (Verm.) Buttler were sampled at three locations in the province of South Holland in the Netherlands. *Epipactis helleborine* was collected at ruderal site 1 (52°0′N, 4°21′E) dominated by *Populus* × *canadensis* Moench. and forest site 2 (52°11′N, 4°29′E at 1 m elevation) dominated by *Fagus sylvatica* L. *Epipactis helleborine* subsp. *neerlandica* was collected at dune site 3 (52°8′N, 4°20′E at 10 m elevation), an open habitat with sandy soil dominated by *Salix repens* L. and *Quercus robur* L. Samples of *E. microphylla* (Ehrh.) Sw. and *E. pupurata* Sm. were collected from two sites (forest sites 4 and 5) with thermophilic oak forest dominated by *Quercus robur* south of Bamberg, north-east Bavaria, Germany (49°50′–49°51′N, 10°52′–11°02′E at 310–490 m elevation). *Epipactis distans* Arv.-Touv., *E. leptochila* (Godfery) Godfery, *E. muelleri* Godfery and *E. neglecta* (Kümpel) Kümpel (Fig. 1a) were collected at four sites (forest sites 6–9) dominated by dense old-growth stands of *Fagus sylvatica* with a sparse cover of understorey vegetation in the Nördliche Frankenalb, north-east Bavaria, Germany (49°35′–49°39′N, 11°23′–11°28′E at 450–550 m elevation). Sampling yielded a total of 45 leaf samples from eight *Epipactis* species and 135 leaf samples from 17 neighbouring autotrophic reference species (Table S1).

To complete the already existing isotope abundance data of fungal fruit bodies, sporocarps of species in the true truffle ascomycete genus *Tuber* were sampled opportunistically at forest sites 7–9 and a further adjacent site dominated by *Fagus sylvatica* (49°40′N, 11°23′E) (Preiss and Gebauer, 2008; Gebauer *et al*., 2016) in December 2014. In total, 27 hypogeous ascocarps in the four ectomycorrhizal species *Tuber aestivum* Vittad. (*n* = 5), *Tuber excavatum* Vittad. (*n* = 19) (Fig. 1c), *Tuber brumale* Vittad. (*n* = 1) (Fig. 1d) and *Tuber rufum* Pico (*n* = 2) were retrieved with the help of a truffle-hunting dog.
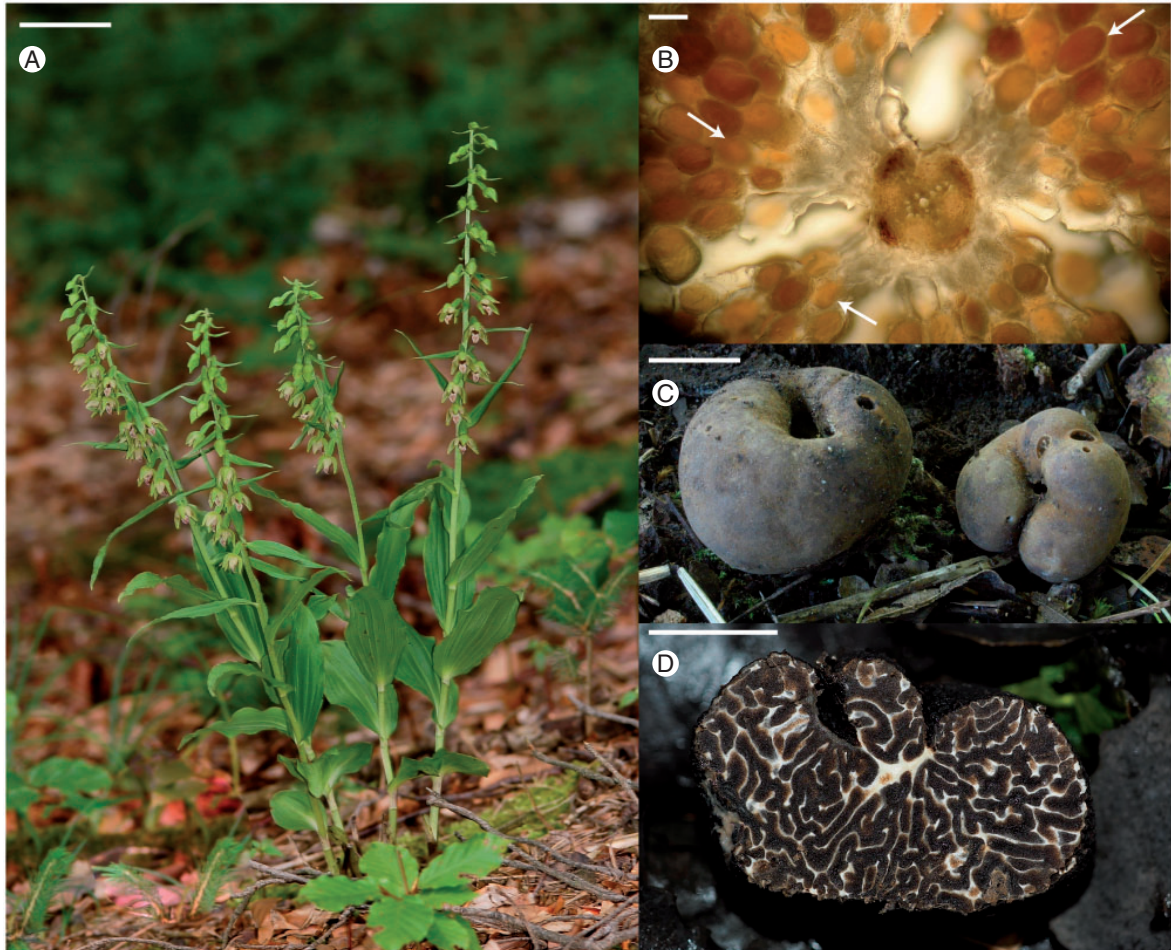
FIG. 1. (A) *Epipactis neglecta* at forest site 9 in the Nördliche Frankenalb in July 2009. Scale bar = 5 cm. Image courtesy of Florian Fraaß. (B) Light micrograph showing a transverse section of a root of *Epipactis neglecta*. Fungal colonization is visible as exodermal, outer and inner cortex cells filled with fungal hyphae, indicated by white arrows. Scale bar = 100 μm. (C) Ascocarps of *Tuber excavatum*. Scale bar = 1 cm. (D) Cross-section of an ascocarp of *Tuber brumale*. Scale bar = 1 cm.

Wherever possible, autotrophic plant species were sampled as references together with the sporocarps ($n = 25$) or were used from the previous sampling of *Epipactis* specimens from the same sites ($n = 45$).

Fungal DNA analysis

Of all species besides *E. helleborine*, two roots per sampled *Epipactis* individual were cut, rinsed with deionized water, placed in CTAB buffer (cetyltrimethylammonium bromide) and stored at −18 °C until further analysis. Root cross-sections (Fig. 1b) were checked for presence and status of fungal pelotons in the cortex cells. Two to six root sections per *Epipactis* individual were selected for genomic DNA extraction and purification with the GeneClean III Kit (Q-BioGene, Carlsbad, CA, USA). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primer combinations ITS1F/ITS4 and ITS1/ITS4-Tul (Bidartondo and Duckett, 2010). All positive PCR products were purified with ExoProStart (GE Healthcare, Amersham, UK) and sequenced bidirectionally with an ABI3730 Genetic Analyser using the BigDye 3·1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. The same protocol was used for molecular analysis of oven-dried fragments of *Tuber* ascocarps. All DNA sequences were checked and visually aligned with Geneious version 7·4·1 (http://www.geneious.com, Kearse *et al.*, 2012) and compared to GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov). GenBank accession numbers for all unique DNA sequences are KX354284–KX354297.

Of all individuals of *E. helleborine*, one root per sampled *Epipactis* individual was cut, rinsed with deionized water, placed in CTAB buffer and stored at −18 °C until further analysis. The entire root of each *Epipactis* individual sampled was used for genomic DNA extraction following the protocol of

Doyle and Doyle (1987). The nuclear ribosomal internal transcribed spacer 2 (nrITS2) region was amplified with the fungal-specific primers fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990). Ion Xpress labels were attached to the primers for individual sample identification. Tags differed from all other tags by at least two nucleotides. Fusion PCRs were performed using the following programme: 98 °C/3 min, 35 cycles of 98 °C/5 s, 55 °C/10 s, 72 °C/30 s, and 72 °C/5 min. One microlitre of DNA template was used in a 25-μL PCR containing 14·3 μL MQ water, 5 μL of 5× buffer, 0·5 μL dNTPs (2·5 mM), 1·25 μL of reverse and forward primers (10 mM), 0·5 μL MgCl₂ (25 mM), 0·75 μL BSA (10 mg mL⁻¹) and 0·5 μL Phire II polymerase (5U μL⁻¹). Primer dimers were removed by using 0·9× NucleoMag NGS Clean-up and Size Select beads (Macherey-Nagel, Düren, Germany) to which the PCR products were bound. The beads were washed twice with 70 % ethanol and resuspended in 30 μL TE buffer. Cleaned PCR products were quantified using an Agilent 2100 Bioanalyzer DNA High Sensitivity Chip. An equimolar pool was prepared of the amplicon libraries at the highest possible concentration. This equimolar pool was diluted according to the calculated template dilution factor to target 10–30 % of all positive Ion Sphere particles. Template preparation and enrichment were carried out with the Ion OneTouch system, using the OT2 400 Kit, according to the manufacturer's protocol 7218RevA0. The quality control of the Ion OneTouch 400 Ion Sphere particles was done using the Ion Sphere Quality Control Kit using a Life Qubit 2·0. The enriched Ion Spheres were prepared for sequencing on a Personal Genome Machine (PGM) with the Ion PGM Hi-Q Sequencing Kit as described in protocol 9816RevB0 and loaded on an Ion-318v2 chip (850 cycles per run). The Ion Torrent reads produced were subjected to quality filtering by using a parallel version of MOTHUR v. 1.32.1 (Schloss *et al.*, 2009) installed at the University of Alaska Life Sciences Informatics Portal. Reads were analysed with threshold values set to $Q \geq 25$ in a sliding window of 50 bp, no ambiguous bases, and homopolymers no longer than 8 bp. Reads shorter than 150 bp were omitted from further analyses. The number of reads for all samples was normalized and the filtered sequences were clustered into operational taxonomic units (OTUs) at 97 % sequence similarity cut-off using OTUPIPE (Edgar *et al.*, 2011). Putatively chimeric sequences were removed using a curated dataset of fungal nrITS sequences (Nilsson *et al.*, 2008). We also excluded all singletons from further analyses. For identification, sequences were submitted to USEARCH (Edgar, 2010) against the latest release of the quality checked UNITE+INSD fungal nrITS sequence database (Kõljalg *et al.*, 2013). Taxonomic identifications were based on the current Index Fungorum classification as implemented in UNITE.

Stable isotope abundance and N concentration analysis

Leaf samples of eight *Epipactis* taxa ($n = 45$) and autotrophic references ($n = 160$) were washed with deionized water and *Tuber* ascocarps ($n = 27$) were surface-cleaned of adhering soil. All samples were dried to constant weight at 105 °C, ground to a fine powder in a ball mill (Retsch Schwingmühle MM2, Haan, Germany) and stored in a desiccator fitted with silica gel until analysis. Relative C and N isotope natural abundances of the leaf and sporocarp samples were measured in dual element analysis mode with an elemental analyser (Carlo Erba Instruments 1108, Milano Italy) coupled to a continuous flow isotope ratio mass spectrometer (delta S Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Thermo Fisher Scientific, Bremen, Germany) as described by Bidartondo *et al.* (2004). Measured relative isotope abundances are denoted as δ values that were calculated according to the following equation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard}-1) \times 1000$ [‰], 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 with respect to international standards (CO_2 vs PDB and N_2 vs N_2 in air) by use of the reference substances ANU sucrose and NBS19 for the carbon isotopes and N1 and N2 for the nitrogen isotopes provided by the IAEA (International Atomic Energy Agency, Vienna, Austria). Reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measuring the laboratory standard acetanilide (Gebauer and Schulze, 1991). Acetanilide was routinely analysed with variable sample weight at least six times within each batch of 50 samples. The maximum variation of $\delta^{13}C$ and $\delta^{15}N$ both within and between batches was always below 0·2 ‰.

Total N concentrations in leaf and sporocarp samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on measurements of the laboratory standard acetanilide with a known N concentration of 10·36 % (Gebauer and Schulze, 1991).

Literature survey

We compiled C and N stable isotope natural abundance and nitrogen concentration data of five additional *Epipactis* species and their autotrophic references from all available publications (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Abadie *et al.*, 2006; Zimmer *et al.*, 2007; Tedersoo *et al.*, 2007; Liebel *et al.*, 2010; Johansson *et al.*, 2014; Gonneau *et al.*, 2014): *Epipactis atrorubens* (Hoffm.) Besser ($n = 11$), *Epipactis distans* Arv.-Touv. ($n = 4$), *Epipactis fibri* Scappat. and Robatsch ($n = 29$), *Epipactis gigantea* Douglas ex. Hook ($n = 5$) and *Epipactis palustris* (L.) Crantz ($n = 4$) and additional data points of *Epipactis helleborine* (L.) Crantz ($n = 21$) and *Epipactis leptochila* (Godfery) Godfery ($n = 4$) yielding a total of 78 further data points for the genus *Epipactis* and 161 data points for 26 species of photosynthetic non-orchid references (Supplementary Data Table S2).

The C and N stable isotope and nitrogen concentration data of 11 species of ectomycorrhizal basidiomycetes ($n = 37$) and four species of saprotrophic basidiomycetes ($n = 17$) sampled opportunistically at forest site 10 were extracted from Gebauer *et al.* (2016) (Table S2).

A separate literature survey was conducted to compile fungal partners forming orchid mycorrhiza with the *Epipactis* species *E. atrorubens*, *E. distans*, *E. fibri*, *E. gigantea*, *E. helleborine*, *E. helleborine* subsp. *neerlandica*, *E. microphylla*, *E. palustris* and *E. purpurata* (from Bidartondo *et al.*, 2004; Selosse *et al.*, 2004; Bidartondo and Read, 2008; Ogura-Tsujita and Yukawa, 2008; Ouanphanivanh *et al.*, 2008; Shefferson *et al.*, 2008; Illyés *et al.*, 2009; Těšitelová *et al.*, 2012; Jacquemyn *et al.*, 2016) (Table S3).

Calculations and statistics

To enable comparisons of C and N stable isotope abundances between the *Epipactis* species sampled for this study, data from the literature and fungal sporocarps, we used an isotope enrichment factor approach to normalize the data. Normalized enrichment factors (ε) were calculated from measured or already published δ values as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$ or $\delta^{15}N$ value of an *Epipactis* individual, a fungal sporocarp or an autotrophic reference plant, and δ_{REF} is the mean value of all autotrophic reference plants by plot (Preiss and Gebauer, 2008). Enrichment factor calculations for sporocarps of ectomycorrhizal ascomycetes (ECM A), ectomycorrhizal basidiomycetes (ECM B) and saprotrophic basidiomycetes (SAP) sampled at forest site 10 were enabled by extracting stable isotope data of autotrophic references from previous studies ($n = 158$) (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Zimmer *et al.*, 2007, 2008; Preiss *et al.*, 2010; Gebauer *et al.*, 2016). The $\delta^{13}C$ and $\delta^{15}N$ values, enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, and N concentrations of eight *Epipactis* species, sporocarps of ECM ascomycetes (ECM A) and autotrophic references from this study and six *Epipactis* species, sporocarps of ECM basidiomycetes (ECM B), saprotrophic basidiomycetes (SAP) and autotrophic references from the literature are available in Tables S1 and Table S2, respectively.

We tested for pairwise differences in isotopic enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) and N concentrations between the *Epipactis* species and their corresponding autotrophic reference plants using a non-parametric Mann–Whitney *U*-test. We repeated the Mann–Whitney *U*-test to test for pairwise differences between fungal sporocarps and autotrophic references in $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentrations. We used the non-parametric Kruskal–Wallis *H*-test in combination with a post-hoc Mann–Whitney *U*-test for multiple comparisons to test for differences in isotopic enrichment factors and N concentrations between sporocarps of ECM A, ECM B and SAP. The *P* values were adjusted using the sequential Bonferroni correction (Holm, 1979). For statistical analyses we used the software environment *R* [version 3.1.2 (Pumpkin Helmet) (R Development Core Team, 2014)] with a significance level of $\alpha = 0.05$.

RESULTS

Fungal DNA analysis

Pelotons apparent as dense coils of fungal hyphae were not visible in all roots of the 31 *Epipactis* individuals examined. Yet for all *Epipactis* species studied here, associations with ectomycorrhizal (ECM) non-rhizoctonia fungi were found. All eight *Epipactis* species investigated here were associated with obligate ECM B [*Inocybe* (Fr.) Fr., *Russula* Pers., *Sebacina epigaea* (Berk. and Broome) Neuhoff] or obligate ECM A (*Tuber*, *Wilcoxina*) (Table 1). *Epipactis helleborine* was associated with both obligate ECM B and ECM A at the two sites, but for its subspecies *neerlandica* only ECM B *Inocybe* could be identified as a fungal partner. The obligate ECM B *Sebacina epigaea* and ECM A *Cadophora* Lagerb. and Melin were associated with *E. microphylla*. The obligate ECM basidiomycetes

Russula heterophylla (Fr.) Fr. and *Inocybe* were detected in the roots of *E. purpurata* at forest site 5. Roots of *E. distans* were colonized by the obligate ECM A *Wilcoxina rehmii* Chin S. Yang and Korf. *Epipactis leptochila* and *E. neglecta* formed orchid mycorrhizas exclusively with the ECM A *Tuber excavatum* and *E. muelleri* associated with *Tuber puberulum* Berk. and Broome.

The species identities of the true truffles determined by macroscopic and microscopic identification could be confirmed by nrITS sequencing and BLAST analysis (Table 2). *Tuber excavatum* extracted from the roots of *E. leptochila* at forest site 7 and *T. excavatum* ascocarps collected from the same site had identical nrITS sequences and could be the same genets. The nrITS sequences of *T. excavatum* var. *intermedium* extracted from the roots of *E. neglecta* at forest site 9 and sporocarps of *T. excavatum* var. *intermedium* from the same site were also identical.

Stable isotope abundance and N concentration analysis

Pairwise Mann–Whitney *U*-tests showed that all *Epipactis* species sampled in this study were significantly enriched in ^{13}C and ^{15}N relative to their respective autotrophic reference species (Fig. 2, Table 3). Enrichment of the *Epipactis* species in this survey varied between 2.07 ± 0.89 ‰ (*E. helleborine* subsp. *neerlandica*) and 6.11 ± 0.91 ‰ (*E. purpurata*) in ^{13}C and between 7.98 ± 2.46 ‰ (*E. helleborine* subsp. *neerlandica*) and 24.60 ± 1.57 ‰ (*E. neglecta*) in $\varepsilon^{15}N$ (Table S1). *Epipactis helleborine*, *E. helleborine* subsp. *neerlandica*, *E. purpurata*, *E. distans*, *E. leptochila*, *E. muelleri* and *E. neglecta* ($\mu = 2.38 \pm 0.44$ mmol g d. wt^{-1}) had significantly higher N concentrations than their respective autotrophic references ($\mu = 1.42 \pm 0.32$ mmol g d. wt^{-1}). N concentrations in the leaves of *E. microphylla* (1.51 ± 0.32 mmol g d. wt^{-1}) were only slightly but not significantly higher than the species' references (1.34 ± 0.25 mmol g d. wt^{-1}) ($U = 48$; $P = 0.395$) (Table 3).

For data of *Epipactis* species extracted from the literature, pairwise tests confirmed significant enrichment of *E. atrorubens*, *E. distans*, *E. fibri*, *E. leptochila* and *E. helleborine* in both $\varepsilon^{13}C$ and ^{15}N relative to their autotrophic references (Table 3). For *E. palustris* a significant enrichment in ^{15}N was detected ($U = 48$; $P = 0.001$) but not for ^{13}C ($U = 26$; $P = 0.862$). *Epipactis gigantea* was significantly depleted in ^{13}C ($U = 14$; $P = 0.017$) and enriched in ^{15}N ($U = 93.5$; $P = 0.003$) relative to autotrophic references. Enrichment of the *Epipactis* species compiled from the literature varied between -1.19 ± 0.66 ‰ (*E. gigantea*) and 4.25 ± 1.77 ‰ (*E. fibri*) in ^{13}C and between 3.15 ± 0.75 ‰ (*E. gigantea*) and 22.16 ± 0.49 ‰ (*E. leptochila*) in ^{15}N (Table S2). The N concentrations of all *Epipactis* species extracted from the literature ($\mu = 2.70 \pm 0.69$ mmol g d. wt^{-1}) were significantly higher than of leaves of their autotrophic reference plant species ($\mu = 1.38 \pm 0.72$ mmol g d. wt^{-1}) (Table 3; Table S2). No N concentration data were available for *E. palustris*.

Pairwise Mann–Whitney *U*-tests showed that sporocarps of ECM A, ECM B and SAP were significantly enriched in ^{13}C

TABLE 1. *Orchid mycorrhizal fungi identified from roots of seven* Epipactis *species from nine sites in Germany and the Netherlands (ECM A = ascomycetes forming ectomycorrhizas, ECM B = basidiomycetes forming ectomycorrhizas);* L *is Ellenberg's light indicator value (Ellenberg* et al., *1991) and* n *is the number of* Epipactis *individuals sampled*

Species	L	Site	n	Pelotons	Mycorrhizal fungi	Type of mycorrhizal fungi	Best match sequence/ accession number (UDB-UNITE, others GenBank)	Identity (%)
Epipactis helleborine (L.) Crantz*	3	Ruderal site 1	1	NA	Helotiales	ECM A	DQ182433 uncul. Helotiales	98·9
				NA	*Inocybe* sp.	ECM B	HE601882·1 uncul. *Inocybe*	99·4
				NA	*Sebacina* sp.	ECM B	UDB013653 *Sebacina*	99·7
Epipactis helleborine (L.) Crantz*	3	Forest site 2	1	NA	Thelephoraceae	ECM B	UDB013578 *Tomentella-Thelephora*	97·7
				NA	Helotiales	ECM A	DQ182433 uncul. Helotiales	98·9
				NA	*Tomentella* sp.	ECM B	AJ879656·1 uncul. Ectomycorrhiza (*Tomentella*)	96·5
				NA	*Inocybe* sp.	ECM B	JX630876 uncul. *Inocybe*	98·5
				NA	*Tuber rufum*	ECM A	EF362475 *Tuber rufum*	100
				NA	*Inocybe* sp.	ECM B	HE601882·1 uncul. *Inocybe*	99·4
				NA	*Tuber* sp.	ECM A	AJ510273 uncul. *Tuber* sp.	99·6
				NA	*Sebacina* sp.	ECM B	UDB007522 *Sebacina*	99·6
Epipactis helleborine subsp. *neerlandica* (Verm.) Buttler	NA	Dune site 3	1	no	*Inocybe* sp.	ECM B	JF908119·1 *Inocybe splendens*	90
Epipactis microphylla (Ehrh.) Sw.	2	Forest site 4	5	yes	*Sebacina epigaea*	ECM B	KF000457·1 *Sebacina epigaea*	100
				yes	*Cadophora* sp.	ECM A	JN859252·1 *Cadophora* sp.	99
Epipactis purpurata Sm.	2	Forest site 5	5	no	*Russula heterophylla*	ECM B	DQ422006·1 *Russula heterophylla*	99
				no	*Inocybe* sp.	ECM B	KF679811·1 *Inocybe* sp.	91
Epipactis distans Arv.-Touv.	NA	Forest site 6	5	yes	*Wilcoxina rehmii*	ECM A	DQ069001·1 *Wilcoxina rehmii*	99
Epipactis leptochila (Godfery) Godfery	3	Forest site 7	5	yes	*Tuber excavatum*	ECM A	HM151977·1 *Tuber excavatum* var. *intermedium*	99
				yes	*Tuber excavatum*	ECM A	HM151993·1 *Tuber excavatum*	99
Epipactis muelleri Godfery	7	Forest site 8	5	yes	*Tuber puberulum*	ECM A	FN433157·1 *Tuber puberulum*	100
							AF106891·1 *Tuber oligospermum*	99
Epipactis neglecta (Kümpel) Kümpel	NA	Forest site 9	5	yes	*Tuber excavatum*	ECM A	HM151977·1 *Tuber excavatum* var. *intermedium*	99

*Data from Ion Torrent sequencing.

TABLE 2. *Molecular identification of* Tuber *sporocarps collected at four forest sites in Germany*

Species	Site	Best match sequence/accession number (UDB-UNITE, others GenBank)	Identity (%)
Tuber aestivum Vittad.	Forest site 8	JF926117·1 *Tuber aestivum*	99
	Forest site 10	JQ348411·1 *Tuber aestivum*	98
Tuber brumale Vittad.	Forest site 10	NA	NA
Tuber excavatum Vittad.	Forest site 7	HM151993·1 *Tuber excavatum*	99
	Forest site 8	HM151982·1 *Tuber excavatum*	99
	Forest site 9	HM151977·1 *Tuber excavatum* var. *intermedium*	99
Tuber rufum Pico	Forest site 8	AF106892·1 *Tuber rufum*	98
	Forest site 10	AF132506·1 *Tuber ferrugineum*	99

and ^{15}N relative to their respective autotrophic reference species (Table 3). Enrichment factors of ascocarps of the obligate ECM A ranged between 3·51 ‰ (*T. brumale*) and 5·90 ± 0·71‰ (*T. excavatum*) for ^{13}C and between 10·12 ± 1·25 ‰ (*T. excavatum*) and 16·74 ‰ (*T. brumale*) for ^{15}N (Table S1). A non-parametric Kruskal–Wallis *H*-test showed that sporocarps of *Tuber* species were significantly more enriched in ^{15}N than the sporocarps of obligate ECM B ($P < 0.001$) and sporocarps of SAP ($P < 0.001$). ^{15}N enrichment of ECM and SAP was not significantly different ($P = 0.61$). Sporocarps of SAP were more enriched in ^{13}C than the sporocarps of both ECM B ($P = 0.008$) and ECM A ($P < 0.001$). The ^{13}C enrichment of sporocarps of ECM B was also

significantly higher than of ECM A ($P < 0.001$). Average enrichment of the sporocarps of obligate ECM A was 5·62 ± 0·93 ‰ in ^{13}C and 10·74 ± 2·18 ‰ in ^{15}N and for the sporocarps of the obligate ECM B was 7·10 ± 1·73 ‰ in ^{13}C and 5·19 ± 4·04 ‰ in ^{15}N. Sporocarps of SAP were enriched by 3·26 ± 2·07 ‰ in ^{15}N and 8·77 ± 1·67 ‰ in ^{13}C.

Sporocarps of all fungal types (ECM A: $\bar{x} = 2·90 ± 0·38$ mmol g d. wt^{-1}; ECM B: $\bar{x} = 2·81 ± 0·95$ mmol g d. wt^{-1}; SAP: $\bar{x} = 4·783 ± 1·854$ mmol g d. wt^{-1}) had significantly higher N concentrations than their autotrophic reference plant species ($\bar{x} = 1·54 ± 0·40$ mmol g d. wt^{-1}) (ECM A: $U = 5549$; $P < 0.001$; ECM B: $U = 4776$; $P < 0.001$; SAP: $U = 2302$; $P < 0.001$) but no significant differences could be detected in the N
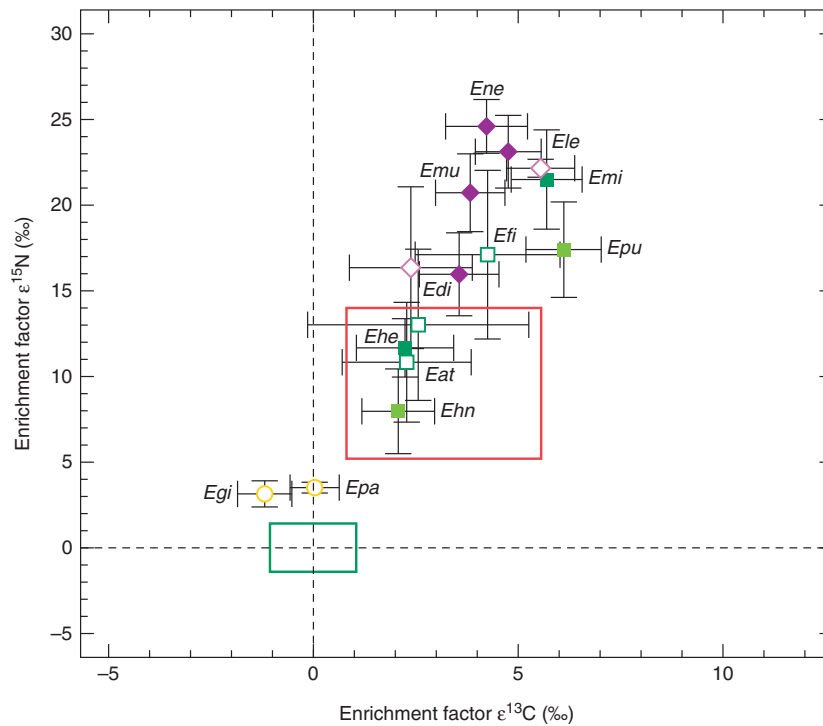
FIG. 2. Mean enrichment factors ε^{13}C and ε^{15}N $\pm$ 1 s.d. of two *Epipactis* species associated with rhizoctonia fungi (yellow circles; *Egi* = *E. gigantea*, *Epa* = *E. palustris*), two *Epipactis* species associated with ECM basidiomycetes (light green squares; *Ehn* = *E. helleborine* ssp. *neerlandica*, *Epu* = *E. purpurata*), four *Epipactis* species associated with ECM ascomycetes and basidiomycetes (dark green squares; *Eat* = *E. atrorubens*, *Ehe* = *E. helleborine*, *Efi* = *E. fibri*; *Emi* = *E. microphylla*) and four *Epipactis* species forming orchid mycorrhizas exclusively with ectomycorrhizal ascomycetes (purple diamonds; *Edi* = *E. distans*, *Ele* = *E. leptochila*, *Emu* = *E. muelleri*, *Ene* = *E. neglecta*). All open symbols indicate isotope data extracted from the literature (Tables S2). The green box represents mean enrichment factors $\pm$1 s.d. for the autotrophic reference plants that were sampled together with the *Epipactis* species (REF, *n* = 296, see Tables S1 and S2) whereas mean ε values of reference plants are zero by definition. The red box represents mean enrichment factors $\pm$1 s.d. of all partially mycoheterotrophic orchid species associated with ectomycorrhizal fungi (ε^{13}C$_{mean}$ = 3·18 $\pm$ 2·38 and ε^{15}N$_{mean}$ = 9·61 $\pm$ 4·40) published since 2003 that were available from the literature (Hynson *et al.*, 2016).

TABLE 3. *Results from pairwise comparisons for enrichment factors ε^{15}N, ε^{13}C and nitrogen concentration (mmol g d. wt^{-1}) between* Epipactis *species and sporocarps of ECM ascomycetes, ECM basidiomycetes and SAP fungi and their autotrophic references using the Mann-Whitney U-test*

Species	ε^{15}N		ε^{13}C		N concentration	
	U	P	U	P	U	P
Epipactis helleborine (L.) Crantz	300	<0·001	265	<0·001	280	<0·001
Epipactis helleborine subsp. *neerlandica* (Verm.) Buttler	75	<0·001	67	0·008	66	0·011
Epipactis microphylla (Ehrh.) Sw.	75	<0·001	75	<0·001	48	0·395
Epipactis purpurata Sm.	75	<0·001	75	<0·001	75	<0·001
Epipactis distans Arv.-Touv.	75	<0·001	75	<0·001	73	<0·001
Epipactis leptochila (Godfery) Godfery	75	<0·001	75	<0·001	75	<0·001
Epipactis muelleri Godfery	75	<0·001	75	<0·001	75	<0·001
Epipactis neglecta (Kümpel) Kümpel	75	<0·001	75	<0·001	75	<0·001
Epipactis atrorubens (Hoffm.) Besser*	275	<0·001	246	<0·001	275	<0·001
Epipactis distans Arv.-Touv.*	48	0·004	47	0·002	45	0·008
Epipactis fibri Scappat. and Robatsch*	348	<0·001	344	<0·001	287·5	0·001
Epipactis gigantea Douglas ex Hook.*	93·5	0·003	14	0·017	99	<0·001
Epipactis helleborine (L.) Crantz*	1596	<0·001	1329	<0·001	1469	<0·001
Epipactis leptochila (Godfery) Godfery*	16	0·029	16	0·029	16	0·029
Epipactis palustris (L.) Crantz*	48	0·001	26	0·862	NA	NA
Sporocarps of ECM ascomycetes	6155	<0·001	6132	<0·001	5549	<0·001
Sporocarps of ECM basidiomycetes	5209	<0·001	5835	<0·001	4776	<0·001
Sporocarps of SAP fungi	2300	<0·001	2686	<0·001	2302	<0·001

**Epipactis* species for which data have been extracted from the literature.

concentrations of sporocarps of obligate ECM A and ECM B (P = 0·199). The N concentrations of sporocarps of SAP were significantly higher than in ECM A ($P = 0·042$) and ECM B ($P = 0·006$).

DISCUSSION

Fungal DNA analysis and stable isotope natural abundances – Epipactis *species*

In this study we provide the first stable isotope data for *E. helleborine* subsp. *neerlandica*, *E. purpurata*, *E. microphylla*, *E. muelleri* and *E. neglecta*. We infer PMH as the nutritional mode of these *Epipactis* species associated with ECM fungi for the first time as they are significantly enriched in ^{13}C relative to their autotrophic reference plants (Fig. 2, Table 3) but show a smaller ^{13}C enrichment than fully mycoheterotrophic Orchidaceae (8·03 ± 0·13 ‰ in Hynson *et al*., 2016). Furthermore, we confirm the PMH shown for *E. distans*, *E. helleborine* and *E. leptochila* in earlier studies (Gebauer and Meyer, 2003; Bidartondo *et al*., 2004; Abadie *et al*., 2006; Liebel *et al*., 2010; Johansson *et al*., 2014; Hynson *et al*., 2016). Differences in ^{13}C enrichment between the individual species might be driven by the respective plant species identity or morphology with, for example, *E. microphylla* having rather narrow leaves and thus a smaller total photosynthetic surface area making this *Epipactis* species more reliant on fungal carbon. Furthermore, the light climate at a respective site is usually mirrored in the ^{13}C enrichment in leaf tissue of orchid species partnering with ECM fungi: ^{13}C enrichment is correlated with decreasing light availability as the proportion of carbon derived from fungi increases in partially mycoheterotrophic orchids associated with ECM fungi (Preiss *et al*., 2010). *Epipactis microphylla* and *E. purpurata*, which were sampled from closed-canopy oak forests, exhibit the highest ^{13}C enrichment and are characterized by a low Ellenberg light indicator value (*L*) of 2 typical for shade plants (Ellenberg *et al*., 1991). The value of *L* ranges from 1 to 9, where 1 indicates plants growing in deep shade (1–30 % light availability relative to irradiance above the forest canopy) and 9 indicates plants growing in full light (>50 % light availability relative to irradiance above the canopy) (Ellenberg *et al*., 1991). *Epipactis leptochila* (*L* 3), *E. neglecta*, *E. muelleri* (*L* 7) and *E. distans* exhibited a slightly lesser enrichment in ^{13}C, mirroring the light-limited conditions of dense *Fagus sylvatica* stands. *Epipactis helleborine* (*L* 3) and *E. helleborine* subsp. *neerlandica* showed only minor enrichment in ^{13}C owing to the relatively open conditions of a ruderal site and a sand dune habitat. The ^{13}C enrichment in *E. distans*, *E. fibri*, *E. helleborine* and *E. atrorubens* (*L* 6) calculated from published data was intermediate with high standard deviations probably owing to sampling at several habitats with different light regimes. *Epipactis gigantea* and *E. palustris* (*L* 8) sampled from open habitats showed no significant enrichment in ^{13}C, reflecting high light availability and rhizotonias as fungal partners (Bidartondo *et al*., 2004; Zimmer *et al*., 2007).

For the observed gradient in ^{15}N enrichment we infer a strong relationship between the specific fungal host group and the respective *Epipactis* species. The ^{15}N enrichment in orchids arises as a result of receiving N mobilized and assimilated by fungi from different sources (Gebauer and Meyer, 2003; Bidartondo *et al*., 2004). We can differentiate the status of ^{15}N

enrichment of *Epipactis* species according to the mycorrhizal fungi associated with the *Epipactis* species.

Epipactis gigantea and *E. palustris*, the only *Epipactis* species solely associated with rhizoctonia fungi, exhibit minor but significant enrichment in ^{15}N (Bidartondo *et al*., 2004; Zimmer *et al*., 2007). *Epipactis helleborine* subsp. *neerlandica* associated with the ECM B *Inocybe* (Table 1) shows a modest enrichment in ^{15}N that lies in the range documented for orchid species associated with ECM fungi in general (Hynson *et al*., 2016). An exception here is *E. purpurata* shown to partner with the ECM B *Russula heterophylla* and *Inocybe* sp., exhibiting high ^{15}N enrichment (Table 1). However, the ECM A *Wilcoxina* has also been documented in a previous study to host *E. purpurata* (Těšitelová *et al*., 2012) and may have been missed here. *Epipactis* species such as *E. atrorubens* and *E. helleborine* associated with a wide array of both ECM A and ECM B (Table 3) show a modest enrichment in ^{15}N in the same range. The ^{15}N enrichment in *E. fibri* and *E. microphylla* that mainly partner with *Tuber* species in addition to a wide array of ECM B and ECM A is even above the so far documented mean ^{15}N enrichment of all orchid species associated with ECM fungi. However, it remains unclear which proportion of fungal N might originate from which exact fungal partner in *Epipactis* taxa that associate with several different mycorrhizal fungi. We detected the highest ^{15}N enrichment in *E. distans*, *E. muelleri*, *E. leptochila* and *E. neglecta* for which we exclusively identified ECM A such as *Wilcoxina rehmii* and *Tuber* (Table 1). Such a high enrichment in ^{15}N has never been documented before for any other orchid species regardless of fungal partner. Nevertheless, both the mean ^{15}N enrichment of 7·5 ‰ of *Epipactis* species exclusively associated with ECM B relative to sporocarps of ECM B and the mean ^{15}N enrichment of 9·6 ‰ of *Epipactis* species exclusively associated with ECM A relative to sporocarps of ECM A exceed by far the estimated increase of 2·2–3·4 ‰ δ^{15}N in the consumer versus its diet per trophic level in usual food chain interactions (VanderZanden and Rasmussen, 2001; McCutchan *et al*., 2003; Fry, 2006).

Still, the observed pattern of ^{15}N enrichment correlating with the presence of ECM A as orchid mycorrhizal fungi in a wide set of *Epipactis* species in our study challenges the conclusion by Dearnaley (2007) that the simple presence of ascomycete fungi in orchid roots does not necessarily indicate a functional association at least in this case study.

Total N concentrations in the leaves of all *Epipactis* species except *E. microphylla* were significantly higher than in the leaves of autotrophic reference species (Table 3; Table S2) and our finding here confirms the overall picture that mean N concentrations in partially mycoheterotrophic Orchidaceae are generally twice as high as in autotrophic plants (Hynson *et al*., 2016).

Stable isotope natural abundances – fungal species

Our results confirm the findings by Hobbie *et al*. (2001) and Mayor *et al*. (2009) that ECM fungi are significantly more enriched in ^{15}N and depleted in ^{13}C than saprotrophic fungi but we here provide further isotopic evidence to distinguish ECM A and ECM B: ECM A are significantly more enriched in ^{15}N and depleted in ^{13}C compared to ECM B (Fig. 3). Possible
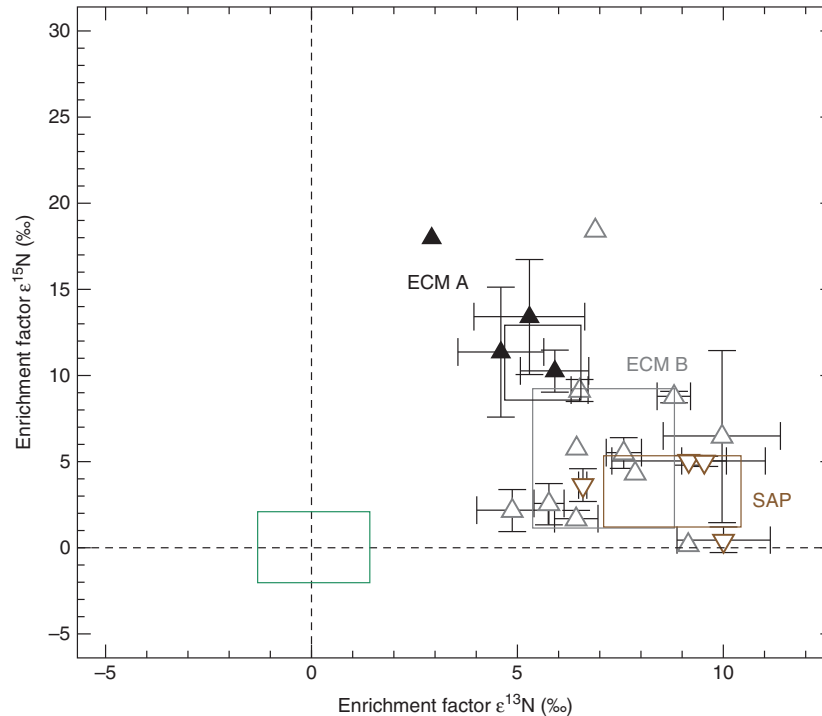
FIG. 3. Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N \pm 1$ s.d. as calculated for sporocarps of four ECM ascomycete *Tuber* species (filled black upward triangles), 11 ECM basidiomycete species (open grey upward triangles) and four saprotrophic basidiomycete species (open brown downward triangles). All open symbols indicate data extracted from Gebauer *et al.* (2016) (Table S2). The green box represents mean enrichment factors ± 1 s.d. for the autotrophic reference plants that were sampled at the same sites as the fungal sporocarps (REF, $n = 228$, see Tables S1 and S2) whereas mean ε values of reference plants are zero by definition. The black, grey and brown boxes represent mean enrichment factors ± 1 s.d. of the ECM ascomycetes (ECM A), ECM basidiomycetes (ECM B) and saprotrophic basidiomycetes (SAP), respectively.

explanations for the observed pattern lie in the truffle genomic traits (Martin *et al.*, 2010). Fungal genomics allows for a reverse ecology approach, enabling the autecology of a fungal species to be predicted from its genetic repertoire. *Tuber melanosporum* Vittad., an example of a true truffle species of high economic value and therefore entirely sequenced, has a large genome (125 Mb) but few protein-coding genes (approx. 7500), exhibiting a low similarity to genomes of other already genetically analysed fungi. In their study on genome size of 172 fungal species, Mohanta and Bae (2015) report an average genome size of 46·48 Mb with a mean number of 15431·51 protein coding genes for basidiomycetes and a mean genome size of 36·91.Mb and 11129·45 protein coding genes for ascomycetes. Furthermore, the sequence similarity of proteins predicted for *T. melanosporum* was only significant for three out of 7496 predicted proteins compared to other ascomycete species (Martin *et al.*, 2010). The ascomycete phylum separated approx. 450 Mya from other ancestral fungal lineages, indicating why truffles (*T. melanosporum* in particular) might have a different enzymatic setup (Martin *et al.*, 2010).

We also find that SAP fungi are more enriched in ^{13}C compared to ECM fungi as they act as decomposers whereas ECM fungi receive carbon from their hosts (Mayor *et al.*, 2009; Gebauer *et al.*, 2016). We furthermore observe here that ECM B are more enriched in ^{13}C than ECM A and explain the

perceived pattern by a possibly wider suite of decomposing enzymes of ECM B compared to ECM A. For example, the ECM A *T. melanosporum* has many fewer glycoside hydrolase-encoding genes compared to saprotrophic fungi (Martin *et al.*, 2010).

Here we showed that ECM A of the genus *Tuber* are significantly more enriched in ^{15}N than ECM B and SAP fungi. Our results confirm the high $\delta^{15}N$ values published by Hobbie *et al.* (2001) for *Tuber gibbosum* Harkn. (15·1 ‰) and the ECM ascomycete *Sowerbyella rhenana* (Fuckel) J. Moravec (17·2 ‰) sampled in Oregon, USA, that are to our knowledge the only so far published stable isotope abundance data for ECM ascomycetes. A relationship between an increase in ^{15}N enrichment with increasing soil depth exploitation of fungi and increase of recalcitrance of soil organic matter has previously been shown and corresponds well with the hypogeous nature of the ECM A species from literature records and findings of this study (Nadelhoffer and Fry, 1988; Gebauer and Schulze, 1991; Taylor *et al.*, 1997). Taylor *et al.* (1997) reported the highest $\delta^{15}N$ values for the ECM B *Suillus bovinus* (L.) Kuntze (11·1 ‰) and *Cortinarius traganus* var. *finitimum* Fr. (15·4 ‰), two species of which ECM was reported to occur throughout the organic layer and down into mineral layers of the subsoil (Taylor *et al.*, 1997; Rosling *et al.*, 2003). Furthermore, we hypothesize the existence of a different set of exoenzymes for

access to recalcitrant N compounds in soil organic matter for ECM A, providing ECM A access to N sources unavailable for most ECM B. Recalcitrant soil organic matter is known to become increasingly enriched in ^{15}N with ongoing N decomposition (Nadelhoffer and Fry, 1988; Gebauer and Schulze, 1991). Different physiology in soil organic matter decomposition by ECM B and ECM A is a matter for future investigations.

CONCLUSIONS

In summary, we highlight a true functional role of ascomycete fungi in the roots of *Epipactis* species. This finding emerged from the unique ^{15}N enrichments found for those *Epipactis* spp. associated solely with ECM A and the simultaneous finding of unique ^{15}N enrichment of ascomycete sporocarps. Based on this finding we also conclude that the linear two-source mixing model approach to estimate N gains from the fungal source requires knowledge of both the fungal identity and N isotope composition. The relationship between fungal types and ^{15}N enrichment of *Epipactis* ssp. appears to be as follows: ^{15}N enrichment in *Epipactis* spp. associated with orchid mycorrhizal rhizoctonias $<$ ^{15}N enrichment in *Epipactis* spp. associated with ECM B $<$ ^{15}N enrichment in *Epipactis* spp. associated with ECM A and B $<$ ^{15}N enrichment in *Epipactis* spp. exclusively associated with ECM A. Thus, we can now no longer exclude that all mycorrhizal orchids, irrespective of the identity of their fungal host, cover all of their N demands through the fungal source. Based on comparisons of ^{15}N enrichments in initially mycoheterotrophic protocorms and partially mycoheterotrophic adults of *E. helleborine*, a full coverage of the N demand by partially mycoheterotrophic orchids was proposed by Stöckel *et al.* (2014). Our findings extend this hypothesis to adults of ten additional species of *Epipactis* and urge for further studies of other orchid genera.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: single and mean δ^{15}N and δ^{13}C values, single and mean enrichment factors ε^{15}N and ε^{13}C, and single and mean total nitrogen concentration data of all original plant and fungal samples in this study. Table S2: single and mean δ^{15}N and δ^{13}C values, single and mean enrichment factors ε^{15}N and ε^{13}C, and single and mean total nitrogen concentration data of all plant and fungal samples extracted from the literature. Table S3: orchid mycorrhizal fungi detected in the roots of *Epipactis* species extracted from all available publications

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Table S1: Single and mean δ^{15}N and δ^{13}C values, single and mean enrichment factors ε^{15}N and ε^{13}C and single and mean total nitrogen concentration data of all original plant and fungal samples in this study.

Species	Functional type	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Epipactis helleborine (L.) Crantz	*Epipactis* species with ECM A & B	ruderal site 1	1	2,366	9,68	-29,17	9,13	2,87
			2	2,642	13,69	-27,03	13,32	4,09
			3	2,856	9,45	-28,91	9,69	3,08
			4	1,941	12,59	-29,23	12,85	2,77
			5	1,902	12,09	-29,97	11,62	-0,15
			mean	**2,34**	**11,50**	**-28,86**	**11,32**	**2,53**
			SD	**0,42**	**1,86**	**1,09**	**1,86**	**1,59**
			n	**5**				
Epipactis helleborine (L.) Crantz	*Epipactis* species with ECM A & B	forest site 2	6	1,687	13,25	-29,77	11,41	1,89
			7	1,734	14,88	-30,01	12,41	1,79
			8	1,851	14,62	-30,45	12,15	1,00
			9	2,271	15,96	-29,28	14,38	2,21
			10	1,965	13,16	-28,35	9,80	2,84
			mean	**1,90**	**14,37**	**-29,57**	**12,03**	**1,94**
			SD	**0,23**	**1,18**	**0,80**	**1,66**	**0,67**
			n	**5**				
Epipactis helleborine (L.) Crantz	*Epipactis* species with ECM A & B	ruderal site 1 & forest site 2	mean	**2,12**	**12,94**	**-29,22**	**11,67**	**2,24**
			SD	**0,40**	**2,11**	**0,98**	**1,70**	**1,19**
			n	**10**				
Epipactis helleborine subsp. *neerlandica* (Verm.) Buttler	*Epipactis* species with ECM B	dune site 3	11	2,200	5,46	-26,54	11,32	2,24
			12	1,584	1,17	-27,56	6,65	1,09
			13	2,005	4,79	-25,25	9,27	3,48
			14	1,806	2,68	-27,17	7,76	1,80
			15	1,619	0,07	-27,22	4,88	1,76
			mean	**1,84**	**2,83**	**-26,75**	**7,98**	**2,07**
			SD	**0,26**	**2,30**	**0,92**	**2,46**	**0,89**
			n	**5**				
Epipactis microphylla (Ehrh.) Sw.	*Epipactis* species with ECM B	forest site 4	16	2,023	21,61	-26,52	25,48	6,22
			17	1,448	18,53	-25,97	21,82	6,84
			18	1,563	19,88	-27,50	22,76	4,65
			19	1,172	15,05	-27,65	18,09	5,15
			20	1,336	16,08	-26,87	19,38	5,61
			mean	**1,51**	**18,23**	**-26,90**	**21,51**	**5,70**
			SD	**0,32**	**2,69**	**0,69**	**2,90**	**0,86**
			n	**5**				
Epipactis purpurata Sm.	*Epipactis* species with ECM B	forest site 5	21	2,626	16,87	-27,24	19,99	5,54
			22	2,585	17,03	-27,27	20,02	5,13
			23	2,326	9,16	-25,91	13,38	6,16
			24	2,965	13,99	-26,45	17,45	7,53
			25	2,873	13,23	-27,28	16,21	6,17
			mean	**2,68**	**14,06**	**-26,83**	**17,41**	**6,11**
			SD	**0,25**	**3,22**	**0,63**	**2,79**	**0,91**
			n	**5**				
Epipactis distans Arv.-Touv.	*Epipactis* species with ECM A	forest site 6	26	2,573	11,20	-26,97	17,31	4,43
			27	2,170	6,77	-28,86	12,40	2,16
			28	1,579	10,94	-27,96	18,72	2,98
			29	2,107	9,74	-26,77	16,66	3,96
			30	2,698	8,20	-27,20	14,78	4,28
			mean	**2,23**	**9,37**	**-27,55**	**15,98**	**3,56**
			SD	**0,44**	**1,88**	**0,86**	**2,45**	**0,97**
			n	**5**				

Table S1 (continued)

Species	Functional type	Site	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]
Epipactis leptochila (Godfery) Godfery	*Epipactis* species with ECM A	forest site 7	31	2,583	20,57	-28,14	24,51	5,73
			32	2,691	16,33	-28,90	20,37	3,96
			33	2,461	18,99	-29,19	22,93	3,92
			34	2,783	21,13	-27,62	25,77	5,15
			35	2,610	17,68	-28,53	21,98	5,03
			mean	**2,63**	**18,94**	**-28,48**	**23,11**	**4,76**
			SD	**0,12**	**1,99**	**0,62**	**2,11**	**0,80**
			n	**5**				
Epipactis muelleri Godfery	*Epipactis* species with ECM A	forest site 8	36	2,812	21,77	-28,61	23,33	4,34
			37	2,751	19,42	-29,04	21,28	4,27
			38	2,964	19,27	-28,62	21,39	4,56
			39	2,885	14,72	-29,97	17,17	2,53
			40	3,123	17,42	-29,58	20,48	3,45
			mean	**2,91**	**18,52**	**-29,16**	**20,73**	**3,83**
			SD	**0,14**	**2,63**	**0,60**	**2,25**	**0,84**
			n	**5**				
Epipactis neglecta (Kümpel) Kümpel	*Epipactis* species with ECM A	forest site 9	41	2,556	21,71	-27,81	24,73	5,61
			42	2,476	19,41	-29,66	22,92	3,55
			43	2,477	21,55	-27,74	24,22	4,25
			44	2,913	21,35	-27,41	23,97	4,70
			45	2,351	23,75	-28,65	27,14	3,03
			mean	**2,55**	**21,55**	**-28,25**	**24,60**	**4,23**
			SD	**0,21**	**1,54**	**0,91**	**1,57**	**1,00**
			n	**5**				
Tuber aestivum Vittad.	obligate ECM Ascomycota	forest site 8	NA	3,389	6,61	-27,18	8,95	5,92
			NA	3,587	4,27	-28,80	6,62	4,30
			NA	3,244	8,92	-27,67	11,27	5,43
		forest site 10	NA	2,674	9,83	-28,14	14,78	3,97
			NA	2,829	7,74	-27,52	12,69	4,59
			mean	**3,14**	**7,47**	**-27,86**	**10,86**	**4,84**
			SD	**0,38**	**2,16**	**0,63**	**3,18**	**0,81**
			n	**5**				
Tuber brumale Vittad.	obligate ECM Ascomycota	forest site 10	NA	3,654	11,79	-28,60	16,74	3,51
			n	**1**				
Tuber excavatum Vittad.	obligate ECM Ascomycota	forest site 7	NA	2,646	7,08	-27,38	10,95	6,11
			NA	2,519	8,08	-27,15	11,95	6,34
			NA	2,213	7,55	-27,24	11,43	6,25
		forest site 8	NA	2,665	8,89	-26,87	11,24	6,23
			NA	3,179	7,27	-27,17	9,62	5,92
			NA	3,407	7,97	-27,80	10,32	5,30
			NA	2,738	7,35	-27,57	9,70	5,53
			NA	3,572	7,11	-27,23	9,46	5,87
		forest site 9	NA	2,804	7,23	-26,43	10,09	6,61
			NA	2,885	5,98	-27,40	8,84	5,64
			NA	2,858	8,06	-27,65	10,92	5,38
			NA	2,961	7,99	-27,40	10,84	5,64
			NA	2,448	6,09	-26,24	8,95	6,80
			NA	2,841	5,12	-26,80	7,97	6,23
			NA	2,581	7,48	-26,48	10,34	6,56
			NA	2,797	6,54	-26,52	9,39	6,51
			NA	2,837	5,24	-26,68	8,10	6,36
			NA	2,482	9,83	-27,90	12,68	5,13
		forest site 10	NA	2,844	4,62	-28,36	9,57	3,75

Table S1 (continued)

Species	Functional type	Site	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]
Tuber excavatum			mean	2,80	7,13	-27,17	10,12	5,90
(continued)			SD	0,32	1,30	0,56	1,25	0,71
			n	19				
	obligate ECM							
Tuber rufum Pico	Ascomycota	forest site 8	NA	3,071	8,68	-26,85	11,03	6,24
		forest site 10	NA	2,459	9,57	-27,18	14,52	4,93
			mean	2,77	9,12	-27,02	12,77	5,59
			SD	0,43	0,63	0,23	2,47	0,93
			n	2				
	ECM A		mean	2,90	7,51	-27,34	10,70	5,59
			SD	0,38	1,71	0,65	2,20	0,91
			n	27				
	autotrophic							
Acer pseudoplatanus L.	reference plant	forest site 2	6	0,899	0,32	-31,89	-1,52	-0,23
			7	0,739	2,11	-32,19	-0,36	-0,39
			8	0,724	1,75	-33,13	-0,72	-1,68
			9	1,283	3,22	-31,18	1,63	0,31
			10	1,192	3,75	-29,96	0,38	1,23
		forest site 8	36	1,233	0,13	-32,30	1,69	0,66
			37	1,039	-1,22	-33,30	0,63	0,01
			38	1,395	-1,29	-32,07	0,83	1,11
			39	0,778	-2,71	-32,02	-0,26	0,49
			40	1,293	-3,96	-32,60	-0,89	0,42
Brachypodium sylvaticum	autotrophic							
(Huds) P.Beauv.	reference plant	forest site 7	NA	1,668	-0,59	-34,34	3,29	-0,85
		forest site 9	NA	1,844	-0,55	-33,26	2,31	-0,22
			NA	1,785	-3,51	-32,82	-0,65	0,22
			NA	1,767	-0,68	-33,50	2,17	-0,46
			NA	2,004	-1,81	-33,15	1,04	-0,11
	autotrophic							
Carex sp.	reference plant	forest site 8	NA	1,374	-2,92	-32,68	-0,58	0,42
		forest site 9	NA	1,560	0,58	-33,41	3,44	-0,38
	autotrophic							
Carpinus betulus L.	reference plant	forest site 5	21	1,693	-3,42	-32,83	-0,29	-0,05
			22	1,631	-3,25	-32,80	-0,26	-0,40
			23	1,497	-3,64	-31,61	0,58	0,46
			24	1,644	-3,90	-33,11	-0,44	0,87
			25	1,655	-3,48	-32,56	-0,50	0,89
	autotrophic							
Circaea lutetiana L.	reference plant	forest site 8	NA	1,016	-2,71	-33,23	-0,36	-0,13
	autotrophic							
Corylus avellana L.	reference plant	forest site 2	6	1,407	3,30	-31,16	1,47	0,50
			7	1,404	4,34	-31,40	1,88	0,40
			8	1,624	5,85	-29,79	3,38	1,65
			9	1,195	-0,57	-32,00	-2,16	-0,51
			10	1,225	3,62	-32,13	0,25	-0,94
		forest site 6	26	1,013	-6,34	-31,01	-0,23	0,39
			27	1,435	-4,96	-30,00	0,67	1,02
			28	1,250	-5,75	-31,68	2,04	-0,74
			29	1,303	-7,33	-29,89	-0,41	0,83
			30	1,248	-6,39	-31,16	0,20	0,32
	autotrophic							
Euphorbia cyparissias L.	reference plant	forest site 6	26	1,708	-6,04	-30,87	0,07	0,53
			27	1,430	-6,69	-30,79	-1,06	0,23
			28	1,337	-9,33	-29,56	-1,54	1,38
			29	1,515	-6,75	-30,14	0,16	0,58
			30	1,473	-6,36	-30,79	0,23	0,69
	autotrophic							
Fagus sylvatica L.	reference plant	forest site 2	6	1,269	1,89	-31,92	0,05	-0,27

Table S1 (continued)

Species	Functional type	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Fagus sylvatica			7	1,207	0,95	-31,80	-1,52	0,00
(continued)			8	1,086	-0,20	-31,42	-2,67	0,03
			9	1,037	2,12	-31,29	0,53	0,20
			10	1,403	2,73	-31,48	-0,63	-0,28
		forest site 4	16	1,698	-2,28	-32,50	1,58	0,25
			17	1,627	-2,08	-32,52	1,21	0,30
			18	1,805	-1,60	-31,51	1,28	0,65
			19	1,776	-1,63	-32,46	1,40	0,34
			20	1,554	-2,33	-32,45	0,97	0,03
		forest site 5	21	1,960	-2,26	-33,67	0,87	-0,89
			22	1,963	-2,17	-33,53	0,82	-1,13
			23	1,903	-4,63	-32,33	-0,41	-0,26
			24	1,873	-2,69	-35,60	0,77	-1,62
			25	2,011	-1,64	-34,54	1,34	-1,09
		forest site 6	26	1,365	-5,96	-32,31	0,15	-0,91
			27	1,600	-5,24	-32,27	0,39	-1,25
			28	1,195	-8,28	-31,58	-0,49	-0,64
			29	1,487	-6,66	-32,14	0,25	-1,41
			30	1,546	-7,02	-32,49	-0,43	-1,01
		forest site 7	31	1,373	-4,34	-35,08	-0,40	-1,20
			32	1,418	-4,32	-33,87	-0,28	-1,01
			33	1,366	-3,78	-33,78	0,16	-0,68
			34	1,405	-4,36	-33,51	0,27	-0,74
			35	1,427	-4,61	-34,38	-0,32	-0,81
		forest site 8	36	1,397	-3,32	-33,90	-1,76	-0,95
			37	1,470	-2,95	-34,07	-1,09	-0,76
			38	1,375	-3,17	-33,88	-1,05	-0,70
			39	1,753	-3,30	-32,07	-0,85	0,43
			40	1,191	-4,33	-33,77	-1,26	-0,74
		forest site 9	41	1,447	-3,46	-33,72	-0,44	-0,31
			42	1,727	-4,22	-33,25	-0,71	-0,05
			43	1,773	-2,86	-32,31	-0,19	-0,32
			44	1,629	-4,02	-32,76	-1,39	-0,65
			45	1,658	-3,22	-32,37	0,18	-0,68
Fragaria vesca L.	autotrophic reference plant	forest site 7	31	1,286	-4,05	-32,41	-0,10	1,47
			32	1,091	-4,08	-32,25	-0,04	0,61
			33	1,047	-4,77	-31,91	-0,83	1,19
			34	0,867	-5,07	-30,91	-0,44	1,86
			35	1,398	-3,95	-32,76	0,35	0,81
		forest site 9	NA	1,596	-4,88	-32,07	-2,02	0,97
Fraxinus excelsior L.	autotrophic reference plant	forest site 4	16	1,251	-5,46	-32,61	-1,59	0,13
			17	1,258	-5,13	-33,16	-1,84	-0,34
			18	1,099	-4,56	-32,62	-1,68	-0,46
			19	1,078	-4,68	-32,79	-1,64	0,01
			20	1,223	-4,53	-32,23	-1,22	0,25
Galium odoratum (L.) Scop.	autotrophic reference plant	forest site 8	NA	1,085	-2,03	-34,91	0,32	-1,82
		forest site 7	NA	1,138	-3,39	-35,32	0,49	-1,83
Galium verum L.	autotrophic reference plant	dune site 3	11	1,437	-5,37	-29,73	0,49	-0,96
			12	1,302	-5,46	-30,52	0,02	-1,87
			13	1,546	-3,31	-30,55	1,17	-1,82
			14	1,258	-5,15	-31,20	-0,06	-2,23
			15	1,361	-3,65	-31,41	1,17	-2,44
Hedera helix L.	autotrophic reference plant	ruderal site 1	1	1,020	0,52	-28,61	-0,04	3,42
			2	1,111	-0,97	-29,41	-1,35	1,71
			3	0,824	-0,64	-29,47	-0,40	2,51
			4	1,044	0,45	-29,90	0,70	2,10
			5	0,783	-1,82	-27,10	-2,29	2,72
		forest site 4	16	1,142	-3,86	-33,12	0,01	-0,38

Table S1 (continued)

Species	Functional type	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Hedera helix			17	1,174	-2,66	-32,77	0,63	0,04
(continued)			18	1,243	-2,48	-32,34	0,40	-0,19
			19	1,310	-2,80	-33,15	0,24	-0,35
			20	1,418	-3,06	-32,76	0,25	-0,28
		forest site 8	NA	1,202	-0,97	-33,64	1,38	-0,54
Impatiens parviflora DC.	autotrophic reference plant	forest site 9	41	1,874	-3,68	-33,13	-0,66	0,28
			42	1,509	-3,11	-33,95	0,41	-0,75
			43	2,080	-1,85	-31,77	0,82	0,22
			44	1,779	0,04	-31,50	2,66	0,61
			45	1,898	-2,52	-30,93	0,87	0,75
Scorzoneroides autumnalis (L.) Moench	autotrophic reference plant	dune site 3	11	1,408	-6,56	-29,49	-0,70	-0,72
			12	1,796	-5,67	-28,38	-0,19	0,28
			13	1,725	-5,58	-28,46	-1,10	0,27
			14	1,903	-4,87	-28,53	0,22	0,44
			15	1,623	-6,17	-28,46	-1,35	0,51
Leontodon sp.	autotrophic reference plant	ruderal site 1	1	1,030	-0,92	-33,45	-1,48	-1,41
			2	1,444	-0,87	-32,06	-1,25	-0,93
			3	1,375	1,19	-32,92	1,43	-0,93
			4	1,769	-0,69	-32,20	-0,44	-0,20
			5	1,595	3,46	-30,95	2,99	-1,13
Lamium sp.	autotrophic reference plant	forest site 9	NA	1,787	-5,74	-35,18	-2,89	-2,14
			NA	2,038	-4,67	-34,24	-1,81	-1,21
			NA	1,436	-3,96	-34,28	-1,10	-1,24
			NA	2,333	-3,38	-33,36	-0,52	-0,33
Oxalis acetosella L.	autotrophic reference plant	forest site 7	NA	1,113	-1,82	-33,15	2,05	0,34
Picea abies (L.) H.Karst	autotrophic reference plant	forest site 8	NA	0,834	-3,75	-32,13	-1,41	0,97
		forest site 7	NA	0,778	-5,24	-34,96	-1,36	-1,47
Quercus robur L.	autotrophic reference plant	forest site 5	21	1,883	-3,71	-31,85	-0,58	0,94
			22	1,920	-3,56	-30,86	-0,56	1,53
			23	1,780	-4,39	-32,26	-0,17	-0,20
			24	1,864	-3,79	-33,23	-0,33	0,75
			25	1,688	-3,81	-33,26	-0,83	0,19
Salix repens L.	autotrophic reference plant	dune site 3	11	1,220	-5,65	-27,10	0,21	1,67
			12	1,228	-5,31	-27,06	0,17	1,59
			13	1,168	-4,54	-27,18	-0,07	1,55
			14	1,110	-5,24	-27,18	-0,16	1,79
			15	1,151	-4,63	-27,04	0,19	1,93
Sorbus aucuparia L.	autotrophic reference plant	forest site 9	43	1,163	-3,30	-31,89	-0,63	0,10
			44	1,112	-3,90	-32,07	-1,27	0,04
			45	1,229	-4,45	-31,75	-1,05	-0,07
Taraxacum officinale (L.) Weber ex F.H.Wigg.	autotrophic reference plant	ruderal site 1	1	1,877	2,08	-34,05	1,52	-2,02
			2	2,213	2,97	-31,91	2,59	-0,78
			3	1,729	-1,27	-33,56	-1,03	-1,58
			4	2,247	-0,52	-33,91	-0,26	-1,90
			5	1,774	-0,22	-31,40	-0,69	-1,59

Table S1 (continued)

Species	Functional type	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Viola sp.	autotrophic reference plant	forest site 7	31	1,568	-3,44	-34,15	0,50	-0,27
			32	1,194	-3,73	-32,46	0,32	0,40
			33	1,330	-3,27	-33,61	0,67	-0,51
			34	1,113	-4,47	-33,89	0,17	-1,12
			35	1,317	-4,32	-33,57	-0,03	0,00
		forest site 8	36	1,480	-1,49	-32,66	0,07	0,30
			37	1,493	-1,41	-32,57	0,45	0,74
			38	1,426	-1,90	-33,58	0,22	-0,41
			39	1,099	-1,35	-33,42	1,11	-0,92
			40	0,961	-0,91	-32,71	2,16	0,32
		forest site 9	41	1,593	-1,92	-33,38	1,10	0,03
			42	1,274	-3,21	-32,40	0,30	0,80
		forest site 8	NA	1,288	-3,73	-33,56	-1,38	-0,46
		forest site 9	NA	1,556	-2,25	-33,83	0,61	-0,80
			NA	1,872	-1,99	-34,13	0,87	-1,09
			NA	1,831	-3,13	-33,58	-0,27	-0,54
			NA	1,855	-2,34	-33,48	0,52	-0,44
			NA	1,938	-1,73	-33,57	1,13	-0,53

Table S2: Single and mean δ¹⁵N and δ¹³C values, single and mean enrichment factors ε¹⁵N and ε¹³C and single and mean total nitrogen concentration data of all plant and fungal samples extracted from literature.

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ¹⁵N [‰]	δ¹³C [‰]	ε¹⁵N [‰]	ε¹³C [‰]
Epipactis atrorubens (Hoffm.) Besser	*Epipactis* species with ECM A & B	Gebauer & Meyer, 2003	NA	20	2,630	3,67	-26,46	9,92	2,56
		Bidartondo *et al.*, 2004	NA	65	2,902	1,11	-29,90	6,96	-0,26
				66	2,175	0,09	-28,35	5,48	1,51
				67	2,447	0,36	-29,14	5,88	0,81
				68	2,608	6,86	-29,06	11,78	0,30
		Tedersoo *et al.*, 2007	NA	330	2,282	8,13	-27,21	12,85	3,83
				331	2,060	6,25	-27,99	12,48	3,81
				332	2,257	8,97	-25,97	13,87	4,68
				333	2,309	9,95	-28,06	16,51	2,54
				334	2,134	5,37	-28,61	10,61	3,24
				335	1,844	6,98	-28,22	12,77	2,05
				mean	**2,33**	**5,25**	**-28,09**	**10,83**	**2,28**
				SD	**0,30**	**3,48**	**1,17**	**3,49**	**1,57**
				n	**11**				
Epipactis distans Arv.-Touv.	*Epipactis* species with ECM A	Bidartondo *et al.*, 2004	NA	53	2,425	1,86	-26,76	20,90	3,34
				54	2,352	12,10	-23,81	11,98	-0,35
				55	2,491	7,31	-23,24	16,21	4,03
				56	2,611	9,89	-24,53	16,33	2,50
				mean	**2,47**	**7,79**	**-24,59**	**16,36**	**2,38**
				SD	**0,11**	**4,41**	**1,54**	**3,64**	**1,92**
				n	**4**				
Epipactis fibri Scappat. & Robatsch	*Epipactis* species with ECM A & B	Gonneau *et al.*, 2014	NA	3	3,007	26,63	-26,02	31,44	6,61
				3	2,700	13,27	-28,11	18,08	4,52
				3	3,243	14,84	-26,97	19,65	5,66
				3	2,364	18,09	-27,06	22,90	5,57
				3	2,936	25,25	-26,09	30,06	6,54
				3	2,929	14,44	-28,07	19,25	4,56
				3	2,764	10,37	-28,64	15,18	3,99
				3	2,414	15,22	-29,76	20,03	2,87
				3	3,164	11,64	-26,69	16,45	5,94
				3	3,250	17,38	-27,04	22,19	5,59
				3	2,850	9,92	-28,52	14,73	4,11
				3	3,050	9,60	-26,48	14,41	6,15
				3	2,871	12,87	-25,76	17,69	6,87
				3	2,971	11,35	-26,40	16,16	6,23
				3	2,664	8,77	-27,43	13,58	5,20
				4	2,179	12,35	-29,58	13,82	2,12
				4	2,093	11,35	-30,41	12,82	1,30
				4	3,300	15,38	-25,61	16,85	6,09
				4	2,721	14,93	-28,44	16,40	3,27
				4	2,536	14,18	-28,65	15,65	3,05
				4	2,836	10,41	-28,97	11,88	2,73
				4	2,957	9,32	-29,70	10,79	2,01
				4	1,471	6,16	-30,67	7,64	1,04
				4	2,829	13,95	-29,06	15,43	2,64
				4	2,821	14,66	-25,70	16,13	6,00
				4	2,200	14,37	-29,81	15,85	1,89
				4	2,314	15,46	-28,83	16,94	2,87
				4	2,700	15,61	-27,27	17,09	4,43
				4	2,714	15,87	-28,41	17,34	3,29
				mean	**2,72**	**13,92**	**-27,94**	**17,12**	**4,25**
				SD	**0,40**	**4,33**	**1,50**	**4,92**	**1,77**
				n	**29**				
Epipactis gigantea Douglas ex Hook.	*Epipactis* species with Rhizoctonia	Zimmer *et al.*, 2007	NA	140	1,893	-0,79	-28,79	3,71	-1,61
				141	2,108	-1,53	-27,48	3,41	-0,26
				142	2,695	-1,83	-27,46	3,13	-0,94
				143	2,755	-2,42	-29,50	1,88	-2,00
				144	2,070	-0,99	-27,97	3,65	-1,12
				mean	**2,30**	**-1,51**	**-28,24**	**3,15**	**-1,19**
				SD	**0,39**	**0,66**	**0,89**	**0,75**	**0,66**
				n	**5**				

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]
Epipactis helleborine (L.) Crantz	*Epipactis* species with ECM A & B	Gebauer & Meyer, 2003	NA	19	2,678	12,51	-25,52	18,76	4,01
		Bidartondo *et al.*, 2004	NA	57	2,648	2,28	-28,04	6,76	1,45
				58	2,562	4,74	-28,14	9,95	1,48
				59	2,641	-0,11	-28,23	4,12	0,65
				60	2,498	4,14	-28,26	7,38	1,26
		Abadie *et al.*, 2006	NA	168	2,511	6,87	-29,39	11,39	-0,84
				169	2,863	14,00	-26,72	18,52	1,83
				170	2,786	12,63	-27,13	17,15	1,42
				171	2,264	10,84	-27,99	15,36	0,56
				172	2,654	7,85	-27,42	12,37	1,13
				173	2,948	12,61	-25,95	17,13	2,60
				174	2,869	13,89	-26,06	18,41	2,49
				175	2,551	7,79	-27,18	12,31	1,37
		Liebel *et al.*, 2010	NA	524	4,080	14,09	-26,16	16,60	8,03
				525	4,079	7,55	-26,10	10,20	8,68
				526	3,094	8,89	-28,86	9,84	5,01
				527	3,763	12,73	-26,70	13,54	6,59
				528	3,381	15,00	-28,06	15,71	4,98
		Johansson *et al.*, 2014	NA	634	1,940	12,19	-29,78	14,79	1,42
				634	1,730	13,77	-30,13	16,37	1,06
				634	1,522	4,13	-32,63	6,73	-1,44
				mean	**2,76**	**9,45**	**-27,83**	**13,02**	**2,56**
				SD	**0,66**	**4,46**	**1,70**	**4,40**	**2,70**
				n	**21**				
Epipactis leptochila (Godfery) Godfery	*Epipactis* species with ECM A	Hynson *et al.*, 2016	NA	629	4,800	17,57	-29,50	22,76	5,02
				630	4,810	17,24	-27,02	21,67	5,96
				631	5,170	17,07	-27,51	21,77	4,68
				632	3,990	17,33	-27,93	22,43	6,48
				mean	**4,69**	**17,30**	**-27,99**	**22,16**	**5,54**
				SD	**0,50**	**0,21**	**1,07**	**0,52**	**0,83**
				n	**4**				
Epipactis palustris (L.) Crantz	*Epipactis* species with Rhizoctonia	Bidartondo *et al.*, 2004	NA	79	1,410	0,21	-28,65	3,72	0,18
				80	2,110	0,88	-28,93	3,81	0,64
				81	2,080	0,45	-29,97	3,14	-0,80
				82	1,950	0,32	-29,42	3,32	0,12
				mean	**1,89**	**0,47**	**-29,24**	**3,50**	**0,03**
				SD	**0,33**	**0,29**	**0,58**	**0,32**	**0,60**
				n	**4**				
Craterellus tubaeformis (Fr.) Quél.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	1,577	-3,38	-25,47	1,57	6,64
				NA	1,793	-3,55	-25,13	1,41	6,98
				NA	1,727	-3,23	-25,49	1,73	6,63
				NA	1,711	-2,47	-26,53	2,49	5,58
				NA	1,679	-3,73	-25,75	1,22	6,36
				mean	**1,70**	**-3,27**	**-25,67**	**1,68**	**6,44**
				SD	**0,08**	**0,49**	**0,53**	**0,49**	**0,53**
				n	**5**				
Russula cyanoxantha (Schaeff.) Fr.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,364	-0,86	-26,96	4,09	5,15
				NA	2,554	-3,89	-26,16	1,06	5,96
				NA	2,055	-1,68	-26,38	3,27	5,73
				NA	2,863	-3,14	-26,16	1,82	5,95
				NA	2,351	-2,58	-26,04	2,37	6,07
				mean	**2,44**	**-2,43**	**-26,34**	**2,52**	**5,77**
				SD	**0,30**	**1,19**	**0,37**	**1,19**	**0,37**
				n	**5**				

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Russula nobilis Velen.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,092	-4,33	-26,90	0,62	5,21
				NA	2,944	-1,85	-28,35	3,10	3,76
				NA	2,728	-2,74	-27,41	2,21	4,70
				NA	2,749	-1,41	-26,00	3,54	6,11
				NA	3,111	-3,68	-27,48	1,27	4,63
				mean	**2,72**	**-2,80**	**-27,23**	**2,15**	**4,88**
				SD	**0,39**	**1,22**	**0,86**	**1,22**	**0,86**
				n	**5**				
Lactarius deterrimus Gröger	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,254	0,87	-24,38	5,82	7,73
				NA	2,760	-0,83	-25,27	4,12	6,84
				NA	2,557	1,07	-24,24	6,02	7,87
				NA	2,786	1,43	-24,23	6,38	7,88
				NA	2,443	0,19	-24,47	5,14	7,64
				mean	**2,56**	**0,55**	**-24,52**	**5,50**	**7,59**
				SD	**0,22**	**0,89**	**0,43**	**0,89**	**0,43**
				n	**5**				
Cortinarius sp.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	5,659	-2,66	-21,15	2,29	10,96
				NA	5,023	-1,95	-21,06	3,00	11,05
				NA	4,949	-1,81	-21,12	3,14	11,00
				NA	2,472	6,22	-23,92	11,17	8,19
				NA	2,599	7,65	-23,46	12,60	8,65
				mean	**4,14**	**1,49**	**-22,14**	**6,44**	**9,97**
				SD	**1,49**	**5,01**	**1,42**	**5,01**	**1,42**
				n	**5**				
Hydnum rufescens Pers.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,703	4,15	-22,84	9,10	9,27
				NA	2,317	3,69	-23,49	8,64	8,62
				NA	2,472	3,51	-23,59	8,46	8,52
				mean	**2,50**	**3,78**	**-23,31**	**8,73**	**8,80**
				SD	**0,19**	**0,33**	**0,41**	**0,33**	**0,41**
				n	**3**				
Inocybe fastigiata (Schiff. ex Fr.) Quél.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	4,953	-0,67	-24,24	4,29	7,88
Laccaria laccata (Scop.) Cooke	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,934	-4,80	-22,95	0,15	9,16
Thelephora sp.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	3,046	0,81	-25,66	5,76	6,45
Tarzetta catinus Holmsk.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	2,748	3,37	-25,52	8,32	6,59
				NA	2,680	3,88	-25,38	8,84	6,73
				NA	2,983	5,03	-25,87	9,98	6,24
				NA	2,634	4,09	-25,74	9,04	6,38
				NA	2,894	4,52	-25,46	9,47	6,66
				mean	**3,11**	**2,03**	**-25,10**	**6,98**	**7,01**
				SD	**0,76**	**3,38**	**1,00**	**3,38**	**1,00**
				n	**5**				
Ramaria formosa (Pers.) Quél.	obligate ECM Basidiomycota	Gebauer *et al.*, 2016	forest site 10	NA	3,796	13,45	-25,22	18,41	6,89
				mean	**2,81**	**0,24**	**-25,01**	**5,19**	**7,10**
				SD	**0,95**	**4,03**	**1,73**	**4,03**	**1,73**
				n	**37**				
Geastrum fimbriatum Fr.	SAP fungus	Gebauer *et al.*, 2016	forest site 10	NA	5,632	-0,097	-21,628	4,86	10,48
				NA	4,380	0,285	-24,264	5,24	7,85
				mean	**5,01**	**0,09**	**-22,95**	**5,05**	**9,16**
				SD	**0,89**	**0,27**	**1,86**	**0,27**	**1,86**
				n	**2**				

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Gymnopus dryophilus (Bull. Fr.) Murrill	**SAP fungus**	Gebauer *et al.*, 2016	forest site 10	NA	2,315	-0,28	-25,39	4,68	6,72
				NA	2,497	-1,65	-25,65	3,30	6,46
				NA	2,207	-0,48	-25,57	4,47	6,54
				NA	2,481	-2,58	-25,53	2,37	6,58
				NA	2,204	-1,64	-25,46	3,31	6,65
				mean	**2,34**	**-1,33**	**-25,52**	**3,63**	**6,59**
				SD	**0,14**	**0,95**	**0,10**	**0,95**	**0,10**
				n	**5**				
Gymnopus fusipes (Bull. Fr.) Gray	**SAP fungus**	Gebauer *et al.*, 2016	forest site 10	NA	5,297	0,30	-23,03	5,25	9,08
				NA	5,386	-0,34	-21,86	4,62	10,25
				NA	5,362	0,47	-22,33	5,42	9,78
				NA	5,262	-0,02	-22,44	4,93	9,67
				NA	4,011	-0,08	-23,19	4,87	8,92
				mean	**5,06**	**0,07**	**-22,57**	**5,02**	**9,54**
				SD	**0,59**	**0,32**	**0,54**	**0,32**	**0,54**
				n	**5**				
Lycoperdon perlatum Pers.	**SAP fungus**	Gebauer *et al.*, 2016	forest site 10	NA	7,182	-5,421	-21,024	-0,47	11,09
				NA	6,673	-4,052	-23,273	0,90	8,84
				NA	7,379	-4,362	-21,336	0,59	10,77
				NA	6,134	-5,083	-21,461	-0,13	10,65
				NA	6,915	-3,678	-23,371	1,27	8,74
				mean	**6,86**	**-4,52**	**-22,09**	**0,43**	**10,02**
				SD	**0,48**	**0,72**	**1,13**	**0,72**	**1,13**
				n	**5**				
	SAP			**mean**	**4,78**	**-1,69**	**-23,34**	**3,26**	**8,77**
				SD	**1,85**	**2,07**	**1,67**	**2,07**	**1,67**
				n	**17**				
Acer pseudoplatanus L.	**autotrophic reference plant**	Zimmer *et al.*, 2008	forest site 10	NA	1,154	-6,73	-33,62	-1,78	-1,51
				NA	1,468	-7,61	-33,37	-2,66	-1,26
				NA	1,039	-6,71	-33,59	-1,76	-1,48
				NA	1,121	-5,45	-32,32	-0,50	-0,21
				NA	NA	-4,13	-32,43	0,82	-0,32
				NA	NA	-5,01	-33,35	-0,06	-1,24
				NA	NA	-5,24	-32,87	-0,29	-0,76
				NA	NA	-4,09	-32,33	0,86	-0,22
				NA	NA	-5,32	-32,92	-0,37	-0,81
		Preiss *et al.*, 2010	forest site 10	NA	1,287	-4,81	-33,71	0,14	-1,60
				NA	1,295	-4,00	-34,31	0,95	-2,19
Aegopodium podagraria L.	**autotrophic reference plant**	Bidartondo *et al.*, 2004	NA	59	1,260	-4,61	-29,86	-0,38	-0,98
				65	1,788	-7,51	-28,81	-1,66	0,83
				66	1,461	-6,07	-28,59	-0,68	1,27
				67	1,836	-6,53	-29,03	-1,01	0,92
				68	1,650	-5,74	-27,49	-0,82	1,87
		Bidartondo *et al.*, 2004	forest site 10	NA	1,260	-4,61	-29,86	0,34	2,25
Arctostaphylos uva-ursi (L.) Spreng.	**autotrophic reference plant**	Tedersoo *et al.*, 2007	NA	330	0,780	-3,72	-31,38	0,99	-0,34
				331	0,707	-5,28	-31,87	0,94	-0,06
				332	0,830	-3,70	-31,13	1,21	-0,48
				333	0,745	-5,29	-30,57	1,27	0,02
				334	0,683	-2,91	-31,79	2,33	0,06
				335	0,734	-5,57	-30,61	0,22	-0,34
Arctostaphylos patula Greene	**autotrophic reference plant**	Zimmer *et al.*, 2007	NA	140	0,974	-3,90	-27,85	0,60	-0,67
				141	0,772	-6,28	-25,94	-1,35	1,28

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Arctostaphylos patula (continued)				142	0,664	-5,02	-26,76	-0,06	-0,24
				143	0,847	-3,35	-25,91	0,95	1,59
				144	0,818	-3,21	-25,47	1,43	1,38
Briza media L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	79	NA	-2,19	-29,61	1,32	-0,78
				80	NA	-2,94	-29,43	-0,01	0,14
				81	NA	-2,73	-29,72	-0,04	-0,55
				82	NA	-3,00	-29,64	0,00	-0,10
Carex flacca Schreb.	autotrophic reference plant	Preiss *et al.*, 2010	forest site 10	NA	1,372	-3,13	-31,38	1,82	0,73
				NA	1,408	-0,51	-31,90	4,44	0,21
				NA	1,535	-0,44	-32,86	4,51	-0,75
				NA	1,682	-2,00	-33,37	2,95	-1,26
				NA	1,624	-1,31	-32,80	3,64	-0,68
				NA	1,534	-2,82	-32,86	2,14	-0,75
				NA	1,385	-1,26	-32,05	3,70	0,06
				NA	1,665	-2,42	-32,55	2,53	-0,44
				NA	1,721	-2,96	-31,70	1,99	0,41
				NA	1,804	-3,29	-31,46	1,66	0,65
				NA	1,352	-0,89	-31,58	4,07	0,53
				NA	1,462	-0,59	-32,31	4,37	-0,19
				NA	1,471	-0,73	-31,64	4,22	0,47
				NA	1,512	-2,20	-32,37	2,75	-0,26
				NA	1,584	-0,70	-33,10	4,26	-0,99
				NA	1,258	-0,92	-33,20	4,03	-1,09
				NA	1,413	-1,67	-32,71	3,28	-0,59
				NA	1,442	-1,50	-33,12	3,45	-1,01
				NA	1,494	-1,37	-32,60	3,58	-0,49
				NA	1,454	-1,42	-31,73	3,53	0,38
				NA	1,613	-0,78	-32,21	4,17	-0,10
				NA	1,285	0,95	-32,09	5,90	0,02
				NA	1,499	1,77	-32,46	6,72	-0,35
				NA	1,749	-1,61	-34,40	3,35	-2,29
Calocedrus decurrens (Torr.) Florin	autotrophic reference plant	Zimmer *et al.*, 2007	NA	140	0,452	-6,65	-27,17	-2,16	0,01
				141	0,534	-6,35	-26,42	-1,42	0,80
				142	0,598	-5,82	-27,02	-0,86	-0,50
				143	0,456	-5,59	-28,04	-1,29	-0,54
				144	0,481	-6,01	-27,35	-1,37	-0,50
Colchicum autumnale L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	79	NA	-4,06	-30,10	-0,55	-1,27
				80	NA	-2,53	-30,54	0,40	-0,97
				81	NA	-2,62	-29,76	0,07	-0,59
				82	NA	-2,06	-29,98	0,94	-0,44
Convallaria majalis L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	57	1,845	-5,57	-27,19	-1,09	2,30
				58	1,551	-5,32	-28,38	-0,11	1,24
				59	1,498	-3,41	-27,17	0,82	1,71
				60	1,411	-3,27	-27,98	-0,03	1,54
		Zimmer *et al.*, 2008	forest site 10	NA	1,816	-8,24	-30,83	-3,29	1,28
				NA	1,578	-5,67	-31,63	-0,72	0,48
				NA	1,491	-6,97	-31,41	-2,02	0,70
				NA	1,924	-8,80	-31,97	-3,85	0,14
				NA	NA	-4,32	-32,23	0,63	-0,12
				NA	NA	-5,87	-32,81	-0,92	-0,70
				NA	NA	-5,60	-32,44	-0,65	-0,33
				NA	NA	-5,39	-32,19	-0,44	-0,08
				NA	NA	-5,63	-32,72	-0,68	-0,61
		Bidartondo *et al.*, 2004	forest site 10	NA	1,845	-5,57	-27,19	-0,62	4,92
				NA	1,551	-5,32	-28,38	-0,37	3,73
				NA	1,498	-3,41	-27,17	1,54	4,94
				NA	1,411	-3,27	-27,98	1,68	4,13

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Convallaria majalis		Zimmer *et al.*, 2007	forest site 10	NA	1,113	-5,19	-28,75	-0,24	3,36
(continued)		Preiss *et al.*, 2010	forest site 10	NA	1,553	-7,41	-31,22	-2,46	0,89
				NA	1,529	-4,81	-31,17	0,14	0,94
				NA	1,265	-5,58	-32,41	-0,63	-0,30
				NA	1,549	-3,83	-32,32	1,13	-0,21
				NA	2,195	0,34	-31,18	5,29	0,93
				NA	1,728	-4,48	-31,27	0,48	0,84
				NA	1,963	-5,57	-31,65	-0,62	0,46
Epilobium angustifolium **L.**	autotrophic reference plant	Zimmer *et al.*, 2007	forest site 10	NA	2,026	-8,03	-32,52	-3,08	-0,41
Euphorbia cyparissias **L.**	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	53	2,533	-9,24	-27,00	-0,44	0,15
				54	1,918	-11,03	-27,70	-0,91	-1,29
				55	1,804	-10,81	-27,43	-1,91	-0,16
				56	1,783	-7,66	-26,98	-1,22	0,05
		Preiss *et al.*, 2010	forest site 10	NA	2,586	-5,44	-29,70	-0,49	2,41
				NA	1,784	-5,76	-31,17	-0,80	0,94
				NA	2,492	-5,50	-30,06	-0,54	2,05
				NA	2,370	-6,27	-30,41	-1,32	1,70
				NA	2,410	-7,28	-30,47	-2,33	1,64
				NA	2,637	-6,29	-34,25	-1,34	-2,14
				NA	2,653	-6,69	-31,77	-1,74	0,35
				NA	2,098	-6,13	-32,14	-1,17	-0,03
				NA	3,140	-3,96	-33,54	0,99	-1,43
				NA	2,286	-1,31	-33,67	3,64	-1,56
				NA	2,035	-5,40	-31,59	-0,45	0,52
				NA	2,227	-5,61	-32,34	-0,65	-0,23
				NA	2,347	-4,09	-32,68	0,86	-0,57
				NA	2,247	-6,63	-32,13	-1,68	-0,02
				NA	2,878	-4,45	-33,90	0,51	-1,79
Fagus sylvatica **L.**	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	66	1,532	-5,11	-30,16	0,28	-0,30
				67	1,612	-5,63	-30,51	-0,11	-0,56
				68	1,814	-4,23	-30,16	0,69	-0,80
				57	1,881	-4,06	-33,05	0,42	-3,56
				58	1,367	-4,74	-31,96	0,47	-2,34
				59	1,792	-4,66	-29,60	-0,43	-0,72
				60	1,538	-1,79	-31,17	1,45	-1,65
		Hynson *et al.*, 2016	NA	629	2,920	-5,19	-34,52	-0,34	-1,00
				630	3,170	-4,43	-32,98	0,43	0,55
				631	3,560	-4,70	-32,19	0,16	1,34
				632	2,520	-5,10	-34,41	-0,24	-0,88
		Zimmer *et al.*, 2008	forest site 10	NA	1,200	-8,83	-32,76	-3,88	-0,65
				NA	1,394	-8,22	-32,68	-3,27	-0,57
				NA	1,430	-6,36	-33,47	-1,41	-1,36
				NA	1,159	-7,83	-33,05	-2,88	-0,94
				NA	NA	-6,42	-33,74	-1,47	-1,63
				NA	NA	-6,91	-32,56	-1,96	-0,45
				NA	NA	-6,62	-32,45	-1,67	-0,34
				NA	NA	-6,59	-34,11	-1,64	-2,00
				NA	NA	-6,86	-33,12	-1,91	-1,01
		Bidartondo *et al.*, 2004	forest site 10	NA	1,881	-4,06	-33,05	0,89	-0,94
				NA	1,367	-4,74	-31,96	0,21	0,15
				NA	1,792	-4,66	-29,60	0,29	2,51
				NA	1,538	-1,79	-31,17	3,16	0,94
		Zimmer *et al.*, 2007	forest site 10	NA	1,330	-7,04	-31,48	-2,09	0,63
				NA	1,555	-6,84	-31,31	-1,89	0,80
				NA	1,476	-6,64	-32,36	-1,69	-0,25
				NA	1,618	-8,14	-31,97	-3,19	0,14
		Preiss *et al.*, 2010	forest site 10	NA	1,191	-5,59	-32,50	-0,64	-0,39
				NA	1,052	-7,18	-32,44	-2,23	-0,33
				NA	0,985	-5,42	-33,17	-0,47	-1,06
				NA	1,196	-7,46	-33,63	-2,51	-1,52

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Fagus sylvatica (continued)				NA	1,381	-7,13	-34,22	-2,18	-2,11
				NA	1,309	-6,61	-34,75	-1,66	-2,64
				NA	1,455	-6,36	-34,74	-1,40	-2,63
				NA	1,147	-7,35	-32,83	-2,39	-0,71
				NA	1,300	-4,76	-32,63	0,19	-0,52
				NA	0,955	-6,27	-32,39	-1,32	-0,27
				NA	1,525	-5,54	-30,98	-0,59	1,13
				NA	1,255	-4,64	-32,92	0,31	-0,81
				NA	1,433	-6,29	-32,26	-1,34	-0,15
				NA	1,174	-8,23	-31,83	-3,28	0,28
				NA	1,368	-6,56	-33,45	-1,60	-1,34
				NA	1,385	-6,89	-32,78	-1,94	-0,67
				NA	1,037	-5,94	-33,97	-0,98	-1,86
				NA	0,956	-7,56	-33,14	-2,61	-1,03
				NA	1,142	-7,41	-33,17	-2,46	-1,06
				NA	1,396	-7,29	-33,45	-2,34	-1,34
				NA	1,118	-5,77	-31,79	-0,82	0,32
				NA	1,163	-4,15	-32,09	0,81	0,02
				NA	1,317	-4,30	-32,18	0,65	-0,07
				NA	1,081	-6,16	-33,88	-1,21	-1,77
				NA	1,135	-4,68	-32,90	0,27	-0,79
Fragaria vesca L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	57	1,251	-3,82	-28,22	0,66	1,27
		Zimmer *et a*l., 2008	forest site 10	NA	1,273	-8,03	-30,90	-3,08	1,21
				NA	1,376	-7,98	-30,79	-3,03	1,32
				NA	1,396	-6,17	-31,17	-1,22	0,94
				NA	1,600	-6,32	-30,64	-1,37	1,47
		Bidartondo *et al.*, 2004	forest site 10	NA	1,251	-3,82	-28,22	1,13	3,89
Frangula alnus Mill.	autotrophic reference plant	Abadie *et al.*, 2006	NA	168-175	2,598	-3,28	-28,50	1,24	0,05
				168-175	1,839	-3,77	-29,32	0,75	-0,77
				168-175	2,219	-3,56	-27,16	0,96	1,39
				168-175	2,611	-3,04	-27,53	1,48	1,02
				168-175	2,379	-3,57	-29,27	0,95	-0,72
				168-175	2,383	-3,43	-30,14	1,09	-1,59
				168-175	2,352	-2,50	-30,03	2,02	-1,48
				168-175	2,219	-3,06	-28,82	1,46	-0,27
Galium verum L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	65	1,549	-4,19	-30,46	1,66	-0,82
				66	1,735	-5,00	-30,83	0,39	-0,97
				67	1,490	-4,39	-30,31	1,13	-0,36
				68	1,429	-4,80	-30,43	0,12	-1,07
Geranium robertianum L.	autotrophic reference plant	Liebel *et al.*, 2010	NA	524	2,114	-4,88	-35,86	-2,37	-1,68
				525	2,897	-6,70	-36,04	-4,05	-1,25
				526	2,156	-1,75	-35,28	-0,80	-1,42
				527	2,540	-1,15	-34,99	-0,34	-1,70
				528	3,277	-3,98	-34,52	-3,28	-1,48
Hedera helix L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	58	1,018	-5,56	-28,51	-0,35	1,11
				60	1,117	-4,65	-29,41	-1,41	0,11
		Liebel *et al.*, 2010	NA	524	1,033	-3,03	-33,92	-0,52	0,27
				525	1,274	-1,73	-34,97	0,92	-0,18
				526	1,935	-0,76	-34,18	0,19	-0,32
				527	1,360	-1,06	-34,36	-0,25	-1,07
				528	1,657	1,74	-33,89	2,44	-0,85
		Bidartondo *et al.*, 2004	forest site 10	NA	1,018	-5,56	-28,51	-0,61	3,60
				NA	1,117	-4,65	-29,41	0,30	2,70
Hieracium sylvaticum (L.) Gouan	autotrophic reference plant	Zimmer *et al.*, 2007	forest site 10	NA	0,924	-6,75	-33,15	-1,80	-1,04

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Juniperus communis **L.**	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	53	0,980	-9,02	-27,02	-0,22	0,13
				54	0,936	-9,19	-25,10	0,93	1,31
		Abadie *et al.*, 2006	NA	168-175	0,450	-7,81	-28,56	-3,29	-0,01
				168-175	0,528	-7,39	-28,51	-2,87	0,04
				168-175	0,468	-7,60	-28,76	-3,08	-0,21
				168-175	0,429	-9,06	-29,06	-4,54	-0,51
				168-175	0,608	-8,79	-28,36	-4,27	0,19
				168-175	0,587	-7,80	-28,43	-3,28	0,12
				168-175	0,484	-7,81	-27,82	-3,29	0,73
				168-175	0,557	-8,11	-28,35	-3,59	0,20
Lysimachia vulgaris **L.**	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	79	NA	-4,29	-26,77	-0,78	2,06
				80	NA	-3,33	-28,74	-0,40	0,83
				81	NA	-2,72	-28,04	-0,03	1,13
				82	NA	-3,94	-28,99	-0,94	0,55
Picea abies **(L.) H.Karst**	autotrophic reference plant	Tedersoo *et al.*, 2007	NA	330	0,632	-5,70	-30,69	-0,99	0,34
				331	0,446	-7,17	-31,74	-0,94	0,06
				332	0,548	-6,11	-30,17	-1,21	0,48
				333	0,444	-7,83	-30,62	-1,27	-0,02
				334	0,544	-7,58	-31,91	-2,33	-0,06
				335	0,481	-6,00	-29,93	-0,22	0,34
		Abadie *et al.*, 2006	NA	168-175	0,365	-5,01	-27,04	-0,49	1,51
				168-175	0,572	-6,30	-26,98	-1,78	1,57
				168-175	0,712	-2,53	-27,40	1,99	1,15
				168-175	0,415	-4,09	-28,37	0,43	0,18
				168-175	0,596	-3,84	-27,95	0,68	0,60
				168-175	0,478	-4,62	-27,87	-0,10	0,68
				168-175	0,462	-2,77	-27,17	1,75	1,38
				168-175	0,390	-5,32	-27,71	-0,80	0,84
		Johansson *et al.*, 2014	NA	634	0,840	-4,05	-30,38	-1,45	0,82
				634	0,826	-4,49	-31,54	-1,89	-0,35
				634	0,709	-4,06	-30,94	-1,46	0,26
				634	0,805	-3,92	-30,70	-1,32	0,50
				634	0,838	-3,44	-30,17	-0,84	1,03
Pinus ponderosa **Douglas ex. C.Lawson**	autotrophic reference plant	Zimmer *et al.*, 2007	NA	140	0,824	-4,73	-27,24	-0,24	-0,06
				141	0,778	-5,88	-27,69	-0,95	-0,47
				142	0,621	-5,44	-26,38	-0,48	0,14
				143	0,658	-5,96	-27,92	-1,66	-0,42
				144	0,653	-6,83	-25,75	-2,19	1,10
Polygala chamaebuxus **L.**	autotrophic reference plant	Gebauer & Meyer, 2003	NA	19	2,000	-5,08	-30,43	1,17	-0,91
				20	1,818	-5,97	-30,90	0,28	-1,89
			forest site 10	NA	2,000	-5,08	-30,43	-0,10	1,61
				NA	1,818	-5,97	-30,90	-1,02	1,21
		Preiss *et al.*, 2010	forest site 10	NA	1,861	-4,25	-32,72	0,71	-0,61
				NA	1,706	-2,04	-30,96	2,91	1,15
				NA	1,410	-3,38	-31,07	1,57	1,04
				NA	1,551	-4,18	-32,03	0,77	0,08
				NA	1,527	-3,98	-32,64	0,97	-0,53
				NA	1,700	-7,03	-32,32	-2,08	-0,21
				NA	1,970	-2,84	-33,10	2,11	-0,99
				NA	2,185	-3,10	-32,45	1,85	-0,34
				NA	1,872	-2,80	-31,72	2,15	0,39
				NA	1,961	-2,37	-33,82	2,58	-1,71
				NA	1,849	-1,46	-33,11	3,50	-1,00
				NA	1,824	-4,94	-32,91	0,01	-0,80
				NA	1,962	-3,29	-31,88	1,66	0,23
				NA	1,807	-2,54	-32,91	2,42	-0,80
				NA	1,365	-4,41	-32,81	0,55	-0,70
				NA	1,674	-2,04	-32,94	2,91	-0,82
				NA	1,684	-3,96	-32,79	0,99	-0,68

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	ε^{15}N [‰]	ε^{13}C [‰]
Polygala chamaebuxus				NA	1,800	-4,48	-35,15	0,47	-3,04
(continued)				NA	1,706	-2,35	-34,19	2,61	-2,08
Potentilla erecta (L.) Raeusch.	autotrophic reference plant	Abadie *et al.*, 2006	NA	168-175	1,893	-2,64	-29,10	1,88	-0,55
				168-175	1,913	-2,29	-28,26	2,23	0,29
				168-175	1,770	-2,67	-29,18	1,85	-0,63
				168-175	1,675	-3,80	-30,38	0,72	-1,83
				168-175	1,799	-2,43	-29,39	2,09	-0,84
				168-175	1,985	-0,95	-29,60	3,57	-1,05
				168-175	1,682	-2,92	-28,93	1,60	-0,38
				168-175	1,826	-2,00	-29,54	2,52	-0,99
Potentilla reptans L.	autotrophic reference plant	Johansson *et al.*, 2014	NA	634	2,034	-0,13	-31,88	2,47	-0,69
				634	2,319	-0,13	-31,07	2,47	0,12
				634	1,571	0,74	-30,32	3,34	0,87
				634	1,914	-0,48	-31,16	2,12	0,04
				634	2,130	-1,19	-30,78	1,41	0,41
Quercus ilex L.	autotrophic reference plant	Liebel *et al.*, 2010	NA	524	0,872	0,37	-32,77	2,88	1,41
				525	0,978	0,48	-33,34	3,13	1,44
				526	0,992	-0,34	-32,13	0,61	1,74
				527	0,992	-0,22	-30,52	0,59	2,77
				528	0,994	0,13	-30,71	0,84	2,33
Quercus robur L.	autotrophic reference plant	Bidartondo *et al.*, 2004	NA	55	1,429	-5,47	-27,18	3,43	0,09
				56	1,373	-4,79	-27,44	1,65	-0,41
		Johansson *et al.*, 2014	NA	634	1,223	-2,73	-33,02	2,08	-4,21
				634	1,535	-2,81	-31,81	-0,21	-0,61
				634	1,253	-3,75	-32,40	-1,15	-1,20
				634	2,153	-4,16	-30,88	-1,56	0,32
				634	1,652	-4,39	-30,90	-1,79	0,30
		Zimmer *et al.*, 2007	forest site 10	NA	1,134	-9,03	-30,25	-4,08	1,86
				NA	1,515	-8,44	-31,08	-3,49	1,03
				NA	1,195	-7,74	-29,77	-2,79	2,34
Rhododendron occidentale (Torr & A. Gray) A. Gray	autotrophic reference plant	Zimmer *et al.*, 2007	NA	140	1,580	-2,70	-26,47	1,80	0,71
				141	1,581	-1,23	-28,82	3,71	-1,60
				142	1,954	-3,55	-25,92	1,41	0,60
				144	1,509	-2,29	-28,14	2,01	-0,64
				144	1,590	-2,51	-28,83	2,13	-1,98
Rubus saxatilis L.	autotrophic reference plant	Zimmer *et al.*, 2007	forest site 10	NA	1,013	-4,90	-30,57	0,05	1,54
Sesleria albicans Kit.	autotrophic reference plant	Gebauer & Meyer, 2003	NA	19	0,869	-7,42	-28,62	-1,17	0,90
				20	1,214	-6,54	-27,13	-0,28	1,89
		Bidartondo *et al.*, 2004	NA	53	1,906	-8,15	-27,43	0,65	-0,28
				54	1,249	-10,15	-26,43	-0,03	-0,02
				55	1,296	-10,42	-27,20	-1,52	0,07
				56	0,950	-6,88	-26,68	-0,44	0,35
		Gebauer & Meyer, 2003	forest site 10	NA	0,869	-7,42	-28,62	-2,47	3,49
				NA	1,214	-6,54	-27,13	-1,59	4,98
Sorbus aucuparia L.	autotrophic reference plant	Zimmer *et al.*, 2008	forest site 10	NA	NA	-5,74	-32,76	-0,79	-0,65
				NA	NA	-5,76	-32,25	-0,81	-0,14
				NA	NA	-5,50	-32,63	-0,55	-0,52
				NA	NA	-5,30	-33,08	-0,35	-0,97
				NA	NA	-6,92	-31,48	-1,97	0,63

Table S2 (continued)

Species	Functional type	Publication	Site	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]
Vaccinium myrtillus **L.**	autotrophic reference plant	Zimmer *et al*., 2007	forest site 10	NA	1,168	-7,13	-32,26	-2,18	-0,15
				NA	1,311	-9,43	-32,88	-4,48	-0,77
				NA	1,145	-7,79	-33,71	-2,84	-1,60
autotrophs *E. fibri*	autotrophic reference plant	Gonneau *et al*., 2014	shaded	3	1,514	-4,71	-34,74	0,10	-2,11
				3	1,486	-3,53	-33,47	1,28	-0,84
				3	1,693	-2,46	-32,25	2,35	0,39
				3	1,693	-3,22	-32,76	1,59	-0,13
				3	2,621	-8,70	-31,45	-3,89	1,18
				3	1,621	-6,25	-31,12	-1,44	1,51
			sunny	4	0,343	-0,86	-31,70	0,61	0,00
				4	0,457	-1,88	-30,62	-0,41	1,08
				4	1,007	-1,84	-32,80	-0,37	-1,10
				4	1,071	-0,61	-32,68	0,86	-0,97
				4	3,114	-1,95	-30,93	-0,47	0,77
				4	2,971	-1,70	-31,48	-0,22	0,22

Table S3: Orchid mycorrhizal fungi detected in the roots of *Epipactis* species extracted from all available publications.

Epipactis species	Mycorrhizal fungi	Type of mycorrhizal fungi	Publication
***Epipactis atrorubens* (Hoffm.) Besser**	*Inocybe*	ECM B	
	Leptodontidium	OM R	
	Phialophora	OM R	
	sebacinoid	**ECM B**	Bidartondo *et al*., 2004[**]
	Tuber	**ECM A**	
	Tulasnella	OM R	
	Wilcoxina	**ECM A**	
	Sebacina	**ECM B**	
	Cenococcum	**ECM A**	Bidartondo & Read, 2008[*]
	Geopora	**ECM A**	
	Wilcoxina	**ECM A**	
	Tulasnellaceae	OM R	
	Trichophaea wollhopeia	**ECM A**	Shefferson *et al*., 2008[*]
	Geopora cooperi	**ECM A**	
	Chalara dualis	**ECM A**	
	Tuber	**ECM A**	Těšitelová et al., 2012[*]
	Wilcoxina	**ECM A**	
***Epipactis distans* Arv.-Touv.**	***Wilcoxina***	**ECM A**	Bidartondo *et al*., 2004[**]
***Epipactis fibri* Scappat. & Robatsch**	**Tuberaceae**	**ECM A**	
	Pyrenomataceae	**ECM A**	Selosse *et al*., (unpubl.) "Mycorrhizal
	Tremellales	**ECM B**	fungi of *Epipactis fibri* at the Ile du
	Debaryomyces	**A**	Beurre Reserve" submission to
	***Inocybe* sp.**	**ECM B**	Genbank, 2013
	***Amanita* sp.**	**ECM B**	
***Epipactis gigantea* Douglas ex Hook.**	*Ceratobasidium*	OM R	
	Leptodontidium	OM R	Bidartondo *et al*., 2004[*]
	Thanatephorus	OM R	
***Epipactis helleborine* (L.) Crantz**	*Ceratobasidium*	OM R	
	sebacinoid	**ECM B**	Bidartondo *et al*., 2004[**]
	Tuber	**ECM A**	
	Wilcoxina	**ECM A**	
	Tuber	**ECM A**	
	Hydnotrya	**ECM A**	
	Helvella	**ECM A**	Ogura-Tsujita & Yukawa, 2008[*]
	Genea	**ECM A**	
	Leptodontidium	OM R	
	Ceratobasidium	OM R	
	Tuber maculatum	**ECM A**	Ouanphanivanh *et al*., 2008[*]
	Wilcoxina	**ECM A**	
	***Genea* sp.**	**ECM A**	
	***Geopyxis* sp.**	**ECM A**	Těšitelová et al., 2012[*]
	***Hymenogaster* sp.**	**ECM B**	
	Thelephoraceae	**ECM B**	
	Inocybe sp.	**ECM B**	
	Helotiales	A	
	Thelephora	**ECM B**	
	Sebacina	OM R	
	Inocybe	**ECM B**	
	Cenococcum	**ECM A**	
	Tuber	**ECM A**	
	Exophiala	A	
	Leptodontidium	OM R	Jacquemyn *et al*., 2016[*]
	Meliniomyces	A	
	Tricholoma	**ECM B**	
	Russula	**ECM B**	
	Helvella	**ECM A**	
	Wilcoxina	**ECM A**	
	Psathyrella	B	
***Epipactis helleborine* subsp. *neerlandica* (Verm.) Buttler**	Helotiales	A	
	Thelephora	**ECM B**	
	Sebacina	OM R	
	Inocybe	**ECM B**	
	Cortinarius	**ECM B**	
	Tuber	**ECM A**	Jacquemyn *et al*., 2016[*]
	Geopora	ECM A	
	Ceratobasidium	OM R	
	Exophiala	A	
	Leptodontidium	OM R	
	Hebeloma	**ECM B**	
	Helvella	**ECM A**	
***Epipactis microphylla* (Ehrh.) Sw.**	***Cortinarius* sp.**	**ECM B**	
	Sebacina epigaea	**ECM B**	
	Tuber excavatum	**ECM A**	Selosse *et al*., 2004[*]
	Tuber uncinatum	**ECM A**	
	Trichocomaceae	**ECM A**	

Table S3 (continued)

Epipactis species	Mycorrhizal fungi	Type of mycorrhizal fungi	Publication
Epipactis microphylla	*Tuber aestivum*	**ECM A**	
(continued)	*Hymenogaster* **sp.**	**ECM B**	
	Pezizales sp.	**ECM A**	
	Wilcoxina mikolae	**ECM A**	Selosse *et al.*, 2004[*]
	Sarcosomataceae	**ECM A**	
	Sebacinaceae	**ECM B**	
	Thelephoraceae	**ECM B**	
	Russula foetens	**ECM B**	
	Tuber excavatum	**ECM A**	Ouanphanivanh *et al.*, 2008[**]
Epipactis palustris (L.) Crantz	*Ceratobasidium*	OM R	
	Leptodontidium	OM R	Bidartondo *et al.*, 2004[**]
	sebacinoid	OM R	
	tulasnelloid	OM R	
	Epulorhiza sp.	OM R	Illyés *et al.*, 2009[*]
	Helotiales	A	
	Thelephora	**ECM B**	
	Sebacina	OM R	
	Inocybe	**ECM B**	
	Ceratobasidium	OM R	Jacquemyn *et al.*, 2016[*]
	Exophiala	A	
	Leptodontidium	OM R	
	Hebeloma	**ECM B**	
	Tulasnella	OM R	
Epipactis purpurata **Sm.**	*Wilcoxina*	**ECM A**	
	Helvella	**ECM**	Těšitelová *et al.*, 2012[*]
	Russula **sp.**	**ECM B**	
	Ceratobasidium sp.	OM R	

[*] fungi
[**] fungi & isotopes

MANUSCRIPT 3

Exploiting mycorrhizas in broad daylight: Partial mycoheterotrophy is a common nutritional strategy in meadow orchids

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G. Gebauer

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RESEARCH ARTICLE

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Exploiting mycorrhizas in broad daylight: Partial mycoheterotrophy is a common nutritional strategy in meadow orchids

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Abstract

1. Partial mycoheterotrophy (PMH) is a nutritional mode in which plants utilize organic matter, i.e. carbon, both from photosynthesis and a fungal source. The latter reverses the direction of plant-to-fungus carbon flow as usually assumed in mycorrhizal mutualisms. Based on significant enrichment in the heavy isotope ^{13}C, a growing number of PMH orchid species have been identified. These PMH orchids are mostly associated with fungi simultaneously forming ectomycorrhizas with forest trees. In contrast, the much more common orchids that associate with rhizoctonia fungi, which are decomposers, have stable isotope profiles most often characterized by high ^{15}N enrichment and high nitrogen concentrations but either an insignificant ^{13}C enrichment or depletion relative to autotrophic plants. Using hydrogen stable isotope abundances recent investigations showed PMH in rhizoctonia-associated orchids growing under light-limited conditions. Hydrogen isotope abundances can be used as substitute for carbon isotope abundances in cases where autotrophic and heterotrophic carbon sources are insufficiently distinctive to indicate PMH.

2. To determine whether rhizoctonia-associated orchids growing in habitats with high irradiance feature PMH as a nutritional mode, we sampled 13 orchid species growing in montane meadows, four forest orchid species and 34 autotrophic reference species. We analysed δ^2H, δ^{13}C, δ^{15}N and δ^{18}O and determined nitrogen concentrations. Orchid mycorrhizal fungi were identified by DNA sequencing.

3. As expected, we found high enrichments in ^{2}H, ^{13}C, ^{15}N and nitrogen concentrations in the ectomycorrhiza-associated forest orchids, and the rhizoctonia-associated *Neottia cordata* from a forest site was identified as PMH. Most orchids inhabiting sunny meadows lacked ^{13}C enrichment or were even significantly depleted in ^{13}C relative to autotrophic references. However, we infer PMH for the majority of these meadow orchids due to both significant ^{2}H and ^{15}N enrichment and high nitrogen concentrations. *Pseudorchis albida* was the sole autotrophic orchid in this study as it exhibited neither enrichment in any isotope nor a distinctive leaf nitrogen concentration.

4. *Synthesis.* Our findings demonstrate that partial mycoheterotrophy is a trophic continuum between the extreme endpoints of autotrophy and full mycoheterotrophy,

ranging from marginal to pronounced. In rhizoctonia-associated orchids, partial mycoheterotrophy plays a far greater role than previously assumed, even in full light conditions.

KEYWORDS

carbon, fungi, hydrogen, nitrogen, Orchidaceae, orchid mycorrhiza, plant-soil (below-ground) interactions, rhizoctonia, stable isotopes, symbiosis

1 | INTRODUCTION

The Orchidaceae is usually referred to as the largest plant family with almost a tenth of described vascular plant species, that is *c.* 28,000 species in 736 genera (Chase et al., 2015; Christenhusz & Byng, 2016). Orchids have a world-wide distribution, and they occur in a variety of habitats only avoiding the polar regions and the driest deserts (Chase et al., 2015; Merckx et al., 2013). Regardless of their appearance as epiphytic, lithophytic, terrestrial or subterranean life-forms, all orchid species share the trait of producing large numbers of tiny, endosperm-less seeds containing only very small amounts of nutrients (Arditti & Ghani, 2000). Consequently, the trophic strategy of initial mycoheterotrophy—the colonization of dust-like seeds by mycorrhizal fungi and their provision of protocorms with nutrients until the photosynthetic seedling stage—is a characteristic feature of all Orchidaceae (Alexander & Hadley, 1985; Merckx et al., 2013; Rasmussen, 1995). Protocorms are the non-photosynthetic, fully mycoheterotrophic (FMH), pre-seedling stages formed after germination of dust seeds colonized by a mycorrhizal fungus. Some orchid species lack chlorophyll at maturity so they satisfy also in the adult stage all nutrient demands by exploiting their mycorrhizal fungi. Due to ongoing taxonomic work and recent discoveries (Suetsugu, 2016, 2017) the number of FMH orchid species is growing continuously from a minimum of 235 species (Merckx et al., 2013). All FMH Orchidaceae species belong to the subfamilies Vanilloideae, Orchidoideae and Epidendroideae and occur mostly in the tropics. They are associated with either ectomycorrhizal fungi or saprotrophic fungi that decompose litter or wood (Bidartondo, Burghardt, Gebauer, Bruns, & Read, 2004; Hynson, Preiss, Gebauer, & Bruns, 2009; Lee, Yang, & Gebauer, 2015; Martos et al., 2009; Ogura-Tsujita, Gebauer, Hashimoto, Umata, & Yukawa, 2009; Zimmer et al., 2007).

Fully mycoheterotrophic species can be identified by their achlorophyllous appearance and also by measuring the stable isotope natural abundances in their tissues. Fungi, being heterotrophic, are enriched in ^{13}C as well as in ^{15}N compared to their substrates and to autotrophic plants (Gebauer & Dietrich, 1993; Gleixner, Danier, Werner, & Schmidt, 1993; Trudell, Rygiewicz, & Edmonds, 2003). Mycoheterotrophic orchids either take up fungal material via hyphal lysis and/or transfer across intact membranes of fungus and orchid (Smith & Read, 2008). Following the systematic increase in the relative abundance of ^{13}C and ^{15}N at each trophic level in a food chain as originally described by DeNiro and Epstein (1978, 1981), orchid tissues consequently mirror the isotopic signature of their associated fungi (Gebauer & Meyer, 2003).

Stable isotope analysis together with the molecular identification of mycorrhizal fungi have become the standard tools for research on trophic strategies in plants, especially orchids (Leake & Cameron, 2010). Using stable isotope natural abundance analysis, a growing number of partially mycoheterotrophic (PMH) species have been identified (Gebauer & Meyer, 2003; Hynson, Schiebold, & Gebauer, 2016; Hynson et al., 2013) that simultaneously utilize both carbon from photosynthesis and a fungal source (PMH sensu Merckx, 2013). Isotopic enrichment in ^{13}C of PMH orchids was considered until recently to be intermediate between autotrophic plants from the same microhabitats (Gebauer & Meyer, 2003) and FMH orchids that rely on their mycorrhizal fungi as sole nutrient source; ^{13}C enrichment varies with light climate (Preiss, Adam, & Gebauer, 2010) and leaf chlorophyll concentration (Stöckel, Meyer, & Gebauer, 2011). There is evidence that FMH and PMH orchid species also meet their complete nitrogen demands through mycorrhizal fungi (Stöckel, Těšitelová, Jersáková, Bidartondo, & Gebauer, 2014) and as ^{15}N enrichment varies with type of fungal symbiont, ^{15}N enrichment of PMH orchids can even exceed the isotopic enrichment in ^{15}N of FMH species (Schiebold, Bidartondo, Karasch, Gravendeel, & Gebauer, 2017).

Until recently, all identified PMH orchid species gaining nutrients from mixed sources were Epidendroideae exclusively associated with ectomycorrhizal fungi or with ectomycorrhizal fungi additionally to typical orchid mycorrhizal rhizoctonias, a polyphyletic group of basidiomycetes (Bidartondo et al., 2004). Nevertheless, the overwhelming majority of chlorophyllous orchid species is associated with rhizoctonia fungi (Dearnaley, Martos, & Selosse, 2012) and was thus assumed to be putatively autotrophic. However, the stable isotope profiles of rhizoctonia-associated orchids are most often characterized by conspicuously significant ^{15}N enrichment and higher leaf total nitrogen concentrations compared to autotrophic plants, and either a lacking or modest but insignificant ^{13}C enrichment, or even depletion relative to autotrophic references (Girlanda et al., 2011; Johansson, Mikusinska, Ekblad, & Eriksson, 2014; Liebel et al., 2010). Thus, the assumed autotrophy of rhizoctonia-associated orchids has been challenged and they have been called "cryptic mycoheterotrophs" (Hynson, 2016; Hynson et al., 2013). High N isotope abundances and high nitrogen concentrations might indicate that in addition to photosynthesis there is carbon gain from fungal sources, that is, nitrogen gain via organic compounds, and thus partial mycoheterotrophy (PMH).

In a comparison between ^{13}C and ^{15}N enrichment of FMH protocorms and mature individuals of the same species, Stöckel et al. (2014)

found that achlorophyllous, FMH seedlings of rhizoctonia-associated orchids were far less enriched in ^{13}C and ^{15}N than protocorms of orchids that associate with ectomycorrhizal fungi. They proposed that especially the ^{13}C enrichment measured in mature chlorophyllous orchids associated with saprotrophic rhizoctonia fungi might be too small to enable the detection of PMH, in contrast to a usually clear ^{13}C enrichment of mature chlorophyllous orchids associated with ectomycorrhizal fungi. In other words, the carbon source isotope abundance of rhizoctonia-associated orchid species, namely rhizoctonia fungi, is too close to the isotope abundance of autotrophic plants to be distinguished (Gebauer, Preiss, & Gebauer, 2016). Stöckel et al. (2014) concluded that the routinely used δ^{13}C ratios might not be sufficient to unequivocally identify PMH orchid species associated with rhizoctonia fungi. Recently, Gebauer et al. (2016) provided clear evidence that the trophic strategy of PMH is far more widespread in forest understorey orchids than previously assumed. They used δ^{2}H in addition to the routinely employed δ^{13}C and δ^{15}N measurements following the reasoning that not only carbon and nitrogen but also hydrogen atoms are present in organic molecules that move from fungus to orchid during peloton digestion and/or by transfer across intact membranes. Consequently, δ^{2}H values could thus also be used as indicators for mycoheterotrophic nutrition. The δ^{2}H approach is based on the finding that secondary heterotrophic organic compounds (i.e. in our case compounds of fungal origin) are enriched in ^{2}H compared to primary photosynthetic organic compounds (Yakir, 1992). This enrichment is substantiated in the increasing exchange of H atoms from hydroxyl groups in organic molecules with H atoms from surrounding tissue water molecules with increasing heterotrophy. The great value of H stable isotope abundances to identify carbon gain of heterotrophic origin was first elucidated in a study on the C and H stable isotope abundances of plant parasites and their hosts by Ziegler (1994). Holoparasites obtain all organic material from their hosts and thus mirror their δ^{13}C values. However, as heterotrophic material is taken up from the host by the holoparasite, the holoparasite is always enriched in δ^{2}H relative to its host. Thus, based on ^{2}H enrichment, that study documents organic matter, i.e. carbon, gain by a parasite from its host independently of any ^{13}C enrichment.

In Gebauer et al.'s (2016) study not only the ectomycorrhiza-associated FMH orchid species *Neottia nidus-avis* and PMH *Cephalanthera damasonium*, *Cephalanthera rubra* and *Epipactis atrorubens* were enriched in ^{2}H simultaneously to ^{13}C and ^{15}N, serving as a proof of concept, but also four rhizoctonia-associated species (*Cypripedium calceolus*, *Neottia ovata*, *Ophrys insectifera* and *Platanthera bifolia*) were positioned in their ^{2}H enrichment between autotrophic plants sampled as references and the FMH *N. nidus-avis*. Thus, justification was provided for substituting H for C stable isotope abundance analysis in cases where C stable isotope abundances of carbon sources are poorly differentiated (Gebauer et al., 2016) and mask the C flow between mycorrhizal fungi and orchids. Consequently, a significant ^{2}H enrichment in plant tissue can serve as an indicator for flow of heterotrophic organic matter, i.e. carbon, from mycorrhizal fungi to plant and thus for PMH. Rhizoctonia-associated orchid species with C isotope abundances close to autotrophic plants can serve as a prime example. The above-mentioned eight orchid species

were sampled in a closed-canopy beech forest with sparse herb understorey, and thus it is assumed that all orchids compensated lacking irradiance for efficient photosynthesis by increasing exploitation of their mycorrhizal fungi. The gain of organic matter via peloton digestion and/or by transfer through membranes is vital for all orchids in their early pre-photosynthetic stages and also for FMH orchids and ectomycorrhiza (ECM)-associated PMH orchids growing under light limitation. However, digestion and/or organic matter transfer might still be relevant for rhizoctonia-associated orchid species. Partial mycoheterotrophy could be a useful trait for chlorophyllous orchid species associated with rhizoctonia fungi to compete against other herbaceous species in open habitats and to help survive unfavourable conditions during adult dormant underground stages (Shefferson, 2009). Whether the overwhelming majority of rhizoctonia-associated orchid species growing in open habitats and thus under light-saturated conditions are PMH remains to be investigated.

Here, we hypothesize that orchids in the subfamilies Epidendroideae and Orchidoideae forming mycorrhizas with rhizoctonia fungi and growing in habitats with high irradiance levels, such as montane meadows, feature PMH as nutritional mode. These plants may be predisposed to mycoheterotrophic nutrition due to initial mycoheterotrophy in the protocorm stage. We test our hypothesis by employing natural abundance analysis of δ^{2}H, δ^{13}C and δ^{15}N and determination of nitrogen concentrations. In addition, we analysed δ^{18}O to exclude a potential bias due to different transpiration by orchids and reference plants.

2 | MATERIALS AND METHODS

2.1 | Study design and site descriptions

We selected an area in the Northern Limestone Alps in the Eastern Alps of Europe with high orchid diversity and sampled a variety of orchid species to test for a general pattern. We measured multi-element stable isotope abundances (δ^{13}C, δ^{15}N, δ^{2}H and δ^{18}O) and identified mycorrhizal fungi with molecular methods. Orchid species with known trophic pathways such as full (*N. nidus-avis* (L.) Rich.) and PMH (*C. rubra* (L.) Rich. and *Epipactis helleborine* (L.) Crantz) associated with ectomycorrhizal fungi and autotrophic non-orchid plant species were sampled as references.

Seventeen orchid species were sampled at six sites in Austria and Germany during July in four consecutive years between 2012 and 2015. The major sampling area was the Marul Valley located in the biosphere park of the Great Walser Valley in the province of Vorarlberg in Western Austria between the village of Garfülla and the alp Laguz and nearby Laguz (N 47.1°–47.2°; E 9.8°–10°, 1,220–1,740 m a.s.l.). At Garfülla we sampled the FMH *N. nidus-avis* (L.) Rich. and the PMH *C. rubra* (L.) Rich. and *E. helleborine* (L.) Crantz (all members of the tribe Neottieae) in a nearby montane mixed deciduous and coniferous forest dominated by *Fagus sylvatica* L. and *Picea abies* (L.) H. Karst. Between Garfülla and alp Laguz, we sampled ten orchid species of the tribe Orchideae (*Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh., *Dactylorhiza incarnata* (L.) Soó, *Dactylorhiza viridis* (L.) R.M.Bateman,

Pridgeon & M.W.Chase, *Gymnadenia conopsea* (L.) R.Br., *Gymnadenia nigra* (L.) Rchb.f., *Herminium monorchis* (L.) R.Br., *Neotinea ustulata* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *P. bifolia* (L.) Rich., *P. albida* (L.) Á.Löve & D.Löve and *Traunsteinera globosa* (L.) Rchb.) from open extensively mown or grazed montane meadows and *Nardus* grasslands (Tables S1 and S2). On the colline foothills of the Great Walser Valley in the Walgau region, we sampled *Liparis loeselii* (L.) Rich. (tribe Malaxideae) in a fen near the municipality of Thüringen and *Spiranthes aestivalis* (Poir.) Rich. (tribus Cranichideae) in a periodically wet *Molinia* meadow near Göfis (N 47.2°, E 9.7°–9.9°, 475 and 745 m a.s.l.). Still in the Northern Limestone Alps but in the Karwendel region in Southern Bavaria, *Malaxis monophyllos* (L.) Sw. was sampled at Schachen (N 47.4°; E 11.1°, 1,800 m a.s.l.) on a montane meadow. The Neottieae *Neottia cordata* (L.) Rich. was sampled in a coniferous forest dominated by *P. abies* near Fichtelsee in the low mountain range Fichtelgebirge in NE Bavaria (N 50.0°; E 11.9°, 760 m a.s.l.). Sampling followed the plot-wise sampling scheme by Gebauer and Meyer (2003); leaf samples from flowering individuals of all orchid species were taken in five replicates (resembling five 1 m^2 plots) together with three autotrophic reference plants under the same growth conditions and in the same microclimate. A broad range of non-orchid autotrophic references representing a variety of functional groups and growth forms including plants with arbuscular mycorrhizas, ectomycorrhizas or ericoid mycorrhizas and non-mycorrhizal species were sampled to depict the variability in autotrophic plants. Only leguminous, parasitic and carnivorous plants were excluded from sampling. Please refer to Gebauer and Meyer (2003) for further details of the sampling method. Sampling yielded a total of 85 samples from 17 orchid species (shoot samples of *N. nidus-avis* and leaf samples of all other orchid species) and 253 leaf samples from 34 neighbouring autotrophic reference species in 85 plots distributed over six sites (Tables S1 and S2). Nomenclature for orchid species and autotrophic references follows www.theplantlist.org (The Plant List, 2013).

2.2 | Analysis of stable isotope abundance and nitrogen concentration

Leaf samples of the 17 orchid species (*n* = 85) and autotrophic references (*n* = 253) were washed with deionized water, dried to constant weight at 105°C, ground to a fine powder in a ball mill (Retsch Schwingmühle MM2, Haan, Germany) and stored in a desiccator fitted with silica gel until analysis. Relative C and N isotope natural abundances of the leaf samples were measured in dual element analysis mode with an elemental analyser (1108; Carlo Erba Instruments, Milano, Italy) coupled to a continuous flow isotope ratio mass spectrometer (delta S, Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Thermo Fisher Scientific, Bremen, Germany) as described in Bidartondo et al. (2004). Relative H and O isotope natural abundances of the leaf samples were measured with thermal conversion through pyrolysis (HTO, HEKAtech, Wegberg, Germany) coupled to a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific) via a ConFlo IV open-split interface (Thermo Fisher Scientific) as described in Gebauer et al. (2016). Due

to memory bias each sample was analysed three times and the first two sample runs were skipped for reliable H isotope abundance determination. In order to minimize bias of post-sampling H atom exchange between organically bound hydroxyl groups in our samples and H_2O in ambient air (Yakir, 1992), we analysed samples of orchids and their respective reference plant samples together in identical sample batches. The O isotope abundances were measured to rule out a transpiration effect as a cause of differences in the H isotope abundance between orchids and non-orchid reference plants (Ziegler, 1988).

Measured relative isotope abundances are denoted as δ values that were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, δ^2H or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. Standard gases (Riessner, Lichtenfels, Germany) were calibrated with respect to international standards (CO_2 vs. PDB, N_2 vs. N_2 in air, H_2 and CO vs. SMOW) with the reference substances ANU sucrose and NBS19 for the C isotopes, N1 and N2 for the N isotopes, CH7, V-SMOW and SLAP for H isotopes and IAEA601 and IAEA602 for the O isotopes, all provided by the IAEA (International Atomic Energy Agency, Vienna, Austria). Reproducibility and accuracy of the C and N isotope abundance measurements were routinely controlled by measuring the laboratory standard acetanilide (Gebauer & Schulze, 1991). In relative C and N isotope natural abundance analyses, acetanilide was routinely analysed with variable sample weight at least six times within each batch of 50 samples. The maximum variation in $\delta^{13}C$ and $\delta^{15}N$ both within and between batches was always below 0.2‰. For relative H and O isotope natural abundance analyses, benzoic acid was routinely analysed with variable sample weight at least six times within each batch of 40 samples. The maximum variation in δ^2H and $\delta^{18}O$ both within and between batches was always below 4‰ for δ^2H and 0.6‰ for $\delta^{18}O$.

Total nitrogen concentrations in leaf and shoot samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on measurements of the laboratory standard acetanilide with a known nitrogen concentration of 10.36% (Gebauer & Schulze, 1991).

2.3 | Molecular identification of mycorrhizal fungi

Mycorrhizal fungi of some of the orchid species under investigation were already known form previous studies. Nonphotosynthetic *N. nidus-avis* shows high fungal specificity for *Sebacina* (ectomycorrhizal clade A) (McKendrick, Leake, Taylor, & Read, 2002; Selosse, Weiß, Jany, & Tillier, 2002). *Cephalanthera rubra* associates with *Leptodontidium*, *Phialophora* and *Tomentella* (Bidartondo et al., 2004). The mycorrhizal communities of *E. helleborine* have been studied thoroughly by several authors and are diverse as they consist of ectomycorrhizal ascomycetes and basidiomycetes and also typical orchid mycorrhizal rhizoctonia fungi; for example *Ceratobasidium*, sebacinoids, *Tuber* (Bidartondo et al., 2004), *Helvella*, *Wilcoxina* (Ogura-Tsujita & Yukawa, 2008), *Inocybe*, *Thelephora* (Těšitelová, Těšitel, Jersáková, Ríhová, & Selosse, 2012) and *Tricholoma* and *Russula* (Jacquemyn, Waud, Lievens, & Brys, 2016). The meadow

orchid *G. conopsea* forms typical orchid mycorrhizae with *Tulasnella*, *Ceratobasidium*, *Thanatephorus* and *Sebacina*, but also with members of the Pezizales (Stark, Babik, & Durka, 2009). Mycorrhizal communities of *P. bifolia* are dominated by Ceratobasidiaceae, but also Tulasnellaceae and Sebacinales (Esposito, Jacquemyn, Waud, & Tyteca, 2016). *Pseudorchis albida* has a diverse fungal community dominated by *Tulasnella* (Jersáková, Malinová, Jeřábková, & Dötterl, 2011; Kohout, Těšitelová, Roy, Vohník, & Jersáková, 2013).

To check whether the prerequisite of orchid mycorrhizas with rhizoctonia was met in the remaining meadow orchids, we identified their mycorrhizal fungi by molecular methods. Of the sampled orchid species, *L. loeselii*, *M. monophyllos*, *N. cordata*, *S. aestivalis*, *D. incarnata*, *D. majalis*, *D. viridis*, *G. nigra*, *H. monorchis*, *N. ustulata* and *T. globosa*, two roots per sampled orchid individual were cut, rinsed with deionized water, placed in CTAB buffer (cetyltrimethylammonium bromide) and stored at −18°C until further analysis. Root cross-sections were checked for presence and status of fungal pelotons in the cortex cells. Two to six root sections per orchid individual were selected for genomic DNA extraction and purification with the GeneClean III Kit (Q-BioGene, Carlsbad, CA, USA). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primer combinations ITS1F/ITS4 and ITS1/ITS4-Tul (Bidartondo & Duckett, 2010). All positive PCR products were purified with ExoProStart (GE Healthcare, Buckinghamshire, UK) and sequenced bidirectionally with an ABI3730 Genetic Analyser using the BigDye 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. All DNA sequences were checked and visually aligned with Geneious version 7.4.1 (http://www.geneious.com, Kearse et al., 2012) and compared to GenBank using BLAST (http://blast.ncbi.nlm.nih.gov). All unique DNA sequences have been submitted to GenBank (accession numbers: KY271858–KY271875).

2.4 | Calculations and statistics

To enable comparisons of C, N, H and O stable isotope abundances between the 17 orchid species sampled at six different sites we used an isotope enrichment factor approach to normalize the data. Normalized enrichment factors (ε) were calculated from measured δ values as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, δ^2H or $\delta^{18}O$ value of an orchid individual or an autotrophic reference plant and δ_{REF} is the mean value of all autotrophic reference plants by plot (Preiss & Gebauer, 2008). The $\delta^{13}C$, $\delta^{15}N$, δ^2H and $\delta^{18}O$ values, enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, ε^2H and $\varepsilon^{18}O$ and nitrogen concentrations of 17 orchid species and autotrophic references are available in Tables S1 and S2.

We tested for pairwise differences in the isotopic enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, ε^2H and $\varepsilon^{18}O$ and nitrogen concentration between the sampled orchid species and all autotrophic reference plants using a nonparametric Mann–Whitney *U* test after significant nonparametric Kruskal–Wallis *H* test. To test for differences between orchids and autotrophic references in nitrogen concentrations, the orchids were grouped according to type of fungal partner and degree of mycoheterotrophy (FMH ECM: fully mycoheterotrophic orchids associated with ECM fungi; PMH ECM: partially mycoheterotrophic orchids associated

with ECM fungi; rhizoctonia: orchids associated with rhizoctonia fungi; autotrophic references). The *p*-values were adjusted using the correction after Benjamini and Hochberg (1995). For statistical analyses we used R version 3.1.2 (R Development Core Team, 2014) with a significance level of α = 0.05.

3 | RESULTS

3.1 | Stable isotope abundances

Mean enrichment in ^{13}C of all orchid species in this study varied between −2.9‰ ± 0.9‰ (*H. monorchis*) and 5.7‰ ± 0.8‰ (*N. nidus-avis*; Figure 1a, Table S1). Post hoc pairwise Mann–Whitney *U* tests after a significant Kruskal–Wallis test (χ^2 = 114.672, *df* = 17, *p* < .001) showed that only *N. nidus-avis* (p_{adj} = .014), *C. rubra* (p_{adj} = .029), *E. helleborine* (p_{adj} = .029) and *N. cordata* (p_{adj} = .016) were significantly enriched relative to autotrophic references in ^{13}C. All other species were either significantly depleted relative to autotrophic references or not distinguishable from them (Table 1).

All species except for *G. nigra* (p_{adj} = .056) and *P. albida* (p_{adj} = .641) were significantly enriched in ^{15}N relative to autotrophic references as shown by post hoc pairwise Mann–Whitney *U* tests after a significant Kruskal–Wallis test (χ^2 = 140.992, *df* = 17, *p* < .001, Table 1). Mean enrichment of the orchid species ranged between 0.3‰ ± 1.4‰ (*P. albida*) and 16.5‰ ± 1.6‰ (*N. nidus-avis*) in ^{15}N (Figure 1a, Table S1).

Mean enrichment in ^2H varied between −5.6‰ ± 7.1‰ (*M. monophyllos*) and 45.5‰ ± 7.0‰ (*N. nidus-avis*; Figure 1b, Table S1). The only species that were not enriched in ^2H relative to autotrophic references, according to post hoc pairwise Mann–Whitney *U* tests after a significant Kruskal–Wallis test (χ^2 = 125.817, *df* = 17, *p* < .001), were *M. monophyllos* (p_{adj} = .214), *D. majalis* (p_{adj} = .159), *P. albida* (p_{adj} = .111) and *T. globosa* (p_{adj} = .781). All other orchid species were significantly enriched relative to autotrophic references (Table 1).

Although the Kruskal–Wallis test to assess differences between orchid species and autotrophic references in $\varepsilon^{18}O$ was significant (χ^2 = 62.123, *df* = 17, *p* < .001), no significant differences between orchid species and autotrophic references could be detected after adjustment of *p*-values (Table S3). The $\varepsilon^{18}O$ values varied between −2.4‰ ± 0.5‰ (*G. conopsea*) and 2.3‰ ± 2.6‰ (*E. helleborine*) and thus ranged around the mean of autotrophic references (−0.2‰ ± 1.7‰; Figure S1, Tables S1 and S2).

Here, we provide the first stable isotope abundance data for *M. monophyllos*, *N. cordata*, *S. aestivalis*, *D. incarnata*, *D. viridis*, *G. nigra* and *H. monorchis* and additionally the first H isotope data for *D. majalis*, *G. conopsea*, *N. ustulata* and *P. albida* (Figure 1, Table 1). Similar to the forest orchid species *N. nidus-avis*, *C. rubra* and *E. helleborine*, *N. cordata* is significantly enriched in ^{13}C, ^{15}N and ^2H.

3.2 | Nitrogen concentrations

Nitrogen concentrations between the three groups of orchids (FMH ECM: fully mycoheterotrophic orchid species associated with ectomycorrhizal fungi, PMH ECM: partially mycoheterotrophic orchid species associated
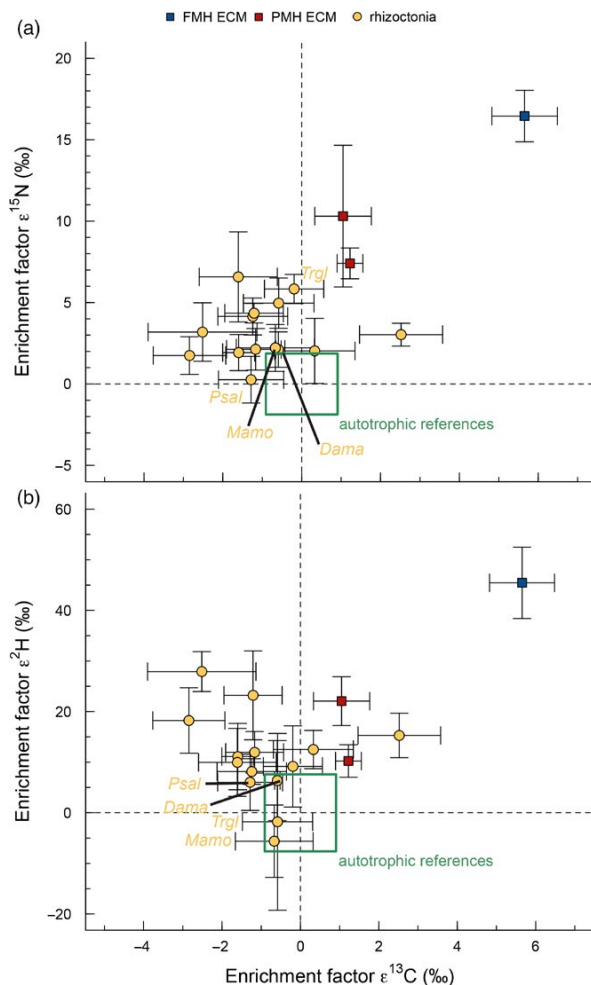
FIGURE 1 Mean enrichment factors (a) ε^2H and ε^{13}C ± 1 *SD* and (b) ε^{15}N and ε^{13}C ± 1 *SD* of 17 orchid species sampled in the Northern Limestone Alps in Austria and Germany and the Fichtelgebirge in NE Bavaria/Germany in July of the four consecutive years 2012–2015. One fully mycoheterotrophic orchid species associated with ectomycorrhizal fungi (FMH ECM: blue squares; *Neottia nidus-avis*), two partially mycoheterotrophic orchid species associated with ectomycorrhizal fungi (PMH ECM: red squares; *Cephalanthera rubra* and *Epipactis helleborine*) and 14 orchid species associated with rhizoctonia fungi (rhizoctonia: yellow circles; *Dama = Dactylorhiza majalis*, *Mamo = Malaxis monophyllos*, *Psal = Pseudorchis albida*, *Trgl = Traunsteinera globosa*; *n* = 5 for each of the 17 orchid species; for a full species list see Table S1). The green box represents mean enrichment factors ± 1 *SD* for the autotrophic reference plants that were sampled together with the orchid species (*n* = 253, see Table S2) whereas mean ε values of reference plants are zero by definition

with ectomycorrhizal fungi, rhizoctonia: orchid species associated with rhizoctonia fungi) and autotrophic references were significantly different (χ^2 = 62.686, *df* = 3, *p* < .001; Figure 2). Pairwise comparisons showed that total nitrogen concentrations were highest in PMH ECM (2.64 ± 0.22 mmol/g_{dw}) and FMH ECM (2.48 ± 0.35 mmol/g_{dw}) and were not significantly different (p_{adj} = .582). Nitrogen concentrations in the leaves of orchids associated with rhizoctonia fungi (1.76 ± 0.35 mmol/

g_{dw}) were significantly lower (p_{adj} < .001) than in the leaves of orchids associated with ECM fungi. Nitrogen concentrations were lowest in the leaves of autotrophic references (1.48 ± 0.48 mmol/g_{dw}; p_{adj} < .001; Figure 2, Tables S1 and S2).

3.3 | Molecular identification of mycorrhizal fungi

Cortex cells in the orchid roots of all 11 investigated orchid species, except *M. monophyllos*, contained pelotons apparent as dense coils of fungal hyphae. The colonization level ranged between 10% and 100% of cortex cells filled with pelotons. Colonization was conspiciously poor in *L. loeselii* where pelotons were only visible in 10% of the cortex cells in the roots of two of the five sampled individuals. Yet, for the majority of species of open meadow habitats we investigated, and *N. cordata* sampled in a coniferous forest, associations with rhizoctonia fungi were found (Table 2). Associations with basidiomycetes matching DNA sequences of the Ceratobasidiaceae genera *Thantephorus* and *Ceratobasidium* and the Tulasnellaceae *Tulasnella* were most frequent (Table 2). The orchid mycorrhizal basidiomycete *Sebacina* (Sebacinaceae) was only detected in one individual of *D. majalis*. Potentially ectomycorrhizal fungi of the ascomycete order Helotiales and the genus *Peziza* (Ascomycota) were only found in a root of one individual of the orchid species *L. loeselii* and *H. monorchis*. An Ascomycota species of unknown mycorrhizal type was detected in two individuals of *D. majalis*. The dark septate endophyte *Phialocephala* was found in the roots of two individuals of *D. majalis*, whereas the dark septate endophyte *Leptodontidium orchidicola* (Ascomycota) was more frequent and found in the roots of *L. loeselii*, *M. monophyllos* and *G. nigra* (Table 2).

4 | DISCUSSION

All rhizoctonia-associated meadow orchid species lack ^{13}C enrichment or even display a significant depletion in ^{13}C relative to autotrophic references (Table 2). However, most of these species are significantly enriched in both ^{15}N and ^{2}H relative to autotrophic species and are thus PMH (Table 1). Average nitrogen concentrations of rhizoctonia-associated orchid species are significantly higher than those of autotrophic references which suggests transfer of fungal material in colonized orchid cells. *Pseudorchis albida* is the sole species in this study that shows neither enrichment in any of ^{13}C, ^{15}N or ^{2}H nor has a distinctive nitrogen concentration that would differentiate this meadow orchid from autotrophic species (Figure 2). Consequently, *P. albida* remains the only species among the investigated orchids that should be categorized as apparently autotrophic (Table 1). *Dactylorhiza majalis* also lacks ^{2}H enrichment but shows a significant enrichment in ^{15}N relative to autotrophic references, though its leaf total nitrogen concentration is not higher than that of autotrophic references (Tables S1 and S2). If at all, *D. majalis* is only marginally PMH. A lacking ^{2}H enrichment, but a pronounced ^{15}N enrichment additionally to significant higher total leaf nitrogen concentrations, characterizes both *M. monophyllos* and *T. globosa*; thus, these two meadow species are

Species	$\varepsilon^{13}C$ U	p_{adjust}	$\varepsilon^{15}N$ U	p_{adjust}	ε^2H U	p_{adjust}
Neottia nidus-avis	**1,265**	**.014**	**1,265**	**.006**	**1,265**	**.007**
Cephalanthera rubra	**1,133.5**	**.029**	**1,261**	**.006**	**1,138**	**.032**
Epipactis helleborine	**1,055**	**.029**	**1,260**	**.006**	**1,260**	**.007**
Liparis loeselii	**37**	**.016**[a]	**1,124**	**.026**	**1,265**	**.007**
Malaxis monophyllos	330.5	.111	**1,094**	**.026**	381	.214
Neottia cordata	**1,208.5**	**.016**	**1,181.5**	**.015**	**1,213**	**.011**
Spiranthes aestivalis	**166**	**.029**[a]	**1,229**	**.007**	**1,249**	**.007**
Dactylorhiza incarnata	**193**	**.029**[a]	**1,069**	**.026**	**1,166**	**.024**
Dactylorhiza majalis	303.5	.081	**1,095**	**.026**	912	.159
Dactylorhiza viridis	**60**	**.016**[a]	**1,061.5**	**.026**	**1,108.5**	**.032**
Gymnadenia conopsea	**110.5**	**.029**[a]	**1,247**	**.006**	**1,062**	**.032**
Gymnadenia nigra	775	.486	993.5	.056	**1,180**	**.020**
Herminium monorchis	**12.5**	**.014**[a]	**1,035**	**.032**	**1,230**	**.009**
Neotinea ustulata	**187.5**	**.029**[a]	**1,214**	**.008**	**1,086**	**.032**
Platanthera bifolia	584	.820	**1,250**	**.006**	**1,024**	**.046**
Pseudorchis albida	**167**	**.029**[a]	730	.641	950	.111
Traunsteinera globosa	374.5	.182	**1,231**	**.007**	571.5	.781
	$\chi^2 = 114.674, df = 17,$ $p < .001$		$\chi^2 = 140.992, df = 17,$ $p < .001$		$\chi^2 = 125.817,$ $df = 17, p < .001$	

[a]Significantly depleted relative to autotrophic references.

TABLE 1   Results from *post hoc* pairwise comparisons for the enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$ and ε^2H between 17 orchid species and autotrophic references using the Mann–Whitney U test after Kruskal–Wallis tests. The p-values were adjusted using the correction after Benjamini and Hochberg (1995). Significant differences between orchids and their autotrophic references are marked in bold. $n = 5$ for each of the 17 orchid species and $n = 253$ for autotrophic reference plants

slightly PMH. *Gymnadenia nigra* is significantly enriched in ^2H, indicating a mycoheterotrophic nutrient gain, but it lacks ^{15}N enrichment and has only a marginally higher nitrogen concentration.
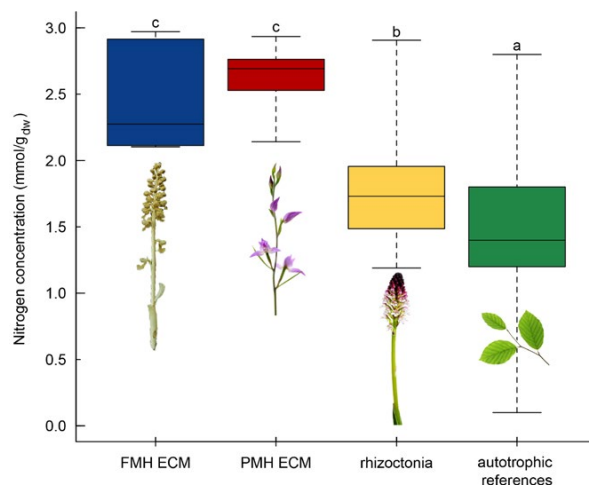
FIGURE 2   Box-and-whisker plot with results from pairwise comparisons in nitrogen concentration data (mmol/g_{dw}) between the three groups of orchids, FMH ECM: fully mycoheterotrophic orchids associated with ectomycorrhizal fungi ($n = 5$), PMH ECM: partially mycoheterotrophic orchids associated with ectomycorrhizal fungi ($n = 10$), rhizoctonia: orchids associated with rhizoctonia fungi ($n = 70$) and autotrophic references ($n = 253$). Different letters indicate significant differences between the groups

Partial mycoheterotrophy is much clearer for the remaining meadow orchid species, *L. loeselii, S. aestivalis, D. incarnata, D. viridis, G. conopsea, H. monorchis, N. ustulata* and *P. bifolia*, sharing the trophic strategy of PMH as expressed by both significantly higher ^{15}N and ^2H enrichment relative to autotrophic references and on average significantly higher nitrogen concentrations (1.76 ± 0.35 vs. 1.48 ± 0.48 mmol/g_{dw}). It is worth noting that the measured nitrogen concentrations lie in the same range as previously reported by Liebel et al. (2010) as 1.67 ± 0.44 mmol/g_{dw} for non-neottioid orchids and 1.40 ± 0.53 mmol/g_{dw} for autotrophic reference species. *Liparis loeselii* shows a similar depletion in ^{13}C as *L. hawaiensis* sampled on the tropical island of Oahu by Hynson (2016). However, *L. loeselii* had a higher ^{15}N enrichment and lower nitrogen concentration than both *L. hawaiensis* and *L. nervosa*, the latter sampled in a warm-temperate evergreen broad-leaved forest in Japan by Motomura, Selosse, Martos, Kagawa, and Yukawa (2010). *Spiranthes aestivalis* shows ^{15}N enrichment and ^{13}C depletion similar to the closely related *S. spiralis* on an open meadow habitat in Italy evaluated by Liebel et al. (2010). The ^{15}N enrichment and ^{13}C depletion in *D. incarnata* and *D. viridis* are similar to the enrichment factors measured in *D. majalis* in a similar habitat, but contrasting *D. incarnata* and *D. viridis* are significantly enriched in ^2H. Both *Gymnadenia* species in this study share a similar ^2H enrichment, but only *G. conopsea* exhibits significant ^{15}N enrichment typical of PMH, and closely related *G. nigra* appears only slightly PMH. However, significant enrichment in ^{15}N and ^2H relative to autotrophic references as observed in *H. monorchis* and *N. ustulata* seem to be common for most rhizoctonia-associated meadow orchids in the Orchidoideae. Summarizing, *P. albida, D. majalis* and *P. bifolia*

TABLE 2 Orchid mycorrhizal fungi identified from roots of 11 orchid species from six sites in Austria and Germany (ECM = fungi forming ectomycorrhizas; printed in bold). *n*: number of orchid individuals in which a fungus was detected. Pelotons in the cortex cells were visible in all species except for *Malaxis monophyllos*

Species	Mycorrhizal fungi (*n*)	Type of mycorrhizal fungi	Best match sequence/ accession number (GenBank)	Identity (%)	Max score	*E*-value
Liparis loeselii	*Leptodontidium orchidicola* (1)	Dark septate endophyte	KF646097.1	99	1,123	0
	Helotiales (1)	ECM	JX001621.1	96	837	0
M. monophyllos	*L. orchidicola* (2)	Dark septate endophyte	KF646097.1	98	1,096	0
	Tulasnella sp. (3)	Rhizoctonia	JF926510.1	94	832	0
Neottia cordata	*Tulasnella* sp. (2)	Rhizoctonia	AB369933.1	98	1,175	0
Spiranthes aestivalis	*Tulasnella calospora* (1)	Rhizoctonia	GU166403.1	98	1,120	0
	Thanatephorus fusisporus (2)	Rhizoctonia	HQ441575.1	95	1,051	0
Dactylorhiza incarnata	*Ceratobasidium* sp. (2)	Rhizoctonia	EU218894.1	99	1,230	0
	Tulasnella sp. (2)	Rhizoctonia	AB369931.1	95	1,033	0
Dactylorhiza majalis	*Phialocephala* sp. (2)	Dark septate endophyte	KF156325.1	97	1,402	0
	Ascomycota sp. (2)	NA	GU566289.1	99	1,146	0
	Tulasnella sp. (4)	Rhizoctonia	AB369933.1	95	1,031	0
	Tulasnella sp. (1)	Rhizoctonia	JN655633.1	99	1,020	0
	Sebacina sp. (1)	Rhizoctonia	AB831798.1	98	1,002	0
Dactylorhiza viridis	*Ceratobasidium* sp. (5)	Rhizoctonia	EU218894.1	99	1,230	0
Gymnadenia nigra	*Ceratobasidium* sp. (1)	Rhizoctonia	EU218894.1	99	1,201	0
	L. orchidicola (1)	Dark septate endophyte	AF486133.1	99	1,158	0
	Tulasnella sp. (3)	Rhizoctonia	AB369931.1	94	968	0
Herminium monorchis	***Peziza* sp.** (1)	ECM	AF491609.1	99	1,059	0
	Ceratobasidiaceae (2)	Rhizoctonia	KC243940.1	97	1,007	0
Neotinea ustulata	*Tulasnella* sp. (1)	Rhizoctonia	KF537641.1	98	1,116	0
	Thanatephorus (5)	Rhizoctonia	AB712278.1	97	1,022	0
	Ceratobasidiaceae (1)	Rhizoctonia	HM141034.1	97	1,022	0
Traunsteinera globosa	*Ceratobasidium* sp. (4)	Rhizoctonia	EU218894.1	99	1,157	0

exhibit similar ^{15}N and ^{13}C enrichments as reported in previous studies (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Johansson et al., 2014; Stöckel et al., 2014; Tedersoo, Pellet, Kõljalg, & Selosse, 2007). Additionally, ^{2}H enrichment in the rhizoctonia-associated orchids *C. calceolus*, *N. ovata*, *O. insectifera* and *P. bifolia* determined in an earlier study by Gebauer et al. (2016) ranged between 20.4 and 41.3‰ and was thus much higher than measured here (−5.6‰ to 27.9‰).

For most rhizoctonia-associated meadow orchids we observed depletion in ^{13}C, enriched ^{15}N and ^{2}H values, and no ^{18}O enrichment (Figure 1 and Figure S1; Table S3). We conclude that the majority of rhizoctonia-associated meadow orchid species is PMH because ^{2}H enrichment can be used as indicator for the gain of C in the form of heterotrophic organic matter and thus as substitute for ^{13}C enrichment in C sources that are insufficiently distinguished in their C isotope abundances. A higher transpiration by orchids compared to autotrophic plants as driver of ^{2}H enrichment can be ruled out here, as the orchids should then be depleted in ^{2}H simultaneously to both depletions in ^{13}C and ^{18}O (Cernusak, Pate, & Farquhar, 2004; Gebauer et al., 2016; Ziegler, 1996), a pattern not observed here.

In this study, we furthermore confirm full mycoheterotrophy for the achlorophyllous forest orchid *N. nidus-avis* that forms mycorrhizas with Sebacina A (McKendrick et al., 2002; Selosse et al., 2002), according to its high enrichment in ^{13}C and ^{15}N, as reported in previous studies (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Preiss et al., 2010; Stöckel et al., 2014), and also based on a high enrichment in ^{2}H (Gebauer et al., 2016; Figure 1). However, it is unclear why we find a less pronounced ^{2}H enrichment (45.5‰ ± 7.0‰) compared to the results of Gebauer et al. (2016) (69.1‰ ± 5.5‰). Still, it is evident that achlorophyllous *N. nidus-avis* gains its entire N, C and (C-H bound) H from a fungal source and ^{13}C, ^{15}N and ^{2}H enrichments are highest compared to all other green-leaved species under study. We also reaffirm PMH for *C. rubra* and *E. helleborine* (Abadie et al., 2006; Bidartondo et al., 2004; Johansson et al., 2014; Preiss et al., 2010; Schiebold, Bidartondo, Karasch et al., 2017), two forest species that associate with a wide spectrum of ectomycorrhizal and rhizoctonia fungi (Bidartondo et al., 2004; Jacquemyn et al., 2016; Ogura-Tsujita & Yukawa, 2008; Těšitelová et al., 2012), due to these species' significant enrichment in ^{13}C and ^{15}N relative to autotrophic references but also due to their intermediate ^{2}H enrichment (Figure 1). The ^{2}H enrichment in *C. rubra* was higher in a previous study by Gebauer et al. (2016) (29.1‰ ± 4.6‰) than in this study (10.2‰ ± 3.2‰) but this might be due to the different light climate at the sampling sites. Here, we sampled *C. rubra* individuals in a more open mixed deciduous and coniferous forest while the previous study was in a closed-canopy beech forest. In addition, the lower ^{13}C enrichment in our study (1.2‰ ± 0.3‰ vs. 2.9‰ ± 1.8‰) supports this reasoning because ectomycorrhiza-associated PMH orchids are known to increase exploitation of fungal carbon as indicated by increasing ^{13}C enrichment with decreasing irradiance (Preiss et al., 2010). The ^{2}H enrichment in *E. helleborine* was in a similar range as previously measured in closely related *E. atrorubens* (22.1‰ ± 4.8‰ vs. 18.8‰ ± 6.0‰). Nitrogen concentrations in all three orchid species predominantly or exclusively associated with ECM fungi (2.64 ± 0.22 mmol/g$_{dw}$ for *C. rubra* and *E. helleborine*, and 2.48 ± 0.35 mmol/g$_{dw}$ for *N. nidus-avis*) confirm the overall picture that mean total nitrogen concentrations in Orchidaceae partnering with ECM fungi are generally twice as high as in autotrophic non-legume plants regardless of their degree of mycoheterotrophy (Hynson et al., 2016). It is noteworthy that the reason for similar nitrogen concentrations in PMH and FMH orchids associated with ECM fungi is likely to due to sampling. While leaf material was used to determine nitrogen concentrations of autotrophic references and of orchid species associated with ECM and rhizoctonia fungi, shoot material was used to analyse nitrogen concentrations of FMH species associated with ECM fungi (*N. nidus-avis*). Shoot material is known to generally have lower nitrogen concentrations than leaf material (Gebauer, Rehder, & Wollenweber, 1988).

Meadow orchid species associated with rhizoctonia fungi appear predisposed to a varying degree of PMH nutrition due to their obligate gain of organic matter via peloton digestion and/or by transfer of compounds across membranes in their early pre-photosynthetic stages. For the most efficient carbon gain, switching fungal partners from rhizoctonia to ECM fungi (Bidartondo et al., 2004) or saprotrophic

non-rhizoctonia fungi (Lee et al., 2015; Ogura-Tsujita et al., 2009) seems to be necessary, and also to sustain full mycoheterotrophy. As far as we know, no FMH orchid species specializes on rhizoctonia. We propose that PMH in rhizoctonia-associated chlorophyllous orchid species is a useful trait to improve competitive success in open habitats and helps survive unfavourable conditions during periods of dormancy. Nevertheless, PMH sensu Merckx (2013) in rhizoctonia-associated orchids does not exclude the occurrence of bi-directional carbon fluxes as shown for the orchid *Goodyera repens* (Cameron, Johnson, Read, & Leake, 2008).

5 | CONCLUSIONS

Our study demonstrates that PMH is a trophic continuum between the extreme endpoints of autotrophy and full mycoheterotrophy. We infer PMH for the meadow orchid species *L. loeselii*, *S. aestivalis*, *D. incarnata*, *D. viridis*, *G. conopsea*, *H. monorchis*, *N. ustulata* and *P. bifolia*, as expressed by both significantly higher ^{2}H and ^{15}N enrichment relative to autotrophic references and on average significantly higher nitrogen concentrations, even though these traits are less pronounced than in the PMH forest species *C. rubra* and *E. helleborine*. Of the meadow orchid species associating with rhizoctonia fungi, *P. albida* is the sole apparently autotrophic orchid species in this study as it shows neither enrichment in ^{13}C, ^{15}N or ^{2}H, nor a distinctive nitrogen concentration. *Dactylorhiza majalis*, *M. monophyllos* and *T. globosa* are only marginally PMH as they all lack ^{2}H enrichment but are characterized by pronounced ^{15}N enrichment and significantly higher leaf total nitrogen concentrations. *Gymnadenia nigra* is on a similar position of this continuum as it is distinctive from autotrophic plants in its ^{2}H enrichment.

Our findings support the hypothesis that orchids in the subfamilies Epidendroideae and Orchidoideae forming orchid mycorrhizas with rhizoctonia fungi and growing in habitats with high irradiance, such as montane meadows, feature PMH as nutritional mode. Significant ^{15}N enrichment and higher total nitrogen concentrations in cryptic mycoheterotrophs can be used as indicators for PMH in rhizoctonia-associated orchid species as recently suggested by Hynson (2016). We conclude that PMH in rhizoctonia-associated orchid species, as elucidated here by pronounced ^{2}H enrichment, plays a far greater role in orchids of open habitats than expected. The implications of our findings for the ecology, evolution and conservation of orchids, mycorrhizal fungi and co-occurring plants, and the function of their ecosystems, now deserve investigation.

AUTHORS' CONTRIBUTIONS

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.3nf8b (Schiebold, Bidartondo, Lenhard et al., 2017) and GenBank (accession numbers: KY271858–KY271875).

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SUPPORTING INFORMATION

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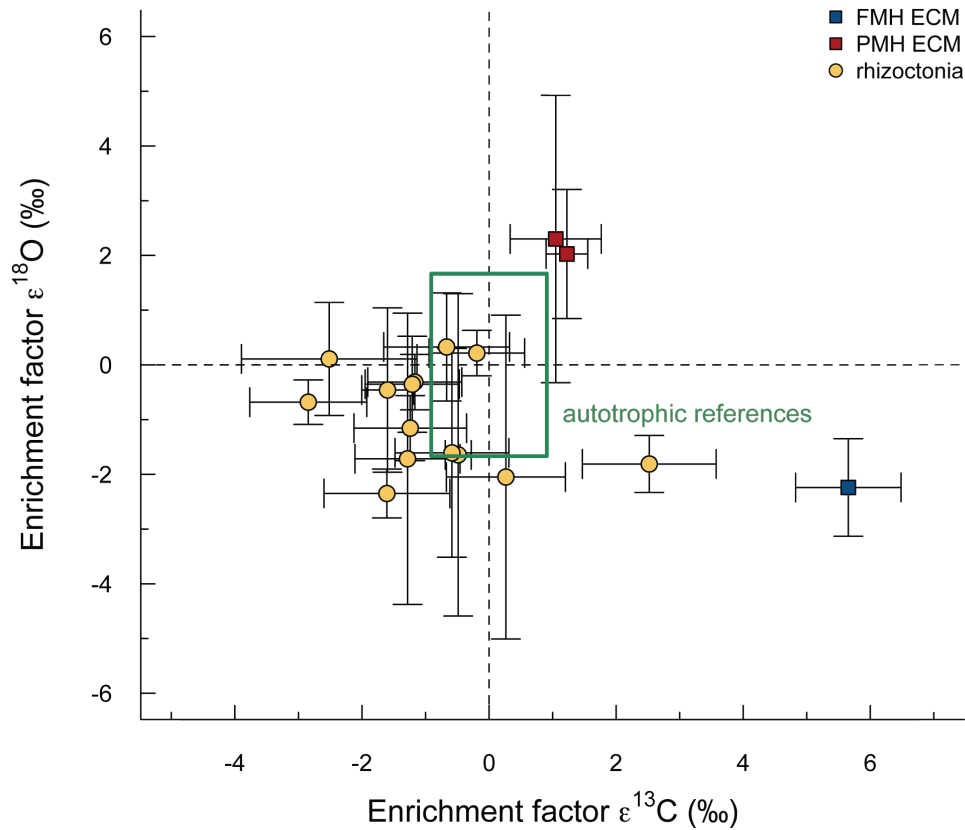
Figure S1. Mean enrichment factors e^{18}O and e^{13}C ± 1 SD of 17 orchid species sampled in the Northern Limestone Alps in Austria and Germany and the Fichtelgebirge in NE Bavaria/Germany in July of the four consecutive years 2012−2015. One fully mycoheterotrophic orchid species associated with ectomycorrhizal fungi (blue squares; *Neottia nidus-avis*), two partially mycoheterotrophic orchid species associated with ectomycorrhizal fungi (red squares; *Cephalanthera rubra* and *Epipactis helleborine*) and 14 orchid species associated with rhizoctonia fungi (yellow circles; *n* = 5 for each of the 17 orchid species; for a full species list see Table S1). The green box represents mean enrichment factors ± 1 s.d. for the autotrophic reference plants that were sampled together with the orchid species (*n* = 253, see Table S2) whereas mean e values of reference plants are zero by definition.

Table S1: Single and mean $\delta^{15}N$, $\delta^{13}C$, δ^2H, $\delta^{18}O$ values, enrichment factors $\varepsilon^{15}N$, $\varepsilon^{13}C$, ε^2H, $\varepsilon^{18}O$ and total nitrogen concentration data of 17 Orchidaceae species.

Functional type	Subfamily	Tribe	Species	Site	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	δ^2H [‰]	$\delta^{18}O$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{13}C$ [‰]	ε^2H [‰]	$\varepsilon^{18}O$ [‰]
FMH ECM	Epidendroideae	Neottieae	*Neottia nidus-avis* (L.) Rich.	Garfülla	296	2,11	9,40	-26,66	-44,58	22,77	14,45	6,07	43,60	-1,97
					297	2,10	12,70	-26,37	-37,29	20,82	15,45	6,92	50,74	-2,88
					298	2,27	8,55	-25,77	-42,51	21,21	16,45	5,17	53,85	-1,28
					299	2,92	7,45	-26,45	-55,24	22,10	17,45	5,13	35,93	-1,64
					300	2,97	7,49	-26,51	-53,09	20,70	18,45	4,97	43,14	-3,43
					mean	**2,48**	**9,12**	**-26,35**	**-46,54**	**21,52**	**16,45**	**5,65**	**45,45**	**-2,24**
					SD	**0,43**	**2,16**	**0,34**	**7,49**	**0,89**	**1,58**	**0,83**	**7,03**	**0,89**
					n	**5**								
PMH ECM	Epidendroideae	Neottieae	*Cephalanthera rubra* (L.) Rich.	Garfülla	973	2,72	3,83	-30,84	-89,69	24,69	8,59	0,81	8,05	0,80
					974	2,53	3,06	-31,61	-90,70	24,01	7,88	1,07	7,01	1,04
					975	2,76	4,36	-31,76	-89,57	25,54	7,64	1,70	13,15	2,88
					976	2,76	1,09	-32,02	-86,52	26,12	6,71	1,31	8,75	3,55
					977	2,83	1,95	-32,62	-83,82	24,26	6,22	1,23	14,16	1,87
					mean	**2,72**	**2,86**	**-31,77**	**-88,06**	**24,92**	**7,41**	**1,22**	**10,22**	**2,03**
					SD	**0,11**	**1,34**	**0,65**	**2,84**	**0,89**	**0,95**	**0,33**	**3,21**	**1,18**
					n	**5**								
PMH ECM	Epidendroideae	Neottieae	*Epipactis helleborine* (L.) Crantz	Garfülla	953	2,93	8,85	-28,99	-104,10	30,66	15,26	2,15	28,81	2,50
					954	2,66	3,58	-29,83	-121,54	25,83	11,44	0,94	18,60	-1,25
					955	2,48	0,66	-30,51	-109,75	29,22	6,06	0,14	20,12	1,21
					956	2,14	6,52	-28,97	-112,76	30,17	13,26	1,01	25,34	5,92
					957	2,62	-1,64	-29,60	-119,15	29,31	5,50	1,02	17,47	3,11
					mean	**2,57**	**3,59**	**-29,58**	**-113,46**	**29,04**	**10,31**	**1,05**	**22,07**	**2,30**
					SD	**0,29**	**4,25**	**0,64**	**7,06**	**1,89**	**4,35**	**0,72**	**4,83**	**2,63**
					n	**5**								
green Epidendroideae rhizoctonia	Epidendroideae	Malaxideae	*Liparis loeselii* (L.) Rich.	Thüringen	918	1,61	1,06	-28,33	-104,12	19,39	2,96	-1,57	31,17	0,12
					919	1,69	1,49	-28,66	-106,76	21,58	5,40	-1,09	28,04	0,56
					920	1,67	-1,32	-29,25	-113,81	20,37	0,44	-3,18	21,14	-1,49
					921	2,01	0,45	-30,67	-104,65	20,70	3,81	-4,55	29,44	0,02
					922	1,69	-0,04	-28,24	-111,04	21,51	3,32	-2,18	29,75	1,33
					mean	**1,73**	**0,33**	**-29,03**	**-108,08**	**20,71**	**3,19**	**-2,52**	**27,91**	**0,11**
					SD	**0,16**	**1,09**	**1,00**	**4,21**	**0,90**	**1,80**	**1,38**	**3,94**	**1,03**
					n	**5**								
green Epidendroideae rhizoctonia	Epidendroideae	Malaxideae	*Malaxis monophyllos* (L.) Sw.	Schachentor	963	2,16	-0,34	-26,57	-115,97	23,05	1,42	1,04	2,30	1,33
					964	1,73	-0,57	-28,67	-120,54	21,68	2,10	-1,19	-1,59	-0,47

Table S1 (continued)

Functional type	Subfamily	Tribe	Species	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	ε^{18}O [‰]
			Malaxis monophyllos (continued)		965	2,14	-0,52	-27,90	-117,39	23,25	1,09	-0,75	-4,54	1,47
					966	1,73	0,23	-28,60	-133,43	21,37	1,76	-0,98	-16,56	-0,53
					967	1,60	1,78	-29,37	-126,94	21,63	4,70	-1,46	-7,69	-0,16
					mean	**1,87**	**0,11**	**-28,22**	**-122,85**	**22,20**	**2,21**	**-0,67**	**-5,62**	**0,33**
					SD	**0,26**	**0,98**	**1,06**	**7,27**	**0,88**	**1,44**	**0,99**	**7,14**	**0,99**
					n	**5**								
green Epidendroideae rhizoctonia	Epidendroideae	Neottieae	*Neottia cordata* (L.) Rich.	Fichtelsee	958	1,75	-3,96	-29,92	-88,38	22,45	2,24	2,91	8,92	-1,01
					959	1,75	-2,24	-30,75	-87,01	21,54	4,01	2,45	12,57	-1,88
					960	1,47	-3,58	-32,30	-80,65	21,30	2,48	0,74	19,53	-2,42
					961	1,59	-2,10	-29,99	-90,58	21,62	3,12	3,36	17,74	-1,71
					962	1,61	-1,99	-30,70	-91,48	20,76	3,30	3,15	17,66	-2,05
					mean	**1,63**	**-2,78**	**-30,73**	**-87,62**	**21,53**	**3,03**	**2,52**	**15,28**	**-1,81**
					SD	**0,12**	**0,93**	**0,96**	**4,28**	**0,61**	**0,70**	**1,05**	**4,40**	**0,52**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Cranichideae	*Spiranthes aestivalis* (Poir.) Rich.	Göfis	928	1,80	1,48	-28,32	-112,02	19,95	4,46	-1,13	21,25	0,17
					929	2,17	3,72	-28,17	-103,82	20,42	4,92	-1,06	15,53	0,63
					930	2,32	2,80	-29,19	-112,60	19,50	3,73	-2,35	15,33	-0,45
					931	1,80	2,49	-27,20	-90,91	19,06	3,71	-0,28	35,92	-0,42
					932	1,82	3,21	-28,52	-96,89	18,67	4,92	-1,22	28,04	-1,70
					mean	**1,98**	**2,74**	**-28,28**	**-103,25**	**19,52**	**4,35**	**-1,21**	**23,21**	**-0,35**
					SD	**0,25**	**0,84**	**0,72**	**9,45**	**0,69**	**0,60**	**0,74**	**8,80**	**0,88**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Dactylorhiza incarnata* (L.) Soó	Laguz	923	1,96	-0,14	-26,61	-112,56	19,22	1,78	-1,67	6,24	-0,89
					924	1,98	-1,53	-26,27	-109,84	18,90	0,18	-0,22	12,75	-0,39
					925	1,91	0,24	-27,05	-103,76	18,59	2,40	-1,32	17,65	-0,01
					926	2,03	1,84	-27,92	-111,39	19,04	3,62	-2,02	12,34	0,38
					927	2,17	-0,56	-27,50	-116,01	17,99	2,66	-0,62	10,77	-0,66
					mean	**2,01**	**-0,03**	**-27,07**	**-110,71**	**18,75**	**2,13**	**-1,17**	**11,95**	**-0,32**
					SD	**0,10**	**1,23**	**0,66**	**4,50**	**0,48**	**1,27**	**0,74**	**4,10**	**0,51**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh.	Garfälla	908	1,88	0,62	-28,13	-113,40	19,72	1,93	-0,50	7,20	-3,97
					909	1,48	1,64	-28,64	-123,64	18,07	1,74	-0,65	-0,12	0,46

Table S1 (continued)

Functional type	Subfamily	Tribe	Species	Site	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	ε^{18}O [‰]
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Dactylorhiza majalis* (continued)		910	1,22	1,76	-28,33	-118,22	17,91	2,13	-0,61	12,35	0,60
					911	1,47	0,42	-28,70	-122,31	18,02	0,89	-0,66	-3,07	-5,63
					912	1,37	2,79	-28,27	-115,96	17,39	3,86	-0,52	15,47	0,32
					mean	**1,48**	**1,44**	**-28,41**	**-118,71**	**18,22**	**2,11**	**-0,59**	**6,37**	**-1,64**
					SD	**0,24**	**0,96**	**0,25**	**4,28**	**0,88**	**1,09**	**0,07**	**7,91**	**2,94**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Dactylorhiza viridis* (L.) R.M.Bateman, Pridgeon & M.W.Chase	Laguz	948	1,84	-2,99	-29,04	-118,65	24,60	1,50	-1,78	1,95	0,90
					949	1,42	-0,68	-28,82	-106,48	24,78	3,03	-1,46	8,91	-0,61
					950	1,31	-2,74	-29,12	-99,34	24,46	1,28	-1,61	16,59	1,13
					951	1,44	-2,05	-28,34	-100,97	21,20	0,67	-1,02	18,27	-2,45
					952	1,61	-1,07	-29,95	-109,74	22,45	3,16	-2,12	9,78	-1,26
					mean	**1,52**	**-1,91**	**-29,05**	**-107,04**	**23,50**	**1,93**	**-1,60**	**11,10**	**-0,46**
					SD	**0,21**	**1,01**	**0,58**	**7,72**	**1,59**	**1,11**	**0,41**	**6,55**	**1,50**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Gymnadenia conopsea* (L.) R.Br.	Garfülla	968	1,59	-0,90	-33,18	-107,43	21,21	3,79	-1,48	-0,06	-2,77
					969	1,39	-0,97	-32,56	-103,04	20,95	4,40	-1,90	6,59	-2,47
					970	1,53	4,29	-31,23	-95,54	20,61	9,51	-0,72	16,49	-1,93
					971	1,90	3,77	-31,47	-100,68	21,27	9,52	-0,79	12,22	-1,83
					972	1,91	-0,19	-33,11	-97,29	20,19	5,66	-3,15	14,55	-2,75
					mean	**1,66**	**1,20**	**-32,31**	**-100,80**	**20,85**	**6,58**	**-1,61**	**9,96**	**-2,35**
					SD	**0,23**	**2,61**	**0,91**	**4,72**	**0,45**	**2,77**	**0,99**	**6,72**	**0,45**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Gymnadenia nigra* (L.) Rchb.f.	Laguz	938	1,89	-0,46	-26,04	-100,03	19,35	4,79	1,51	7,43	-3,60
					939	1,75	-2,78	-27,49	-101,85	18,23	2,92	1,05	10,57	-3,23
					940	1,49	-0,67	-28,33	-107,48	16,08	2,27	-1,13	12,23	-4,00
					941	1,63	-2,53	-27,45	-106,78	16,36	0,19	-0,02	16,90	-2,58
					942	1,36	-3,29	-27,67	-101,26	21,77	0,00	0,24	15,37	3,16
					mean	**1,62**	**-1,95**	**-27,39**	**-103,48**	**18,36**	**2,03**	**0,33**	**12,50**	**-2,05**
					SD	**0,21**	**1,29**	**0,84**	**3,41**	**2,34**	**2,00**	**1,02**	**3,78**	**2,96**
					n	**5**								

Table S1 (continued)

Functional type	Subfamily	Tribe	Species	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	ε^{18}O [‰]
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Herminium monorchis* (L.) R.Br.	Garfülla	913	1,56	0,31	-30,20	-103,33	18,84	1,38	-2,77	24,39	-1,18
					914	1,30	-0,34	-30,80	-104,75	18,50	0,08	-3,07	11,57	-0,40
					915	1,58	1,68	-31,19	-106,60	18,47	2,03	-4,12	16,57	-1,07
					916	1,48	0,77	-29,05	-90,11	18,52	2,00	-1,54	25,64	-0,35
					917	1,33	1,16	-30,66	-100,05	18,80	3,25	-2,73	13,00	-0,41
					mean	**1,45**	**0,71**	**-30,38**	**-100,97**	**18,62**	**1,75**	**-2,85**	**18,23**	**-0,68**
					SD	**0,13**	**0,78**	**0,83**	**6,52**	**0,18**	**1,15**	**0,92**	**6,47**	**0,41**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Neotinea ustulata* (L.) R.M.Bateman, Pridgeon & M.W.Chase	Garfülla	903	2,20	3,64	-29,65	-111,93	19,81	2,43	-1,77	8,04	-1,02
					904	2,16	3,74	-28,38	-116,15	19,75	3,56	-0,29	4,66	-0,43
					905	1,34	4,69	-30,38	-108,96	18,93	4,70	-2,48	11,05	-1,80
					906	1,89	2,73	-27,59	-116,29	19,51	4,89	-0,64	10,04	-0,79
					907	2,25	4,05	-28,32	-102,54	19,25	5,15	-1,02	6,95	-1,73
					mean	**1,97**	**3,77**	**-28,86**	**-111,17**	**19,45**	**4,15**	**-1,24**	**8,15**	**-1,16**
					SD	**0,38**	**0,71**	**1,12**	**5,72**	**0,37**	**1,13**	**0,89**	**2,53**	**0,59**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Platanthera bifolia* (L.) Rich.	Garfülla	898	1,92	4,20	-27,38	-100,47	20,62	4,71	0,22	20,77	0,73
					899	1,98	4,94	-28,41	-105,65	19,37	7,11	-1,43	14,18	-0,08
					900	1,83	4,56	-26,85	-112,75	20,50	6,16	0,36	5,08	0,50
					901	1,87	4,61	-27,65	-112,38	19,50	5,37	-0,38	1,73	-0,29
					902	1,43	4,67	-27,40	-109,25	19,92	5,83	0,26	4,06	0,21
					mean	**1,81**	**4,59**	**-27,54**	**-108,10**	**19,98**	**5,84**	**-0,19**	**9,16**	**0,22**
					SD	**0,22**	**0,27**	**0,57**	**5,13**	**0,56**	**0,90**	**0,75**	**8,03**	**0,42**
					n	**5**								
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Pseudorchis albida* (L.) Á.Löve & D.Löve	Laguz	943	1,52	-3,90	-28,36	-115,14	21,39	1,49	-2,12	-2,40	-2,38
					944	1,53	-5,47	-27,57	-113,11	17,14	1,67	-0,81	9,43	-4,89
					945	1,61	-2,76	-27,58	-112,30	21,20	0,25	-0,75	8,83	-1,18
					946	1,41	-5,19	-28,60	-115,31	24,91	-0,26	-2,24	11,06	2,38
					947	1,19	-6,06	-27,12	-111,85	23,43	-1,85	-0,50	3,18	-2,49
					mean	**1,45**	**-4,68**	**-27,84**	**-113,54**	**21,61**	**0,26**	**-1,28**	**6,02**	**-1,71**
					SD	**0,16**	**1,33**	**0,62**	**1,60**	**2,93**	**1,43**	**0,83**	**5,56**	**2,66**
					n	**5**								

Table S1 (continued)

Functional type	Subfamily	Tribe	Species	Site	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	ε^{18}O [‰]
green Orchidoideae rhizoctonia	Orchidoideae	Orchideae	*Traunsteinera globosa* (L.) Rchb.	Laguz	933	2,27	2,31	-27,82	-118,82	21,67	3,30	-1,25	-24,16	-3,85
					934	2,10	2,19	-27,36	-125,88	23,04	3,39	-1,28	-10,83	-1,52
					935	2,72	2,99	-26,79	-107,10	24,59	5,92	-0,24	-4,94	-1,74
					936	2,46	3,98	-27,45	-106,14	22,73	6,71	-0,98	11,59	-2,32
					937	2,91	3,86	-26,16	-96,60	27,07	5,51	0,83	19,39	1,39
					mean	2,49	3,06	-27,12	-110,91	23,82	4,96	-0,58	-1,79	-1,61
					SD	0,33	0,84	0,65	11,50	2,10	1,54	0,90	17,46	1,91
					n	5								

Table S2: Single and mean δ15N, δ13C, δ2H, δ18O values, enrichment factors ε15N, ε13C, ε2H, ε18O and total nitrogen concentration data of all autotrophic reference plant species.

*mycorrhizal status according to Wang B. & Qiu Y. (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza, 16, 299-363; AM = arbuscular mycorrhiza, ECM = ectomycorhiza, EEM = ectendomycorrhiza, ERM = ericoid mycorrhiza, NM = nonmycorrhiza; mycorrhizal status in brackets indicates characterisation of a species in the same genus

Family	Species	mycorrhizal status*	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ15N [‰]	δ13C [‰]	δ2H [‰]	δ18O [‰]	ε15N [‰]	ε13C [‰]	ε2H [‰]	ε18O [‰]
Apiaceae	*Astrantia major*	AM + NM	898	2,22	-1,27	-26,80	-113,58	19,52	-0,75	0,80	7,67	-0,37
			899	2,20	-2,64	-26,87	-116,35	19,99	-0,47	0,11	3,48	0,53
			900	1,83	-2,14	-26,55	-111,04	21,17	-0,55	0,66	6,78	1,18
			901	2,36	0,09	-28,27	-105,48	19,71	0,85	-1,00	8,63	-0,07
			902	2,47	-1,20	-27,05	-109,69	19,78	-0,03	0,62	3,62	0,08
			903	2,15	0,60	-26,68	-115,41	21,13	-0,60	1,19	4,56	0,30
			904	2,80	0,69	-27,01	-112,62	20,19	0,51	1,08	8,19	0,01
			905	2,66	0,51	-26,73	-117,61	20,34	0,52	1,17	2,40	-0,38
			906	1,96	-3,19	-26,04	-119,24	19,41	-1,03	0,92	7,09	-0,89
			907	2,35	-1,90	-26,41	-110,08	21,62	-0,80	0,89	-0,58	0,63
			913	1,81	-1,74	-27,04	-123,58	19,26	-0,67	0,39	4,14	-0,76
			914	1,31	0,18	-26,43	-116,82	18,55	0,60	1,31	-0,50	-0,35
			915	1,81	-0,96	-26,29	-116,85	17,07	-0,61	0,79	6,31	-2,47
			916	2,15	-1,04	-26,63	-115,53	19,26	0,20	0,87	0,22	0,40
			917	2,19	-2,17	-27,17	-112,00	19,47	-0,08	0,76	1,06	0,26
Aristolochia-ceae	*Asarum europaeum*	AM + NM	973	1,49	-6,08	-32,96	-92,90	22,74	-1,32	-1,31	4,83	-1,15
			974	1,40	-6,03	-34,58	-93,53	22,58	-1,21	-1,90	4,19	-0,39
			975	1,18	-4,78	-35,81	-94,30	22,51	-1,50	-2,35	8,42	-0,15
			976	1,14	-6,26	-35,73	-96,92	23,22	-0,65	-2,40	-1,65	0,64
			977	1,58	-5,47	-35,77	-96,57	21,39	-1,20	-1,92	1,42	-1,00
Asteraceae	*Alchemilla nitida*	(AM + NM)	963	1,39	-2,47	-26,73	-123,91	23,15	-0,71	0,88	-5,64	1,43
			964	1,13	-3,23	-27,75	-122,98	21,95	-0,55	-0,27	-4,03	-0,20
			965	1,19	-1,74	-27,06	-111,30	22,51	-0,13	0,10	1,55	0,72
			966	1,26	-2,85	-27,25	-115,68	23,57	-1,31	0,37	1,18	1,66
			967	1,13	-2,99	-27,30	-126,02	22,56	-0,07	0,61	-6,76	0,77
	Centaurea angustifolia	(AM)	928	1,10	-2,55	-26,45	-137,55	19,64	0,43	0,74	-4,28	-0,13
			929	1,28	-3,69	-27,75	-116,57	19,04	-2,49	-0,64	2,79	-0,75
			930	1,45	-2,67	-27,34	-129,20	19,10	-1,74	-0,50	-1,27	-0,86
			931	1,16	-4,19	-27,51	-130,56	18,59	-2,96	-0,59	-3,73	-0,90
			932	1,31	-5,09	-27,55	-123,70	20,69	-3,37	-0,24	1,23	0,31
	Centaurea jacea	AM	898	1,54	0,40	-28,33	-132,57	20,29	0,91	-0,72	-11,33	0,40
			899	1,56	-2,97	-26,86	-121,99	18,93	-0,80	0,12	-2,16	-0,53
			900	1,59	-0,80	-27,63	-120,59	19,31	0,80	-0,42	-2,76	-0,68
			901	1,70	-0,69	-27,27	-116,02	19,18	0,07	0,00	-1,92	-0,61
			902	1,90	-0,48	-27,91	-120,24	19,85	0,68	-0,24	-6,93	0,15
			903	2,08	1,86	-28,28	-124,16	21,04	0,65	-0,40	-4,20	0,21
			904	2,63	1,14	-28,70	-123,75	19,63	0,97	-0,61	-2,94	-0,55
			905	2,31	0,62	-28,32	-123,76	20,47	0,63	-0,42	-3,75	-0,25
			906	1,54	-1,83	-27,92	-136,09	20,33	0,33	-0,96	-9,76	0,03
			907	2,79	-0,46	-27,49	-101,90	20,83	0,64	-0,20	7,60	-0,15
			913	1,78	0,26	-27,68	-136,46	21,21	1,33	-0,25	-8,74	1,19
			914	2,23	-0,30	-28,77	-116,01	18,36	0,11	-1,03	0,31	-0,54
			915	1,43	0,34	-27,54	-132,37	20,48	0,69	-0,47	-9,21	0,95
			916	1,78	-0,19	-27,51	-116,40	18,54	1,04	-0,01	-0,65	-0,32
	Prenanthes purpurea	NA	953	2,71	-4,17	-32,14	-138,33	27,24	2,24	-1,00	-5,42	-0,91
Campanula-ceae	*Campanula cochleariifolia*	(AM + NM)	298	1,37	-3,10	-31,29	-102,12	21,37	0,51	-0,34	-5,76	-1,12
			299	1,45	-4,99	-32,73	-99,19	24,00	0,97	-1,15	-8,02	0,26
			300	2,19	-5,33	-32,05	-106,92	24,62	1,17	-0,57	-10,70	0,49
Caprifoliaceae	*Succisa pratensis*	AM	921	1,05	-2,66	-25,48	-137,57	19,77	0,70	0,64	-3,47	-0,90
			922	0,75	-1,83	-26,32	-134,15	18,08	1,53	-0,26	6,64	-2,10
Cyperaceae	*Carex flava*	AM + NM	933	1,27	4,42	-26,59	-89,34	26,23	5,41	-0,02	5,32	0,72
			934	0,65	1,30	-26,20	-131,60	25,68	2,50	-0,13	-16,55	1,12

Table S2 (continued)

Family	Species	mycorrhizal status*	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	ε^{18}O [‰]
	Carex flava		935	1,39	0,05	-27,34	-103,42	24,40	2,98	-0,79	-1,26	-1,93
	(continued)		936	1,11	-0,18	-26,42	-117,15	22,98	2,55	0,05	0,58	-2,06
			937	0,97	0,33	-27,47	-119,55	24,13	1,97	-0,48	-3,56	-1,55
	Rhynchospora alba	(AM + NM)	918	1,37	0,28	-27,24	-137,87	18,20	2,17	-0,48	-2,58	-1,07
			919	0,81	-0,22	-28,01	-135,93	21,39	3,69	-0,44	-1,13	0,37
			920	1,20	2,15	-26,28	-123,30	21,69	3,91	-0,21	11,66	-0,17
	Trichophorum cespitosum	AM + NM	923	1,59	-1,10	-24,71	-110,02	21,83	0,81	0,23	8,78	1,72
			924	1,03	0,20	-26,22	-119,47	20,50	1,91	-0,17	3,12	1,21
			925	1,51	-1,03	-25,11	-122,16	19,46	1,13	0,62	-0,74	0,85
			926	1,17	-0,89	-25,51	-134,29	20,54	0,90	0,39	-10,57	1,88
			927	1,30	-1,76	-27,05	-119,86	20,71	1,45	-0,17	6,93	2,06
			943	1,34	1,53	-25,77	-101,31	22,67	6,92	0,48	11,43	-1,10
			945	1,54	2,70	-26,64	-116,04	24,30	5,71	0,20	5,09	1,93
			946	1,33	0,04	-24,92	-122,93	22,91	4,97	1,43	3,44	0,39
			947	1,46	2,70	-25,83	-117,48	27,86	6,91	0,78	-2,44	1,94
Ericaceae	*Calluna vulgaris*	ERM	948	1,08	-3,73	-27,60	-125,06	23,90	0,76	-0,34	-4,46	0,20
			949	1,33	-2,40	-27,38	-114,26	27,38	1,31	-0,02	1,12	1,98
			950	1,71	-4,90	-26,47	-118,75	22,35	-0,89	1,04	-2,82	-0,98
			951	1,23	-1,51	-26,56	-120,67	25,48	1,21	0,76	-1,43	1,83
			952	1,18	-3,19	-27,85	-121,49	25,18	1,04	-0,02	-1,96	1,46
	Vaccinum myrtillus	ERM	938	1,04	-6,03	-27,55	-97,12	20,96	-0,78	-0,01	10,34	-1,99
			939	1,10	-7,46	-28,39	-105,83	23,77	-1,77	0,15	6,59	2,31
			940	1,38	-1,08	-28,51	-110,20	21,45	1,86	-1,31	9,51	1,38
			941	1,44	-2,44	-27,85	-113,13	18,96	0,29	-0,42	10,55	0,02
			942	1,35	-1,73	-28,91	-113,24	18,74	1,56	-1,01	3,39	0,13
			948	1,05	-5,38	-28,65	-113,32	24,06	-0,89	-1,39	7,28	0,36
			949	1,18	-4,92	-28,55	-112,99	24,73	-1,22	-1,19	2,39	-0,66
			950	1,09	-4,95	-28,70	-111,32	23,97	-0,93	-1,19	4,62	0,64
			951	1,20	-2,02	-28,09	-106,17	22,56	0,70	-0,77	13,07	-1,09
			952	1,18	-4,91	-28,71	-113,87	24,28	-0,68	-0,88	5,66	0,56
			958	1,19	-4,70	-34,36	-97,15	22,76	1,50	-1,54	0,15	-0,70
			959	1,18	-5,48	-34,23	-99,24	22,70	0,77	-1,03	0,34	-0,72
			960	1,10	-4,92	-34,25	-99,96	23,08	1,14	-1,22	0,21	-0,64
			961	1,27	-4,78	-34,51	-103,13	22,12	0,43	-1,15	5,18	-1,21
			962	1,18	-2,97	-34,44	-115,88	22,52	2,32	-0,60	-6,75	-0,29
	Vaccinum uliginosum	ERM	943	1,43	-8,79	-27,59	-119,91	24,05	-3,40	-1,35	-7,17	0,27
			944	1,29	-5,79	-27,05	-124,22	21,88	1,36	-0,30	-1,68	-0,16
			945	1,27	-5,26	-27,33	-124,14	21,06	-2,25	-0,50	-3,01	-1,32
			946	1,40	-7,04	-27,21	-132,02	22,43	-2,11	-0,85	-5,66	-0,09
			947	1,43	-7,50	-26,74	-114,89	24,76	-3,29	-0,13	0,15	-1,16
	Vaccinum vitis-idea	ERM	958	0,76	-6,48	-30,99	-88,62	23,36	-0,28	1,84	8,68	-0,10
			959	0,66	-5,52	-31,47	-95,10	24,25	0,73	1,72	4,48	0,84
			960	0,66	-5,07	-31,91	-98,12	23,53	0,99	1,12	2,05	-0,19
			961	0,71	-4,57	-32,40	-111,92	22,86	0,65	0,95	-3,60	-0,47
			962	0,74	-4,93	-32,34	-102,60	23,62	0,36	1,51	6,53	0,81
	Rhododendron hirsutum	(ERM)	963	1,72	-4,48	-26,70	-125,31	20,67	-2,73	0,92	-7,04	-1,05
			964	1,36	-5,77	-27,55	-132,94	21,42	-3,09	-0,07	-13,99	-0,73
			965	1,52	-4,50	-26,68	-126,86	21,16	-2,89	0,47	-14,01	-0,63
			966	1,50	-4,48	-27,87	-123,41	20,36	-2,95	-0,25	-6,54	-1,54
			967	1,49	-3,97	-28,03	-128,30	20,24	-1,05	-0,12	-9,05	-1,54

Table S2 (continued)

Family	Species	mycorrhizal status[*]	Plot	N conc. [mmol g_{dw}^{-1}]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ϵ^{15}N [‰]	ϵ^{13}C [‰]	ϵ^{2}H [‰]	ϵ^{18}O [‰]
Euphorbia-ceae	*Mercurialis perennis*	AM + NM	296	2,04	-3,32	-32,79	-73,43	23,69	1,22	-0,07	14,75	-1,04
			297	2,72	-3,96	-33,34	-70,20	22,14	-0,01	-0,04	17,83	-1,56
			298	2,01	-1,79	-30,88	-78,64	24,25	1,82	0,06	17,72	1,76
			299	1,91	-5,67	-30,17	-72,84	23,73	0,29	1,41	18,33	-0,01
			300	1,74	-7,07	-31,51	-85,14	23,62	-0,57	-0,03	11,09	-0,51
Lamiaceae	*Prunella vulgaris*	AM + NM	908	0,84	-1,61	-29,27	-140,93	17,04	-0,30	-1,38	-20,33	-6,66
			909	2,16	-0,42	-29,38	-119,49	17,70	-0,32	-1,38	4,03	0,10
			910	1,78	-0,83	-28,61	-130,03	17,19	-0,46	-0,89	0,54	-0,11
			911	1,29	-1,05	-29,41	-130,80	16,53	-0,58	-1,37	-11,56	-7,12
			912	1,01	-2,72	-29,62	-147,58	16,08	-1,64	-1,88	-16,15	-0,99
			968	1,62	-5,29	-33,66	-122,76	21,65	-0,60	-1,97	-15,39	-2,33
			969	1,59	-5,59	-32,82	-120,93	21,22	-0,23	-2,16	-11,29	-2,20
			970	1,52	-5,41	-33,33	-122,62	21,16	-0,18	-2,82	-10,59	-1,38
			971	1,78	-4,75	-30,13	-117,81	23,71	1,01	0,55	-4,91	0,61
			972	1,48	-6,93	-31,31	-123,00	22,63	-1,08	-1,35	-11,16	-0,31
Oxalidaceae	*Oxalis acetosella*	AM + NM	953	1,99	-8,60	-30,56	-132,59	32,77	-2,19	0,58	0,32	4,62
			954	1,86	-9,03	-30,94	-134,62	29,71	-1,17	-0,17	5,52	2,63
			955	2,31	-6,16	-30,24	-125,94	32,68	-0,75	0,41	3,93	4,67
			957	1,85	-7,74	-30,09	-136,98	28,42	-0,59	0,53	-0,36	2,22
			296	2,09	-5,16	-32,15	-94,52	26,69	-0,62	0,57	-6,34	1,96
			297	2,36	-4,70	-32,93	-97,55	25,83	-0,76	0,36	-9,53	2,12
Pinaceae	*Picea abies*	ECM + EEM	933	0,62	-4,25	-26,02	-96,53	28,16	-3,26	0,55	-1,87	2,64
			934	0,78	-3,80	-24,85	-104,54	26,28	-2,60	1,22	10,51	1,72
			935	0,94	-4,30	-25,92	-96,33	29,69	-1,37	0,64	5,83	3,37
			936	0,85	-5,97	-25,63	-108,16	26,73	-3,23	0,85	9,57	1,68
			937	1,00	-1,56	-26,72	-111,66	28,20	0,08	0,28	4,33	2,53
			956	0,74	-7,62	-30,86	-128,39	24,64	-0,88	-0,88	9,72	0,39
			958	0,58	-7,43	-33,13	-106,13	24,26	-1,22	-0,30	-8,83	0,80
			959	0,64	-7,74	-33,89	-104,41	23,29	-1,49	-0,69	-4,83	-0,12
			960	0,60	-8,20	-32,94	-102,43	24,55	-2,13	0,09	-2,26	0,83
			961	0,60	-6,30	-33,16	-109,90	25,00	-1,08	0,20	-1,58	1,68
			962	0,73	-7,97	-34,75	-108,91	22,30	-2,68	-0,91	0,22	-0,51
Plantagina-ceae	*Plantago lanceolata*	AM	898	1,71	-0,67	-27,68	-117,58	19,85	-0,16	-0,08	3,66	-0,03
			899	1,42	-0,90	-27,21	-121,16	19,46	1,27	-0,23	-1,32	0,00
			900	1,29	-1,84	-27,44	-121,84	19,49	-0,25	-0,24	-4,02	-0,50
			901	1,19	-1,69	-26,28	-120,81	20,46	-0,92	0,99	-6,71	0,68
			902	1,32	-1,82	-28,05	-110,00	19,49	-0,65	-0,38	3,31	-0,22
			903	1,26	1,16	-28,67	-120,32	20,31	-0,05	-0,79	-0,35	-0,51
			904	1,75	-1,30	-28,56	-126,07	20,73	-1,48	-0,47	-5,26	0,54
			905	1,91	-1,15	-28,65	-118,66	21,36	-1,15	-0,75	1,35	0,63
			906	1,11	-1,46	-26,92	-123,66	21,17	0,70	0,04	2,67	0,86
			907	1,36	-0,95	-27,99	-116,52	20,51	0,16	-0,69	-7,02	-0,48
			908	0,78	-1,76	-28,18	-120,38	17,87	-0,44	-0,55	0,22	-5,83
			909	1,19	-1,90	-28,96	-133,88	17,54	-1,80	-0,96	-10,36	-0,07
			910	1,01	-2,14	-28,51	-129,92	18,55	-1,77	-0,80	0,65	1,25
			911	1,06	-2,46	-28,77	-117,31	17,66	-1,99	-0,72	1,93	-5,99
			912	1,24	-2,22	-28,24	-129,79	18,45	-1,15	-0,49	1,64	1,38
			913	1,38	-1,73	-27,56	-123,12	19,59	-0,66	-0,13	4,60	-0,43
			914	1,63	-1,13	-28,01	-116,14	19,80	-0,71	-0,27	0,19	0,89
			915	0,99	-0,43	-27,39	-120,27	21,06	-0,08	-0,32	2,89	1,52
			916	1,36	-2,48	-28,38	-115,32	18,78	-1,24	-0,87	0,43	-0,08
			917	1,23	-2,01	-28,69	-114,11	18,94	0,08	-0,76	-1,05	-0,26

Table S2 (continued)

Family	Species	mycorrhizal status[*]	Plot	N conc. [mmol g_{dw}^{-1}]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	δ^{18}O [‰]	ϵ^{15}N [‰]	ϵ^{13}C [‰]	ϵ^{2}H [‰]	ϵ^{18}O [‰]
	Veronica urticifolia	(AM + NM)	957	1,74	-5,68	-30,55	-146,04	25,64	1,47	0,07	-9,42	-0,56
Poaceae	*Briza media*	AM + NM	938	0,09	-4,41	-27,54	-119,13	24,49	0,84	0,01	-11,68	1,55
			939	1,41	-4,80	-30,10	-123,25	20,36	0,89	-1,55	-10,83	-1,10
			940	1,32	-4,04	-27,50	-129,74	17,79	-1,10	-0,30	-10,03	-2,29
			941	1,39	-1,58	-28,35	-135,37	18,71	1,15	-0,92	-11,69	-0,23
			942	1,33	-3,99	-28,08	-123,41	18,41	-0,70	-0,18	-6,78	-0,20
	Molinia caerulea	AM	918	0,99	-3,21	-26,38	-135,08	20,77	-1,32	0,37	0,21	1,50
			919	1,06	-5,28	-26,67	-139,41	21,71	-1,37	0,91	-4,61	0,69
			920	0,98	-2,77	-25,47	-147,45	22,45	-1,01	0,60	-12,50	0,59
			921	1,08	-3,01	-26,13	-138,53	21,95	0,35	-0,01	-4,43	1,28
			922	1,15	-4,27	-25,73	-137,01	21,60	-0,91	0,33	3,78	1,42
			954	2,48	-6,27	-30,86	-134,41	30,98	1,59	-0,10	5,73	3,90
Ranuncula-ceae	*Aquilegia atrata*	NA	973	2,25	-3,81	-30,63	-88,45	25,37	0,95	1,02	9,28	1,48
			974	2,12	-2,61	-31,78	-92,50	23,02	2,21	0,90	5,21	0,04
			975	1,90	-0,47	-32,59	-97,74	22,01	2,81	0,87	4,98	-0,64
			976	1,37	-6,88	-31,53	-89,71	21,82	-1,27	1,80	5,57	-0,75
			977	2,41	-3,45	-32,64	-94,53	23,08	0,81	1,22	3,46	0,69
	Caltha palustris	AM + NM	908	1,16	-0,57	-25,44	-134,86	17,70	0,74	2,48	-14,25	-6,00
			909	1,93	2,02	-25,65	-117,18	17,58	2,12	2,34	6,34	-0,03
			910	2,21	1,88	-26,04	-131,75	16,17	2,24	1,68	-1,18	-1,14
			911	2,18	2,10	-25,96	-122,77	15,66	2,57	2,08	-3,53	-7,99
			912	2,12	1,72	-25,38	-116,92	16,68	2,79	2,37	14,51	-0,39
			923	1,55	-0,31	-25,02	-123,21	18,36	1,60	-0,08	-4,41	-1,75
			924	1,71	0,05	-25,38	-121,55	18,07	1,77	0,67	1,04	-1,22
			925	1,53	-5,52	-25,70	-121,29	17,62	-3,36	0,03	0,13	-0,98
			926	1,70	-5,93	-26,08	-107,23	17,28	-4,15	-0,18	16,49	-1,38
			927	1,36	-5,67	-26,31	-126,27	16,83	-2,45	0,57	0,52	-1,82
	Trollius europaeus	AM	968	2,61	-4,13	-31,28	-90,33	24,76	0,56	0,42	17,04	0,79
			969	1,97	-4,63	-28,79	-91,30	24,55	0,74	1,86	18,33	1,13
			970	1,38	-5,62	-28,77	-101,70	22,30	-0,39	1,74	10,33	-0,25
			971	1,47	-7,09	-30,27	-102,48	22,86	-1,34	0,42	10,42	-0,24
			972	1,49	-4,09	-28,81	-96,82	22,59	1,76	1,14	15,02	-0,35
Rosaceae	*Fragaria vesca*	AM + NM	954	1,52	-8,28	-30,50	-151,38	20,55	-0,42	0,27	-11,25	-6,53
			955	1,52	-5,22	-30,63	-138,71	24,00	0,19	0,02	-8,85	-4,01
			956	1,17	-6,76	-28,63	-158,67	20,71	-0,01	1,35	-20,56	-3,53
			975	1,37	-4,59	-31,99	-116,11	23,45	-1,31	1,48	-13,39	0,79
Rosaceae	*Potentilla erecta*	AM + NM	918	0,98	-2,74	-26,65	-132,93	18,84	-0,85	0,11	2,37	-0,43
			919	1,20	-6,22	-28,03	-129,06	19,96	-2,31	-0,46	5,74	-1,06
			920	1,00	-4,66	-26,46	-134,12	21,43	-2,90	-0,39	0,84	-0,43
			921	1,12	-4,41	-26,75	-126,19	20,31	-1,05	-0,63	7,90	-0,37
			922	1,09	-3,98	-26,13	-151,22	20,86	-0,62	-0,07	-10,42	0,68
			923	1,87	-4,33	-25,09	-123,18	20,13	-2,42	-0,15	-4,38	0,02
			924	1,42	-5,40	-26,55	-126,75	19,31	-3,68	-0,50	-4,16	0,02
			925	1,06	0,08	-26,38	-120,80	18,73	2,24	-0,65	0,62	0,13
			926	0,84	1,46	-26,12	-129,65	18,17	3,24	-0,22	-5,93	-0,50
			927	1,50	-2,22	-27,28	-134,23	18,42	0,99	-0,40	-7,45	-0,24
			928	1,06	-2,78	-27,81	-135,41	19,47	0,20	-0,62	-2,14	-0,31
			929	1,14	-2,86	-27,11	-117,33	18,79	-1,67	0,00	2,02	-1,00
			930	1,14	-2,80	-27,38	-132,46	19,24	-1,87	-0,54	-4,52	-0,71

Table S2 (continued)

Family	Species	mycorrhizal status[*]	Plot	N conc. [mmol g_{dw}^{-1}]	$\delta^{15}N$ [‰]	$\delta^{13}C$ [‰]	$\delta^{2}H$ [‰]	$\delta^{18}O$ [‰]	$\epsilon^{15}N$ [‰]	$\epsilon^{13}C$ [‰]	$\epsilon^{2}H$ [‰]	$\epsilon^{18}O$ [‰]
	Potentilla erecta (continued)		931	1,02	2,15	-26,15	-137,41	19,09	3,38	0,77	-10,58	-0,40
			932	1,24	-3,76	-27,95	-129,40	18,74	-2,04	-0,65	-4,47	-1,64
			933	1,68	-3,15	-27,10	-98,11	22,16	-2,15	-0,53	-3,46	-3,36
			934	1,68	-1,10	-27,17	-109,01	21,72	0,10	-1,10	6,04	-2,84
			935	1,49	-4,54	-26,39	-106,73	24,88	-1,60	0,16	-4,57	-1,44
			936	2,25	-2,06	-27,37	-127,89	25,42	0,68	-0,90	-10,16	0,38
			937	1,56	-3,69	-26,80	-116,76	24,70	-2,05	0,20	-0,77	-0,98
			938	1,30	-5,31	-27,55	-106,12	23,38	-0,06	-0,01	1,34	0,44
			939	1,85	-4,81	-27,14	-108,19	20,25	0,88	1,40	4,23	-1,21
			940	1,84	-3,71	-25,59	-119,20	20,99	-0,77	1,61	0,52	0,92
			941	1,62	-4,15	-26,08	-122,54	19,15	-1,43	1,34	1,14	0,21
			942	2,17	-4,16	-26,72	-113,24	18,68	-0,86	1,18	3,39	0,07
			943	1,44	-8,90	-25,37	-117,00	24,60	-3,52	0,87	-4,26	0,83
			944	1,40	-8,50	-26,46	-120,87	22,19	-1,36	0,30	1,68	0,16
			945	1,61	-6,47	-26,53	-123,21	21,76	-3,46	0,30	-2,08	-0,61
			946	1,62	-7,79	-26,94	-124,14	22,23	-2,86	-0,58	2,22	-0,29
			947	1,36	-7,83	-27,27	-112,75	25,14	-3,62	-0,66	2,29	-0,78
			948	1,52	-4,36	-25,53	-123,43	23,14	0,13	1,73	-2,82	-0,56
			949	1,88	-3,80	-26,15	-118,90	24,08	-0,09	1,21	-3,52	-1,32
			950	1,32	-2,20	-27,36	-117,74	23,67	1,82	0,15	-1,80	0,34
			951	1,63	-4,63	-27,31	-130,87	22,91	-1,91	0,01	-11,63	-0,74
			952	1,75	-4,60	-26,93	-123,22	21,69	-0,36	0,90	-3,70	-2,03
			968	1,90	-4,65	-30,15	-109,02	25,52	0,04	1,54	-1,65	1,54
			969	1,82	-5,88	-30,36	-116,67	24,49	-0,51	0,30	-7,04	1,07
			970	1,93	-4,65	-29,43	-111,76	24,18	0,58	1,08	0,26	1,63
			971	1,83	-5,43	-31,66	-118,41	22,73	0,33	-0,97	-5,51	-0,37
			972	1,67	-6,54	-29,75	-115,71	23,59	-0,69	0,21	-3,86	0,65
			973	1,70	-4,39	-31,37	-111,85	23,57	0,37	0,29	-14,11	-0,32
			974	1,78	-5,82	-31,67	-107,11	23,32	-1,00	1,01	-9,39	0,35
			976	1,97	-3,70	-32,73	-99,19	22,69	1,92	0,60	-3,92	0,11
			977	2,05	-3,87	-33,16	-102,86	22,70	0,39	0,70	-4,87	0,31
	Sanguisorba major	(AM)	928	1,47	-3,61	-27,32	-126,84	20,21	-0,63	-0,13	6,43	0,44
			929	1,18	2,97	-26,47	-124,17	21,53	4,16	0,64	-4,81	1,75
			930	1,17	2,68	-25,80	-122,14	21,52	3,61	1,04	5,79	1,57
			931	1,27	-1,65	-27,10	-112,52	20,78	-0,42	-0,18	14,32	1,30
			932	0,89	3,70	-26,42	-121,68	21,70	5,42	0,89	3,25	1,33
	Sorbus aucuparia	AM + ECM + NM	953	1,38	-6,47	-30,71	-127,82	24,45	-0,05	0,43	5,09	-3,71
Sapindaceae	*Acer pseudoplatanus*	AM + ECM + NM	955	2,09	-4,85	-31,07	-124,95	27,35	0,56	-0,42	4,91	-0,66
			956	1,66	-5,86	-30,80	-127,26	27,39	0,89	-0,82	10,85	3,15
			957	1,10	-8,01	-31,22	-126,84	24,54	-0,87	-0,60	9,78	-1,66
Tofieldiaceae	*Tofieldia calyculata*	(AM)	963	0,97	1,68	-29,41	-105,59	21,35	3,44	-1,80	12,68	-0,38
			964	1,19	0,97	-27,14	-100,93	23,09	3,64	0,34	18,02	0,93
			965	0,79	1,40	-27,71	-100,39	21,69	3,01	-0,56	12,46	-0,09
			966	1,01	2,73	-27,75	-111,51	21,79	4,26	-0,13	5,36	-0,12
			967	1,07	-1,81	-28,41	-103,44	22,56	1,11	-0,49	15,81	0,77
Violaceae	*Viola biflora*	(AM + NM)	296	1,64	-5,13	-33,24	-96,59	23,82	-0,59	-0,51	-8,41	-0,91
			297	2,04	-3,18	-33,62	-96,33	23,13	0,77	-0,32	-8,30	-0,57
			298	1,33	-5,94	-30,66	-108,32	21,85	-2,33	0,28	-11,96	-0,64
			299	1,48	-7,23	-31,84	-101,49	23,50	-1,27	-0,26	-10,31	-0,25
			300	1,58	-7,09	-30,89	-96,62	24,15	-0,59	0,59	-0,39	0,02
			mean	**1,47**	**-3,17**	**-28,55**	**-116,97**	**21,85**	**0,00**	**0,00**	**-0,19**	**-0,16**
			SD	0,47	2,79	2,54	14,46	2,89	1,87	0,91	7,61	1,67
			n	253								

Table S3: Results from post hoc pairwise comparisons for the enrichment factor e18O between 17 orchid species and autotrophic references using the Mann-Whitney U-test after a significant Kruskal-Wallis-test (X^2 = 62.123, df = 17, P < 0.001). P values were adjusted using the correction after Benjamini & Hochberg (Benjamini & Hochberg, 1995). No significant differences between orchids and their autotrophic references were detected. $n = 5$ for each of the 17 orchid species and $n = 15$ autotrophic reference plants for each orchid species (except for autotrophic references of *Herminium monorchis, n* = 14).

Species	$\varepsilon^{18}O$	
	U	P_{adjust}
Neottia nidus-avis	99.5	0.060
Cephalanthera rubra	1135.5	0.060
Epipactis helleborine	991.5	0.124
Liparis loeselii	714	0.772
Malaxis monophyllos	733	0.707
Neottia cordata	123	0.060
Spiranthes aestivalis	543	0.752
Dactylorhiza incarnata	530.5	0.707
Dactylorhiza majalis	531	0.707
Dactylorhiza viridis	535.5	0.719
Gymnadenia conopsea	78	0.060
Gymnadenia nigra	289	0.129
Herminium monorchis	373	0.250
Neotinea ustulata	245.5	0.092
Platanthera bifolia	763.5	0.598
Pseudorchis albida	314	0.164
Traunsteinera globosa	292	0.132

MANUSCRIPT 4

Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi

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Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi

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Abstract

1. Orchids produce dust seeds dependent on the provision of organic carbon by mycorrhizal fungi for their early development stages. Hence, all chlorophyllous orchids experience a dramatic switch in trophic strategies from initial mycoheterotrophy to either autotrophy or partial mycoheterotrophy during ontogeny. Yet, the degree to which partially mycoheterotrophic orchids gain carbon from their mycorrhizal fungi is unclear based on existing approaches.

2. Here, we propose a novel approach to quantify the fungal-derived organic matter gain of chlorophyllous mature orchids mycorrhizal with rhizoctonia fungi, using the stable isotope signatures of their fully mycoheterotrophic (FMH) seedlings in a linear two-source mixing model.

3. We conducted a field germination experiment with seven orchid species and measured carbon, nitrogen and hydrogen stable isotope natural abundances and nitrogen concentrations of mature orchids, underground seedlings, and autotrophic references.

4. After in situ burial for 19–30 months, germination rates varied considerably among five orchid species and failed for two. On average, underground seedlings were enriched in ^{13}C and ^{15}N relative to mature orchids and had higher nitrogen concentrations. Using the mean enrichment factors ε^{13}C and ε^{2}H of seedlings as FMH endpoint, the organic matter gain derived by mature orchids from mycorrhizas was *c.* 20%.

5. Chlorophyllous orchids mycorrhizal with rhizoctonias are predisposed to partially mycoheterotrophic nutrition due to their initially mycoheterotrophic seedling stage. We show that the carbon and hydrogen isotope abundances of underground seedlings can be used in an improved mixing-model to identify a significant proportion of fungal-derived organic matter in mature orchids.

KEYWORDS
carbon (C), fungi, hydrogen (H), mycoheterotrophy, orchid mycorrhiza, orchid seedlings, protocorm, rhizoctonia

1 | INTRODUCTION

Among plant families with initially mycoheterotrophic species, that is, plants dependent on fungi for their supply of organic matter during the early stages of development (Merckx, 2013), the Orchidaceae family has been studied most intensively. With *c.* 28,000 species in 736 genera the Orchidaceae is considered the largest angiosperm family; one in every ten plant species is an orchid (Chase et al., 2015; Christenhusz

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& Byng, 2016). Orchids are distributed worldwide in a wide variety of habitats only excluding the polar regions and the driest deserts (Chase et al., 2015; Merckx et al., 2013).

Bernard (1899) and Burgeff (1909) were the first to describe pelotons (intracellular coils of fungal hyphae) as the distinctive structures of orchid mycorrhizas and to realise that fungi are essential for successful orchid seed germination (Bernard, 1899; Burgeff, 1909; Smith & Read, 2008), however, the complex of requirements determining germination and establishment is still under study (Rasmussen, Dixon, Jersáková, & Těšitelová, 2015). Orchid seeds are very small ranging in length from *c.* 0.05 to 6.0 mm with a width of 0.01 to 0.9 mm and extremely light weighing from 0.31 to 24 µg, so they are the prime example of "dust seeds" (Arditti & Ghani, 2000). Unlike the seeds of other plants, mature orchid seeds lack endosperm; a transparent hydrophobic testa encloses an embryo that sometimes consists of only a few cells and limited food reserves such as oil droplets and starch grains (Arditti & Ghani, 2000; Eriksson & Kainulainen, 2011; Rasmussen, 1995).

Orchids are also extraordinarily prolific, an individual seed capsule can contain up to four million dust seeds (Arditti & Ghani, 2000). Charles Darwin noted that *Notwithstanding the astonishing numbers of seeds produced by Orchids, it is notorious that they are sparingly distributed* and concluded there must be limitations in the germination of orchid seeds and seedling establishment, rather than their dispersal (Darwin, 1877). A reason for this notable discrepancy is requiring colonisation by certain groups of fungi that successfully supply nutrients to the fully mycoheterotrophic initial life stage. The early life stages of terrestrial orchids are elusive bottleneck stages as the young seedlings (protocorms) remain underground and fully mycoheterotrophic for months or even years (Rasmussen & Whigham, 1998) after colonisation by mycorrhizal fungi. Protocorms are provisioned with fungal carbohydrates and mineral nutrients until the above-ground photosynthetic seedling stage. This mode of obtaining full nutrition through mycorrhizas likely imposes strong selection pressures on the underground stages (Rasmussen, 1995), especially if mycorrhizal specificity occurs during symbiotic development (Bidartondo & Read, 2008). At least 235 orchid species lacking leaves and chlorophyll remain entirely dependent on their mycorrhizal fungi at adulthood, making them fully mycoheterotrophic (Merckx et al., 2013). A range of partially mycoheterotrophic orchid species have the ability to obtain carbon at maturity simultaneously through photosynthesis and ectomycorrhizal fungi, thus exhibiting all intermediate stages of carbon acquisition between the extreme trophic endpoints of autotrophy and full mycoheterotrophy (Gebauer & Meyer, 2003; Hynson, Schiebold, & Gebauer, 2016; Hynson et al., 2013; Merckx, 2013). Characteristic for partially mycoheterotrophic orchid species associated with ectomycorrhizal fungi is an intermediate isotopic enrichment in ^{13}C and ^{2}H positioned between autotrophic plants and fully mycoheterotrophic orchids (Gebauer & Meyer, 2003; Gebauer, Preiss, & Gebauer, 2016; Schiebold, Bidartondo, Lenhard, Makiola, & Gebauer, 2018), a ^{15}N enrichment that varies with the type of mycorrhizal fungi (Schiebold, Bidartondo, Karasch, Gravendeel, & Gebauer, 2017) and significantly higher N concentrations than autotrophic plants (Gebauer & Meyer, 2003; Hynson et al., 2013, 2016). Carbon stable isotope abundances

have been used to calculate the proportion of carbon derived from mycorrhizal fungi in partially mycoheterotrophic orchids, using a linear two-source mixing model. The mean ^{13}C enrichment factor of the full mycoheterotroph *Neottia nidus-avis* (Bidartondo, Burghardt, Gebauer, Bruns, & Read, 2004; Gebauer & Meyer, 2003) or 11 fully mycoheterotrophic orchid species associated with ectomycorrhizal fungi (Hynson et al., 2013) served as fully mycoheterotrophic endpoint. With this approach the fungal-derived carbon varied between 14 ± 1% (*Epipactis helleborine*) (Bidartondo et al., 2004) and 84 ± 12% (*Corallorhiza trifida*) (Hynson et al., 2013) in partially mycoheterotrophic orchids associated with ectomycorrhizal fungi. Whether mature chlorophyllous orchids associated with typical orchid-mycorrhizal rhizoctonia fungi use these fungi to supplement their carbon demands has been a long and ongoing debate. Baylis (1975) argued that the morphology of the orchids' root systems with few, generally fleshy and unbranched roots has all the characteristics of plants with a high dependency on mycorrhizal fungi to gain mineral nutrients (Peterson, Massicotte, & Melville, 2004). Furthermore, there is also supporting evidence from stable isotope natural abundance analyses and measurements of nitrogen concentrations that chlorophyllous orchid species associated with rhizoctonia fungi are partially mycoheterotrophic (Gebauer et al., 2016; Girlanda et al., 2011; Schiebold et al., 2018). Most orchid species inhabiting sunny meadows lack ^{13}C enrichment or are even significantly depleted in ^{13}C relative to autotrophic references. However, significant ^{2}H and ^{15}N enrichment, and high leaf total nitrogen concentrations in the tissue of the majority of rhizoctonia-associated orchid species growing under light-saturated conditions led to concluding that partial mycoheterotrophy is the nutritional mode for these orchids (Schiebold et al., 2018). However, the proportion of organic matter that these chlorophyllous orchid species gain from their mycorrhizal rhizoctonia fungi is still unknown and might be underestimated, using fully mycoheterotrophic orchid species that form mycorrhizas with ectomycorrhizal fungi as mixing model endpoints (Hynson et al., 2013) because no mature fully mycoheterotrophic orchids with rhizoctonia fungi are known. Thus, mixing-model calculations almost always yielded negative carbon gains from fungi or those not significantly different from zero for Epidendroideae (e.g. −14 ± 8%, *Epipactis gigantea*; 6 ± 20%, *Neottia ovata*) and for Orchidoideae species (e.g. −13 ± 1%, *Anacamptis laxiflora*) associated with rhizoctonia fungi (Bidartondo et al., 2004; Girlanda et al., 2011; Hynson et al., 2013).

Alternatively, protocorms that germinate from seed with rhizoctonia fungi in situ represent a fully mycoheterotrophic state of an otherwise photosynthetic orchid and could thus serve as a suitable fully mycoheterotrophic endpoint in linear mixing-model calculations. What has been done only in one case each in the Orchidaceae and Ericaceae is to compare the carbon and nitrogen stable isotope compositions of mycoheterotrophic seedlings and mature chlorophyllous plants (Johansson, Mikusinska, Ekblad, & Eriksson, 2014; Stöckel, Těšitelová, Jersáková, Bidartondo, & Gebauer, 2014), but protocorm stable isotope abundances have not been used yet in mixing-model calculations. Here, we propose a novel approach to quantify the fungal-derived organic matter gain of chlorophyllous mature orchid species mycorrhizal with rhizoctonia fungi using the stable isotope signatures of fully

TABLE 1 Orchid species and sites chosen for the germination experiment, numbers of seed packets buried per orchid species, time of burial and results of the germination experiment (number of protocorms obtained per orchid species, mean dry weights of protocorms ± *SD*, and germination rates)

Orchid species	Site characteristics	Latitude/longitude	Seed packets	Burial in situ (months)	Protocorms	Protocorm dry weight (mg)	Germination rate (%)
Dactylorhiza majalis	Wet meadow	49.9°N/11.6°E	100	19	>1,021	0.028 ± 0.099	10.2
Epipactis palustris	Wet meadow	49.8°N/11.6°E	500	30	0	NA	0
Gymnadenia conopsea	Dry calcareous grassland	50.1°N/11.2°E	504	19	52	0.106 ± 0.082	0.1
Orchis militaris			500	19	36	0.351 ± 0.352	0.1
Neottia ovata			500	19	0	NA	0
Ophrys insectifera	Beech forest	49.7°N/11.4°E	100	19	7	10.352 ± 1.349	0.1
Platanthera bifolia			500	29	>1,607	2.006 ± 4.560	3.2

mycoheterotrophic protocorms of the same orchid species germinated in situ in a linear two-source mixing model.

2 | MATERIALS AND METHODS

2.1 | Study sites and sampling scheme

Seven orchid species known to be associated with rhizoctonia fungi as adults (Bidartondo et al., 2004; Esposito, Jacquemyn, Waud, & Tyteca, 2016; Jacquemyn, Brys, Cammue, Honnay, & Lievens, 2011; Jacquemyn et al., 2017; Oja, Kohout, Tedersoo, Kull, & Kõljalg, 2014; Stark, Babik, & Durka, 2009; Těšitelová et al., 2015) were chosen for the in situ seed sowing experiment and sampled together with autotrophic references at four sites in North-East Bavaria for stable isotope natural abundance analysis (Table 1): *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. and *Epipactis palustris* (L.) Crantz growing on two seasonally flooded *Molinia* meadows, *Gymnadenia conopsea* (L.) R.Br., *Orchis militaris* L. (Figure 1a) and *Neottia ovata* (L.) Bluff. & Fingerh. growing sympatrically on a dry calcareous grassland, and *Ophrys insectifera* L. (Figure 1b) and *Platanthera bifolia* (L.) Rich. growing in a mixed forest dominated by *Fagus sylvatica* L. and *Picea abies* (L.) H.Karst.

Sampling of flowering individuals of all seven orchid species took place between 2012 and 2013 during monitoring of the orchid populations to assess the right timing for seed capsule harvesting following the scheme by Gebauer and Meyer (2003): Samples of the target species (orchids) were taken in five replicates (resembling five 1 m^2 plots) together with three autotrophic non-orchid, non-leguminous reference plant species each (listed in Table S2). We yielded a total of 35 leaf samples from seven adult orchid species and 105 leaf samples from 15 neighboring autotrophic reference species sampled on 35 plots distributed over four sites (Tables S1 and S2). Nomenclature for orchid species and autotrophic references follows www.theplantlist.org (The Plant List, 2013).

For DNA analysis of orchid mycorrhizal fungi, two roots per sampled adult orchid individual were cut, rinsed with deionised water, placed in CTAB buffer (cetyltrimethylammonium bromide) and stored at −18°C.

2.2 | Sowing experiment and protocorm preparation

The orchid populations were monitored over the growing season to make sure only mature dehiscent seed capsules were harvested for the experiment. About 5–10 seed capsules per species originating from natural pollination events were detached from the inflorescences of adult orchids and placed into paper bags. The seeds were carefully released from their capsules and transferred into 2 × 1.5 cm nylon net packets of 48-μm aperture mesh (Plastok Associates Ltd., Birkenhead, UK) with *c.* 100 seeds per seed packet. This specific nylon mesh was chosen to ensure the dry orchid seeds are retained and because it is penetrable for water and fungal hyphae and durable for several years in the soil (Rasmussen et al., 2015). The packets were sealed with an impulse sealer (ME-300HC; MoFix GmbH, Bad Rappenau, Germany). The seeds received no cold or chemical stratification and were processed within 30 days to avoid effects on natural ontogenesis. Between 100 and 500 seed packets per species were manufactured (2,704 total) and the packets threaded on mason's lacing cord with 20–25 packets per string (Table 1). The seed packets of each species were reintroduced to their natural sites and buried 2 cm below the soil surface in close proximity to adult plants of the same species.

In May and June 2015, after 19–30 months of burial in situ, the seed packets were excavated and stored at 4°C for up to 1 week until further processing. Seed packets were cut open under a dissecting microscope and inspected for germinated orchid seeds (protocorms). If a seed packet contained protocorms, they were counted and a subset was transferred to microcentrifuge tubes containing 300 μl CTAB buffer (*Dactylorhiza majalis*: n = 38; *Gymnadenia conopsea*: n = 5; *Ophrys insectifera*: n = 7; *Orchis militaris*: n = 9; *Platanthera bifolia*: n = 28) and stored at −18°C for fungal DNA analysis. The remaining protocorms were transferred to preweighed tin or silver capsules and yielded 92 samples for determination of biomass (protocorm dry weight), N concentrations and stable isotope abundances after drying to constant weight at 105°C.

2.3 | Identification of mycorrhizal fungi and germination mycobionts

Root cross-sections were checked for presence and status of fungal pelotons in the cortex cells. Two to six root sections per orchid
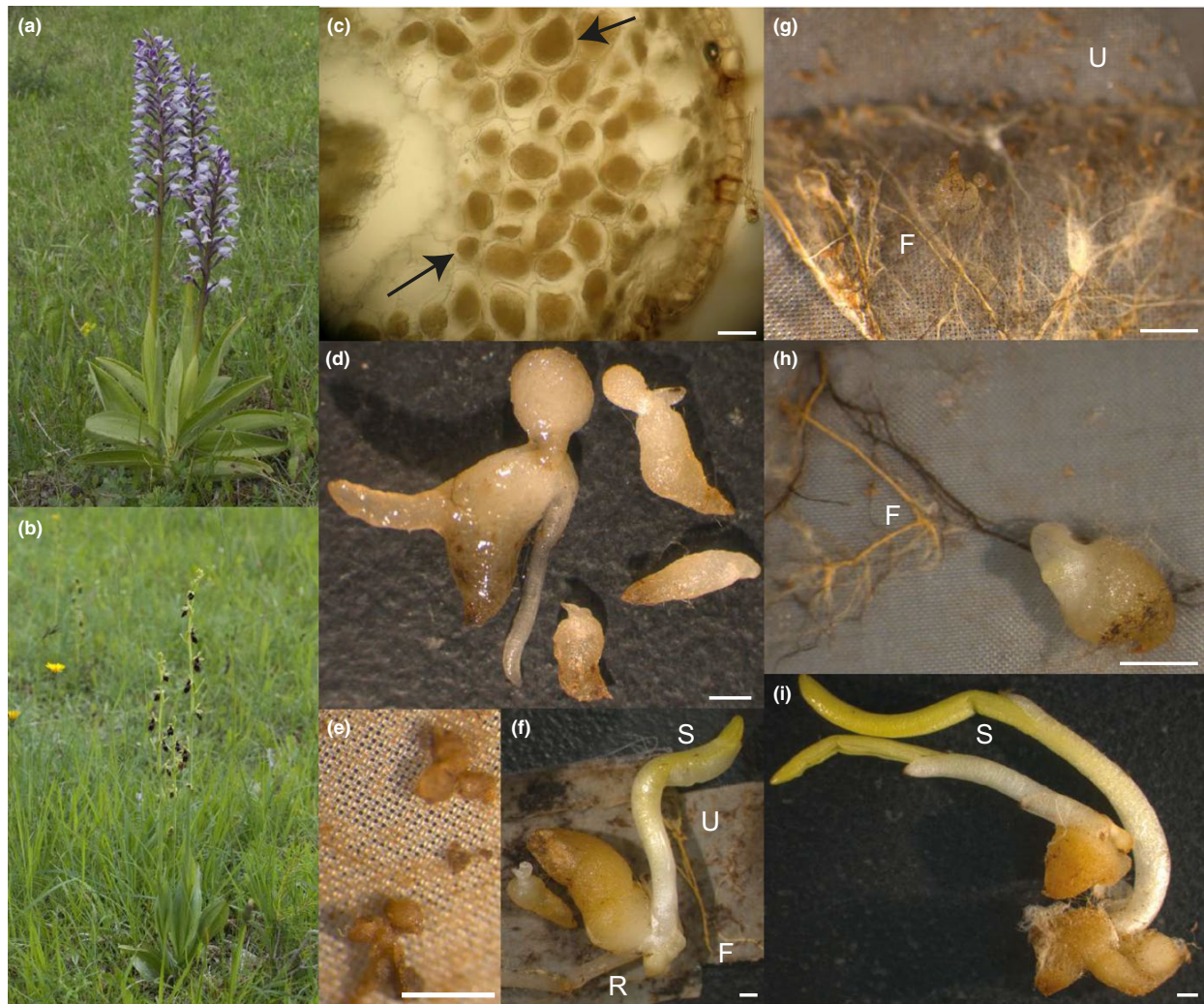
FIGURE 1 Mature flowering individuals of (a) *Orchis militaris* and (b) *Ophrys insectifera* at a dry calcareous grassland in NE Bavaria. (c) Light micrograph showing a transverse section of a root of *Platanthera bifolia*. Fungal colonisation is visible as outer and inner cortex cells filled with coils of fungal hyphae (pelotons) in different stages of degradation, indicated by arrows. Scale bar = 100 μm. Orchid seedlings (protocorms) of (d) *Orchis militaris*, (e) *Dactylorhiza majalis*, (f) *Platanthera bifolia*, (g–i) *Ophrys insectifera* after 19 (*D. majalis*, *O. insectifera*, *O. militaris*) and 29 (*P. bifolia*) months in situ incubation in the top soil layer at three sites with orchid populations in NE Bavaria, Germany (Table 1). U indicates ungerminated seeds, F indicates fungi, R indicates roots emerging from protocorms, and S indicates shoots emerging from protocorms. Scale bar for all protocorms = 1 mm. Note the well-developed leafy shoots of *O. insectifera* and *P. bifolia* seedlings (f, i), the root primordia in the *P. bifolia* seedling (f), the fungal hyphae enclosing the protocorms of *P. bifolia* and *O. insectifera* just after opening the seed packets, and the non-germinated orchid seeds in the background (f–h)

individual and all protocorm samples were selected for genomic DNA extraction and purification with the GeneClean III Kit (Q-BioGene, Carlsbad, CA, USA). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primer combinations ITS1F/ITS4 and ITS1/ITS4-Tul (Bidartondo & Duckett, 2010). All positive PCR products were purified with ExoProStart (GE Healthcare, Buckinghamshire, UK) and sequenced bidirectionally with an ABI3730 Genetic Analyser using the BigDye 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. All DNA sequences were checked and visually aligned with Geneious version 7.4.1 (http://www.geneious.com, Kearse et al., 2012) and compared to GenBank using BLAST (http://blast.ncbi.nlm.nih.gov). All unique DNA sequences have been submitted to GenBank (accession numbers: KY630470–KY630480).

2.4 | Analysis of stable isotope abundance and N concentration

Leaf samples of the seven adult orchid species (*n* = 35) and autotrophic references (*n* = 105) were washed with deionised water, dried to constant weight at 105°C and ground to a fine powder in a

ball mill (Retsch Schwingmühle MM2, Haan, Germany). Protocorms were either analysed individually, or combined to a minimum sample weight of 0.5 mg if their individual dry weight was too small, or measured separately for shoot and root protocorm fractions if these had developed. All samples were stored in a desiccator fitted with silica gel until analysis. Relative C and N isotope natural abundances of the leaf and protocorm samples were measured in dual element analysis mode with an elemental analyzer (Carlo Erba Instruments 1108, Milano, Italy) coupled to a continuous flow isotope ratio mass spectrometer (delta S; Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Thermo Fisher Scientific, Bremen, Germany) as described in Bidartondo et al. (2004). Relative H isotope natural abundances of the leaf and protocorm samples were measured with thermal conversion through pyrolysis (HTO; HEKAtech, Wegberg, Germany) coupled to a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific) via a ConFlo IV open-split interface (Thermo Fisher Scientific) as described in Gebauer et al. (2016). Due to memory bias each sample was analysed three times and the first two sample runs were skipped for reliable H isotope abundance determination. To minimise bias of post-sampling H atom exchange between organically bound hydroxyl groups in   our samples and H_2O in ambient air (Yakir, 1992), we analysed samples of orchids and their respective reference plant samples together in identical sample batches.

Measured relative isotope abundances are denoted as δ values that were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$ or δ^2H = $(R_{sample}/R_{standard} − 1) × 1,000$ [‰], 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 with respect to international standards (CO_2 vs. PDB, N_2 vs. N_2 in air, H_2 vs. SMOW) with the reference substances ANU sucrose and NBS19 for the carbon isotopes, N1 and N2 for the nitrogen isotopes, CH7, V-SMOW and SLAP for hydrogen isotopes, all provided by the International Atomic Energy Agency, Vienna, Austria. Reproducibility and accuracy of the C and N isotope abundance measurements were routinely controlled by measuring the laboratory standard acetanilide (Gebauer & Schulze, 1991). In relative C and N isotope natural abundance analyses, acetanilide was routinely analysed with variable sample weight at least six times within each batch of 50 samples. The maximum variation in $\delta^{13}C$ and $\delta^{15}N$ both within and between batches was always below 0.2‰. For relative H isotope natural abundance analyses, benzoic acid was routinely analysed with variable sample weight at least six times within each batch of 40 samples. The maximum variation in δ^2H both within and between batches was always below 4‰ for δ^2H.

Total N concentrations in leaf and protocorm samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on measurements of the laboratory standard acetanilide with a known N concentration of 10.36% (Gebauer & Schulze, 1991).

2.5 | Calculations and statistics

To enable comparisons of C, N and H stable isotope abundances between the seven orchid species sampled at four different sites we used an isotope enrichment factor approach to normalise the data. Normalised enrichment factors (ε) were calculated from measured δ values as $\varepsilon = \delta_S − \delta_{REF}$, where δ_S is a single $\delta^{13}C$, $\delta^{15}N$ or δ^2H value of an orchid individual or an autotrophic reference plant and δ_{REF} is the mean value of all autotrophic reference plants by plot (Preiss & Gebauer, 2008). The $\delta^{13}C$, $\delta^{15}N$ and δ^2H values, enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$ and ε^2H and N concentrations of seven orchid species and their autotrophic references are available in Tables S1 and S2.

We tested for pairwise differences in the isotopic enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, and N concentration, between the adult orchid species and all autotrophic reference plants, and between the sampled adult orchid species and protocorms of the same orchid species using a nonparametric Mann–Whitney U-test. The p values were adjusted using sequential Bonferroni correction (Holm, 1979). We furthermore tested for pairwise differences in the isotopic enrichment factor ε^2H between samples of the adult orchid *Platanthera bifolia* (n = 5), protocorms of *P. bifolia* (n = 5) and autotrophic references (n = 15) using the Mann–Whitney U-test after a significant nonparametric Kruskal–Wallis H-test.

The percentage of organic matter derived from fungi (% x_{df}) in the leaf tissue of the two adult photosynthetic orchid species *Ophrys insectifera* and *Platanthera bifolia* was calculated using the linear two-source mixing model: % $x_{df} = (\varepsilon_{PMH}/\varepsilon_{MH}) × 100$, where ε_{PMH} is the enrichment factor of an adult partially mycoheterotrophic orchid individual, and ε_{MH} the mean enrichment factor of the fully mycoheterotrophic endpoint. We used δ^2H to assess its value as a substitute for $\delta^{13}C$. For ε_{MH} the fully mycoheterotrophic protocorms of the same orchid species was used. For statistical analyses R version 3.3.2 (R Core Team, 2016) with a significance level of α = 0.05 was used.

3 | RESULTS

3.1 | Germination experiment

Germination rate, protocorm size and biomass (dry weight) varied greatly between the investigated species (Figure 1d–i, Table 1). After 19 months of burial in soil, numerous protocorms had germinated for *Dactylorhiza majalis* (n > 1,021) and *Gymnadenia conopsea* (n = 52), but the protocorms were comparably small with dry weights between 0.028 and 0.106 mg (Figure 1e, Table 1). The germination rate of 0.1% of *Orchis militaris* (n = 36) was similar to *G. conopsea*, but the protocorms were larger (0.351 ± 0.352 mg_{dw} per protocorm) (Figure 1d). More than 1,607 seeds (3.2%) of *Platanthera bifolia* had germinated after 29 months' burial and the protocorms varied in size (Figure 1f, Table 1). We retrieved only seven protocorms for *Ophrys insectifera*, but most of these were very large (10.352 ± 1.349 mg_{dw} per protocorm) (Figure 1g–i). Well-developed leafy shoots were present in seedlings of *O. insectifera* and *P. bifolia* (Figure 1f,i). Of all investigated species, the germination rate was highest for *D. majalis* (>10.2%). Germination of seeds of *Epipactis palustris* and *Neottia ovata* failed after in situ burial for 30 and 19 months, respectively.

TABLE 2 Fungi identified from roots (r) and protocorms (p) of five orchid species (ECM, ectomycorrhizal; NM, not known to form mycorrhizas)

Orchid species	Fungi identified from protocorm (p) or root (r)	Colonisation level (%)	Fungi (n)	Type of fungi	Best match sequence/accession number (GenBank/UNITE)	Identity (%)	Max score
Dactylorhiza majalis	r	60–100	Uncult. *Phialocephala* (1)	Dark septate endophyte	KF660561.1	94	1,321
			Uncult. *Sebacina* (1)	Rhizoctonia	JX317492.1	95	1,040
			Uncult. Tulasnellaceae (2)	Rhizoctonia	KJ188446.1	98	1,140
	p*	n.d.	Uncult. Ceratobasidiaceae (8)	Rhizoctonia	AY634129	99	1,182
			Craterellus lutescens (2)	ECM	AY082606	92	906
			Hydnum magnorufescens (4)	ECM	KC293545	99	1,142
			Uncult. Tulasnellaceae (6)	Rhizoctonia	EU490419	99	1,195
			Uncult. Tulasnellaceae (5)	Rhizoctonia	KF871201	99	1,162
Gymnadenia conopsea	r	50–90	Uncult. *Exophiala* (1)	Dark septate endophyte	HG936562.1	96	1,026
			Mortierella humilis (1)	NM	JF439486.1	97	941
			Uncult. Tulasnellaceae (1)	Rhizoctonia	GQ241750.1	97	1,077
	p*	n.d.	Uncult. Pleosporales (1)	NM	HQ212361	96	829
Ophrys insectifera	r*	80–90	Uncult. Tulasnellaceae (9)	Rhizoctonia	KF871201.1	98	1,055
	p*	n.d.	Uncult. Ceratobasidiaceae (3)	Rhizoctonia	KC243940.1	100	1,227
			Ilyonectria destructans (1)	NM	KC989076	91	728
Orchis militaris	r	100	Uncult. Tulasnellaceae (5)	Rhizoctonia	GQ907266.1	99	1,192
	p	n.d.	Uncult. Ceratobasidiaceae (2)	Rhizoctonia	KC243940	97	1,130
			Uncult. Helotiales (1)	NM	HG936811.1	91	536
Platanthera bifolia	r	90–100	*Thanatephorus fusisporus* (5)	Rhizoctonia	HQ441575.1	97	1,123
		n.d.	Uncult. Ceratobasidiaceae (3)	Rhizoctonia	KC243940	99	1,187
	p*		Uncult. *Thanatephorus* (3)	Rhizoctonia	AB712278	99	1,122
			Uncult. Tulasnellaceae (1)	Rhizoctonia	KC243933	99	1,171

Asterisks (*) indicate newly investigated stages of an orchid species; *n*, number of orchid individuals in which a fungus was detected. Pelotons in the cortex cells were visible in all species. The *E* value for all DNA matched sequences was 0.

n.d., no data.

3.2 | Identification of mycorrhizal fungi and germination mycobionts

The roots of all investigated orchid species were well-colonised by mycorrhizal fungi with 50%–100% of the inner and outer cortex cells containing pelotons visible as dense coils of fungal hyphae with different levels of degradation (Figure 1c). Colonisation was especially high in *O. militaris* where 100% of the root cortex cells of all observed cross-sections showed fungal colonisation.

The fungi found to form orchid mycorrhizas in the roots of the orchid species that showed successful protocorm germination were associated with fungi from the polyphyletic rhizoctonia group (Table 2). Flowering individuals of *D. majalis*, *G. conopsea*, *O. insectifera* and *O. militaris* all associated with Tulasnellaceae, and *P. bifolia* associated with the Ceratobasidiaceae *Thanatephorus fusisporus*. The orchid mycorrhizal basidiomycete genus *Sebacina* (Sebacinaceae) was only detected in one individual of *D. majalis*. The germination mycobionts of *D. majalis* matched with the mycorrhizal fungi found in the roots of adult plants for Tulasnellaceae but protocorms were also colonised by Ceratobasidiaceae and the ectomycorrhizal fungi *Hydnum magnorufescens* Vizzini, Picillo & Contu and *Craterellus lutescens* (Fr.) Fr. Germination mycobionts of *O. insectifera*, *O. militaris* and *P. bifolia* were Ceratobasidiacae, and we found Tulasnellaceae additionally for *P. bifolia* protocorms. Despite the large number of protocorms it was difficult to obtain clean sequences for *G. conopsea* protocorms and only one member of the ascomycete order Pleosporales as possible germination mycobiont could be identified (Table 2).

3.3 | Stable isotope abundances and nitrogen concentrations

Mean enrichment in ^{13}C (ε) of all adult orchids in this study with successfully germinated protocorms varied between 0.17 ± 0.54‰ (*O. militaris*) and 1.90 ± 0.33‰ (*P. bifolia*) (Figure 2, Table S1). Pairwise Mann–Whitney *U*-tests showed that only adults of *O. insectifera* (p_{adj} = .004) and *P. bifolia* (p_{adj} = .001) were significantly enriched relative to all autotrophic references in ^{13}C whereas adults of *D. majalis*, *G. conopsea* and *O. militaris* were not distinguishable from autotrophic references (Table 3). The protocorms of all orchid species exhibited a mean ^{13}C enrichment of 6.93 ± 2.22‰ and the protocorms of *O. insectifera*, *O. militaris* and *P. bifolia* were significantly enriched relative to their respective adult orchid species (p < .001) (Figure 2). This test could not be conducted for *D. majalis* and *G. conopsea* as less than three protocorms were available for stable isotope analyses for these two species that produced smaller protocorms, but their enrichment is higher than zero (Figure 2, Table S1).

All mature orchid species in this study, except *P. bifolia* (p_{adj} = .117), were significantly enriched in ^{15}N as adults relative to autotrophic references as shown by pairwise Mann–Whitney *U*-tests (Table 1). Mean enrichment in ^{15}N of these orchid species ranged between 2.48 ± 0.91‰ (*D. majalis*) and 8.08 ± 1.51‰ (*G. conopsea*) (Figure 2, Table S1). On average, the protocorms of all orchid species exhibited a mean ^{15}N enrichment of 7.65 ± 1.88‰ but only the protocorms of *O. insectifera* and
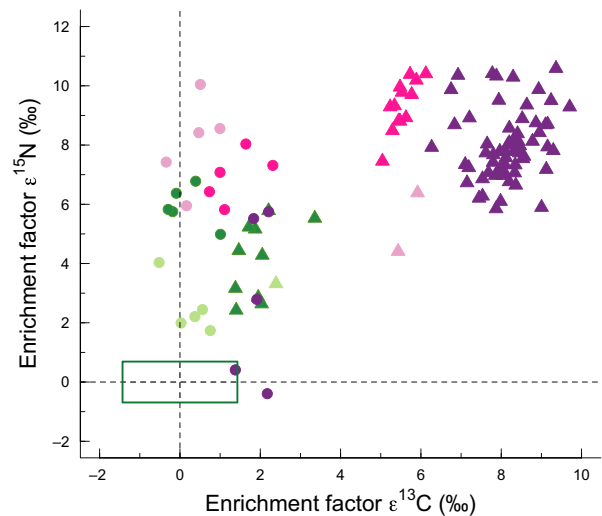
FIGURE 2 Mean enrichment factors ε^{15}N and ε^{13}C ± 1 *SD* of adult green leaves (circles) and fully mycoheterotrophic protocorms (triangles) of five orchid species sampled in NE Bavaria/Germany: *Dactylorhiza majalis* (light green), *Gymnadenia conopsea* (rose), *Ophrys insectifera* (pink), *Orchis militaris* (dark green) and *Platanthera bifolia* (purple) (for details see Table S1). The green box represents mean enrichment factors ±1 *SD* for the autotrophic reference plants that were sampled together with the orchid species (*n* = 75, see Table S2) whereas mean ε values of reference plants are zero by definition

P. bifolia were significantly enriched in ^{15}N relative to their respective adult orchid species (p < .001); the protocorms of *O. militaris* were significantly depleted in ^{15}N relative to their adult counterparts. Again this test could not be conducted for *D. majalis* and *G. conopsea* because less than three protocorms were available for these two species, but their enrichment is clearly higher than zero (Figure 2, Table S1).

The protocorms of *D. majalis* and *O. militaris*, and of *G. conopsea*, *O. insectifera* and *P. bifolia*, respectively, form two separate clusters due to their enrichments in both ^{13}C (1.98 ± 0.56‰ vs. 7.65 ± 1.23‰) and ^{15}N (4.07 ± 1.24‰ vs. 8.17 ± 1.31‰). The cluster composed of the protocorms of *G. conopsea*, *O. insectifera* and *P. bifolia* is significantly more enriched in ^{13}C and ^{15}N than the cluster composed of the protocorms of *D. majalis* and *O. militaris* (ε^{13}C: *U* = 836, *p* < .001; ε^{15}N: *U* = 831, *p* < .001).

The N concentrations in the leaf tissue of adult individuals of *D. majalis* (1.91 ± 0.17 mmol/g_{dw}) and *O. insectifera* (2.28 ± 0.13 mmol/g_{dw}) were significantly higher than those of all autotrophic references (1.28 ± 0.37 mmol/g_{dw}), whereas the leaves of *G. conopsea*, *O. militaris* and *P. bifolia* exhibited similar N concentrations as autotrophic plants (Tables S1 and S2). The protocorms of all orchid species exhibited a mean N concentration of 3.46 ± 1.08 mmol/g_{dw} and N concentrations of the *O. insectifera*, *O. militaris* and *P. bifolia* protocorms were significantly higher than in the leaves of their conspecific adult orchid individuals (Tables 3 and S1). Strinkingly, the cluster composed by protocorms of *G. conopsea*, *O. insectifera* and *P. bifolia*, that is significantly more enriched in ^{13}C and ^{15}N than the cluster composed by protocorms of *D. majalis* and *O. militaris*, also exhibits significantly higher

TABLE 3 Results from pairwise comparisons for the enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{13}C$ and N concentrations between the leaves of five adult orchid species and autotrophic references ("a vs. Ref") and between the leaves of five adult orchid species and fully mycoheterotrophic protocorms("a vs. p") using the Mann–Whitney U-test

Species	Test	$\varepsilon^{13}C$		$\varepsilon^{15}N$		N conc.	
		U	p	U	p	U	p
Dactylorhiza majalis	a vs. Ref	233	1	**351**	**.006**	**355**	**.004**
	a vs. p	NA	NA	NA	NA	NA	NA
Gymnadenia conopsea	a vs. Ref	248	1	**375**	**.001**	66	.081
	a vs. p	NA	NA	NA	NA	NA	NA
Ophrys insectifera	a vs. Ref	**372**	**.004**	**375**	**.001**	**355**	**.001**
	a vs. p	**0**	**<.001**	**1**	**<.001**	**0**	**<.001**
Orchis militaris	a vs. Ref	216	1	**375**	**.001**	148	1
	a vs. p	**0**	**<.001**	**45**	**.013**	**2**	**.002**
Platanthera bifolia	a vs. Ref	**373**	**.001**	302	.117	155	1
	a vs. p	**0**	**<.001**	**0**	**<.001**	**0**	**<.001**

The p values resulting from pairwise tests between adult orchids and autotrophic references were adjusted using the Bonferroni correction. Significant differences between the tested groups are marked in bold. $n = 5$ for each of the five orchid species, $n = 15$ for autotrophic reference plants for each orchid species, and n is between 1 and 61 protocorms per orchid species. n.d., $n < 3$ protocorms of *D. majalis* and *G. conopsea*.

N concentrations $(1.58 \pm 0.27\ \text{mmol/g}_{dw}$ vs. $3.81 \pm 0.90\ \text{mmol/g}_{dw}$; $U = 834$, $p < .001$).

Due to the development of large seedlings for *O. insectifera* and *P. bifolia*, it was possible to analyse the stable isotope abundances and the nitrogen concentrations separately for the emerging shoot (not colonised by mycorrhizal fungi) and root organs (Figure 1f,i, Table S1). For the protocorms of both species, the emerging shoots were significantly more enriched in ^{15}N than the emerging roots (*O. insectifera*: $p = .035$; *P. bifolia*: $p < .001$) while the roots had significantly higher nitrogen concentrations than the shoots (*O. insectifera*: $p = .001$; *P. bifolia*: $p = .012$). For $\varepsilon^{13}C$ no such differentiation between protocorm parts was detectable (*O. insectifera*: $p = .181$; *P. bifolia*: $p = .4$).

The ^{2}H enrichment for adults and protocorms of *P. bifolia*, and the respective autotrophic references *Carex* sp., *Fagus sylvatica* (L.), *Fragaria vesca* (L.) and *Picea abies* (L.) H.Karst. was significantly different ($\chi^2 = 16.118$, $df = 2$, $p < .001$). The *P. bifolia* protocorms exhibited the highest enrichment ($\varepsilon^2H = 95.78 \pm 6.22‰$) followed by adult *P. bifolia* individuals ($\varepsilon^2H = 20.48 \pm 10.93‰$) and finally by the group of autotrophic references ($\varepsilon^2H = 0.0 \pm 11.38‰$) (Figure 3, Table S1 and S2).

3.4 | Linear two-source mixing model

Linear two-source mixing-model calculations for estimates of percent carbon gains from the fungal source were only possible for the adult individuals of *O. insectifera* and *P. bifolia* as these were the only ones significantly enriched relative to all autotrophic references in ^{13}C (Figure 2, Table 3). Using the mean $\varepsilon^{13}C$ values of their protocorms as fully mycoheterotrophic endpoint, the percentage of organic matter derived from mycorrhizal fungi ranged between $23.3 \pm 11.3\%$ (*P. bifolia*) and $24.5 \pm 11.3\%$ (*O. insectifera*) (Figure 4) and was significantly different from zero ($U = 75$; $p < .001$). The fungal-derived organic matter gains of *O. insectifera* and *P. bifolia* were not significantly different from each other ($U = 12$, $p = 1$). If
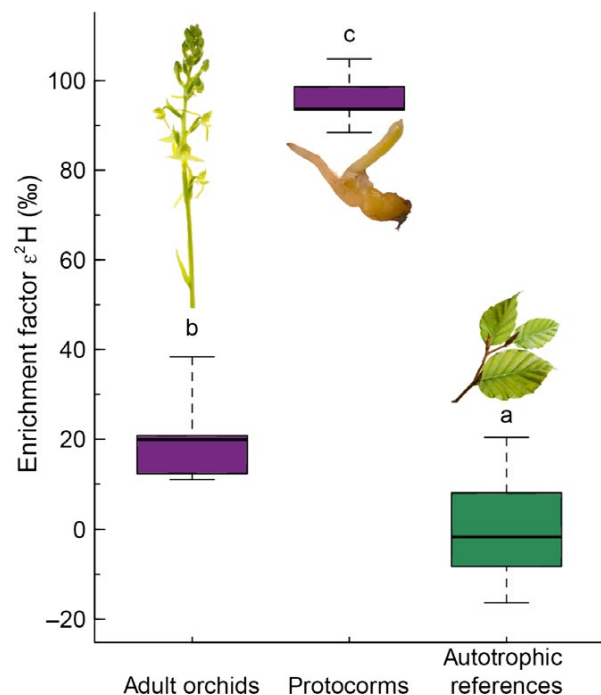
FIGURE 3 Box-and-whisker plot with results from pairwise comparisons in enrichment factor ε^2H between adult green leaves ($n = 5$) and protocorms ($n = 5$) of *Platanthera bifolia* and autotrophic references ($n = 15$). Different letters indicate significant differences between the groups

the enrichment factor ε^2H was used as a substitute for $\varepsilon^{13}C$ in *P. bifolia*, the fungal-derived organic matter gain in the adult orchids' leaf tissue was $21.4 \pm 11.4\%$ (Figure 4) and was also significantly different from zero ($U = 75$; $p < .001$). The percent carbon gain of *P. bifolia* calculated with the mean $\varepsilon^{13}C$ values of the protocorms

and the fungal-derived organic matter gain calculated with the mean ε^2H values were almost identical (U = 18; p = .31).

4 | DISCUSSION

4.1 | Mycorrhizal fungi and germination mycobionts

The prerequisite of our study that the investigated orchid species form orchid mycorrhizas with rhizoctonia fungi was fulfilled. Furthermore, we found the fungi identified from roots of mature orchid individuals and germination mycobionts of fully mycoheterotrophic protocorms to overlap widely between species and ontogenetic stages. Overall, Tulasnellaceae and Ceratobasidiaceae were the most abundant fungi in both roots of mature orchids and protocorms (Table 2). These two basidiomycete fungal families are common mycorrhizal symbionts of both terrestrial and epiphytic orchids worldwide (Dearnaley, Martos, & Selosse, 2012; Těšitelová et al., 2013). However, it was not always the case that the germination mycobionts identified from protocorms were a subset of the fungi from roots of mature plants of the same species (Rasmussen et al., 2015). The largely overlapping but not identical composition of fungi between protocorms and mature roots can result from some de novo colonisation of the roots by mycorrhizal fungi in the soil, instead of all roots being colonising from the protocorm (Peterson et al., 2004). Differences in the sets of fungi detected between developmental stages may reflect differences in how

fungi colonise protocorms versus adults (e.g. symbiont shifts, gains or losses) and merit further investigation.

We found Tulasnellaceae and *Sebacina* in *Dactylorhiza majalis* as in previous studies (Bidartondo et al., 2004; Jacquemyn, Deja, De hert, Cachapa Bailarote, & Lievens, 2012; Kristiansen, Taylor, Kjøller, Rasmussen, & Rosendahl, 2001). However, in addition to Tulasnellaceae, Ceratobasidiaceae and two ectomycorrhizal basidiomycete species in *D. majalis* protocorms were identified. Tulasnellaceae were also present in the roots of mature *Gymnadenia conopsea* individuals which is in agreement with the findings by Stark et al. (2009) and Těšitelová et al. (2013). As no other potentially mycorrhizal families than Tulasnellaceae and Ceratobasidiaceae were found in *G. conopsea* seedlings by Těšitelová et al. (2013), we might have missed these important taxa in *G. conopsea* protocorms here as only the ascomycete order Pleosporales as possible germination mycobiont could be identified. Tulasnellaceae were found in mature *Ophrys insectifera* and *Orchis militaris* individuals and Ceratobasidiaceae in the protocorms of the same species (Table 2). Jacquemyn et al. (2011) found six OTUs matching Tulasnellaceae and one OTU matching Ceratobasidiaceae in the roots of *Orchis militaris* and a subset of these OTUs in *O. militaris* protocorms. Roots of *Platanthera bifolia* were colonised by the Ceratobasidiaceae *Thanatephorus fusisporus* as in Esposito et al. (2016), and the germination mycobionts of *P. bifolia* overlapped with *Thanatephorus* but also hosted Ceratobasidiaceae and Tulasnellaceae.

4.2 | Stable isotope abundances, nitrogen concentrations and mixing-model calculations

Stable isotope natural abundance data of protocorms germinated in situ are scarce. Here, we present the first stable isotope natural abundance data and N concentrations for protocorms of the green-leaved orchid species *D. majalis*, *G. conopsea*, *O. insectifera*, *O. militaris* and *P. bifolia*. Generally, all protocorms were more enriched in ^{13}C than mature orchid individuals which is typical for full mycoheterotrophy. With the exception of *O. militaris* protocorms, all protocorms were also enriched in ^{15}N relative to mature orchid individuals of the same species (Figure 2, Table 3). Interestingly, ^{15}N and ^{13}C enrichment grouped the protocorms of several species together. Protocorms of *O. insectifera*, *P. bifolia* and *G. conopsea* were both highly enriched in ^{15}N and ^{13}C and within the range of protocorms associated with ectomycorrhizal fungi (*Neottia nidus-avis* and *Epipactis helleborine*) as shown by Stöckel et al. (2014). They found that achlorophyllous and thus fully mycoheterotrophic seedlings of two rhizoctonia-associated orchid species were far less enriched in ^{15}N and ^{13}C than protocorms of orchids with ectomycorrhizal fungi. Another group consisting of *D. majalis* and *O. militaris* protocorms was located in the moderate range of ^{15}N and ^{13}C enrichment already known from the rhizoctonia-associated protocorms of *Serapias parviflora* and *Pseudorchis albida* (Stöckel et al., 2014) and fully mycoheterotrophic seeedlings of the Pyroloideae (Ericaceae) *Moneses uniflora* and *Pyrola chlorantha* (Johansson et al., 2014). What causes differences according to ^{15}N and ^{13}C enrichment among orchids with rhizoctonias is unknown. However, it is possible that some rhizoctonia fungi lead to more pronounced ^{15}N and ^{13}C enrichments
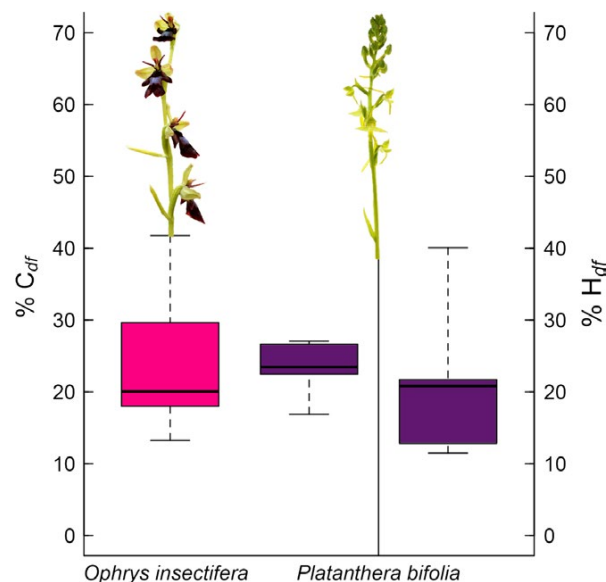
FIGURE 4 Box-and-whisker plot with results from linear two-source mixing-model calculations illustrating the percentage of organic matter derived from mycorrhizal fungi (*df*) for *Ophrys insectifera* (pink bars) and *Platanthera bifolia* (purple bars). %C$_{df}$: calculated with δ^{13}C values; % organic matter$_{df}$: calculated with δ^2H values that can serve as a substitute for δ^{13}C. Protocorms of *O. insectifera* and *P. bifolia* (plain bars) were used as fully mycohetereotrophic endpoints

than others. The fully mycoheterotrophic state of protocorms is also characterised by the extraordinary ^{2}H enrichment measured for *P. bifolia*, an even higher enrichment than the fully mycoheterotrophic ectomycorrhiza-associated *Neottia nidus-avis* (57.3 ± 13.8‰ vs. 95.78 ± 6.22‰) (Gebauer et al., 2016; Schiebold et al., 2018).

Consistent with the only previous study on isotopic signatures of orchid protocorms (Stöckel et al., 2014) are the strikingly high N concentrations of orchid protocorms (3.46 ± 1.08 mmol/g$_{dw}$) that exceed those of fully mycoheterotrophic orchids and match the N concentrations of fungal fruiting bodies (Gebauer & Dietrich, 1993; Gebauer & Taylor, 1999; Hynson et al., 2016; Schiebold et al., 2017). We propose that the fungal biomass present in the protocorms is responsible for this pattern. Our measurements of individual protocorm organs support this hypothesis. The emerging roots of *O. insectifera* and *P. bifolia* protocorms had significantly higher N concentrations than the emerging shoots probably due to the high proportion of fungal biomass in the protocorm's emerging root. In contrast, the fungus-free emerging shoots of both orchid species' protocorms were significantly more enriched in ^{15}N than the emerging roots indicating transfer of fungal derived N into the leaves likely to prepare the emerging shoots for photosynthesis.

We confirm partial mycoheterotrophy as trophic strategy for mature individuals of *O. insectifera* and *P. bifolia* which were the only two orchid species sampled in forests for which mature individuals were significantly enriched in ^{13}C relative to autotrophic references (Table 3). Nevertheless, partial mycoheterotrophy sensu Merckx (2013) as the "the ability of a plant to obtain carbon simultaneously through autotrophy and mycoheterotrophy" in rhizoctonia-associated orchids does not exclude the occurrence of bi-directional carbon fluxes as shown for the orchid *Goodyera repens* (Cameron, Johnson, Read, & Leake, 2008). In addition, they were also either enriched in ^{15}N (*O. insectifera*) or had significantly higher N concentrations (*P. bifolia*) than autotrophic plants. Gebauer et al. (2016) furthermore categorised these two orchid species as partially mycoheterotrophic due to their significant ^{2}H enrichment. The ^{15}N and ^{13}C enrichments and N concentrations in mature *O. insectifera* and *P. bifolia* individuals were in a similar range as in previous studies (Abadie et al., 2006; Gebauer & Meyer, 2003; Gebauer et al., 2016; Johansson et al., 2014; Schiebold et al., 2018; Tedersoo, Kõljalg, & Selosse, 2007). Also as in previous studies (Liebel et al., 2010; Schiebold et al., 2018), all rhizoctonia-associated meadow orchid species lacked ^{13}C enrichment, but were significantly enriched in ^{15}N relative to autotrophic reference species (Table 3). However, most of these species were categorised as partially mycoheterotrophic, as they were also enriched in ^{2}H and thus no longer "cryptic mycoheterotrophs" sensu Hynson et al. (2013). By following the suggestion made by Hynson (2016) that significant ^{15}N enrichment and higher total N concentrations can be used as indicators for partial mycoheterotrophy in rhizoctonia-associated orchid species, we furthermore infer partial mycoheterotrophy for *D. majalis* and at least marginal partial mycoheterotrophy for *G. conopsea* and *O. militaris*. In mature *D. majalis* individuals a marginally higher ^{13}C enrichment and significantly higher N concentrations than in two previous studies were detected, while the ^{15}N enrichment was nearly identical (Bidartondo et al., 2004; Schiebold et al., 2018). In *G. conopsea* we

detected more pronounced ^{15}N and ^{13}C enrichments in the leaves of mature individuals compared to previous studies (Abadie et al., 2006; Schiebold et al., 2018). However, here ^{13}C enrichment and N concentration of *D. majalis* and *G. conopsea* were not distinguishable from autotrophic reference plants. To our knowledge, we present the first stable isotope data and N concentrations for *O. militaris*. Closely related *Orchis purpurea* sampled in Italian grasslands exhibited similar ^{15}N and ^{13}C enrichments (Girlanda et al., 2011; Liebel et al., 2010).

As *O. insectifera* and *P. bifolia* were the only two orchid species displaying significant ^{13}C enrichment, these were also the only two species used to calculate the fungal derived organic matter gain via mycorrhizal fungi using the two-source linear mixing model. Using protocorms as fully mycoheterotrophic endpoint the percentage of organic matter derived from mycorrhizal fungi was significantly different from zero and ranged around 20% for both species. Additionally, it worked well to use the enrichment factor ε^2H as a substitute for ε^{13}C in *P. bifolia* as with both approaches a nearly identical organic matter gain was calculated. We thus show that it is possible to quantify the fungal-derived organic matter gain of chlorophyllous mature orchid species with the C and H stable isotope natural abundances of fully mycoheterotrophic protocorms. On this basis, we conclude that H isotope abundances of protocorms are valuable to estimate the fungal-derived organic matter gain of orchids with inconspicuous ^{13}C enrichments. We, therefore, emphasise the value of H isotope abundance to identify a heterotrophic lifestyle in plants (Gebauer et al., 2016; Schiebold et al., 2018). Proportional carbon gains from the fungal source in the order of 20% as found here for the rhizoctonia-mycorrhizal *O. insectifera* and *P. bifolia* are in the lower range reported for partially mycoheterotrophic orchids (Bidartondo et al., 2004; Girlanda et al., 2011; Hynson et al., 2013). It appears that saprotrophic rhizoctonia fungi are well suited to fully support germination and development of orchids in the early seedling stage; however, they are less suited than fungi simultaneously forming ectomycorrhizas with forest trees or wood-decay fungi to effectively support the carbon demand of partially or fully mycoheterotrophic orchids in the adult stage. Partially mycoheterotrophic orchids associated with ectomycorrhizal fungi were shown to have in most cases higher fungal-derived carbon gains that varied between 14 ± 1% (*Epipactis helleborine*) (Bidartondo et al., 2004) and 84 ± 12% (*Corallorhiza trifida*), whereas fully mycoheterotrophic orchids meet 100% of their carbon demands via mycorrhizal fungi (Hynson et al., 2013).

Our calculations using the enrichment factors of fully mycoheterotrophic protocorms associated with rhizoctonia fungi represent a significant improvement in contrast to mixing-model calculations employing enrichment factors of adult fully mycoheterotrophic orchids (incl. albinos) associated with ectomycorrhizal fungi as fully mycoheterotrophic endpoint. Previous mixing-model calculations almost always yielded negative or insignificant carbon gains from fungi for orchids associated with rhizoctonia fungi (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Girlanda et al., 2011; Hynson et al., 2013). We emphasise that the proportion of organic matter that chlorophyllous orchid species associated with rhizoctonia gain from their mycorrhizal fungi has been underestimated until today due to the employment of fully mycoheterotrophic orchid species that form mycorrhizas with ectomycorrhizal

fungi as mixing model endpoints (Hynson et al., 2013). As no mature fully mycoheterotrophic orchids associated with rhizoctonia fungi are known, protocorms that germinated in situ from seed with rhizoctonia fungi represent a fully mycoheterotrophic state of an otherwise photosynthetic orchid. We conclude that enrichment factors of fully mycoheterotrophic protocorms serve as a suitable endpoint in linear mixing-model calculations and encourage more research on the fascinating fully mycoheterotrophic stage of orchid protocorms.

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AUTHORS' CONTRIBUTIONS

G.G. had the idea for this investigation; J. S. conducted the seed baiting experiment, collected the plant samples, prepared all samples for stable isotope analysis, conducted the molecular analysis of mycorrhizal fungi, performed the data analysis and drafted the manuscript; M.I.B. supervised the molecular analysis of mycorrhizal fungi; G.G. supervised the sample isotope abundance analysis. All co-authors contributed critically to the drafts of the manuscript and gave final approval for publication.

DATA ACCESSIBILITY

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.h1123 (Schweiger, Bidartondo, & Gebauer, 2018) and GenBank (accession numbers: KY630470–KY630480).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1: Single and mean δ ^{15}N, δ ^{13}C, δ^{2}H values, enrichment factors ε^{15}N, ε^{13}C, ε^{2}H and total nitrogen concentration data of adult green leaves of 7 Orchidaceae species and fully mycoheterotrophic protocorms of 5 Orchidaceae species .

Species	Site	adult orchid (a)/ protocorm (p)	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	protocorm part: entire (e), root (r), shoot (s)
Dactylorhiza majalis (Rchb.) P.F.Hunt & Summerh.	wet meadow 1	a	1	2,11	2,25	-29,67	NA	1,99	0,03	NA	NA
		a	2	1,94	1,90	-29,66	NA	1,74	0,76	NA	NA
		a	3	1,71	4,46	-30,47	NA	4,04	-0,52	NA	NA
		a	4	1,78	3,14	-29,63	NA	2,45	0,56	NA	NA
		a	5	2,01	2,69	-29,42	NA	2,21	0,37	NA	NA
		mean		**1,91**	**2,89**	**-29,77**	**NA**	**2,48**	**0,24**	**NA**	
		SD		**0,17**	**1,00**	**0,40**	**NA**	**0,91**	**0,50**	**NA**	
		p	NA		1,42	3,72	-27,62	NA	3,32	2,39	NA	e
Epipactis palustris (L.) Crantz	wet meadow 2	a	26	1,70	1,93	-29,16	NA	3,94	0,75	NA	NA
		a	27	2,07	1,89	-28,76	NA	4,38	1,13	NA	NA
		a	28	1,88	2,67	-28,85	NA	5,05	1,37	NA	NA
		a	29	1,81	2,27	-29,37	NA	4,68	0,68	NA	NA
		a	30	1,73	2,49	-29,48	NA	5,46	0,89	NA	NA
		mean		**1,84**	**2,25**	**-29,12**	**NA**	**4,70**	**0,97**	**NA**	
		SD		**0,15**	**0,34**	**0,31**	**NA**	**0,58**	**0,28**	**NA**	
Gymnadenia conopsea (L.) R.Br.	dry calcareous grassland	a	6	1,17	4,00	-28,24	NA	8,56	1,00	NA	NA
		a	7	0,98	2,72	-29,51	NA	7,43	-0,34	NA	NA
		a	8	0,92	3,83	-29,04	NA	8,42	0,47	NA	NA
		a	9	0,82	6,03	-29,28	NA	10,05	0,51	NA	NA
		a	10	0,93	1,44	-30,12	NA	5,95	0,16	NA	NA
		mean		**0,97**	**3,60**	**-29,24**	**NA**	**8,08**	**0,36**	**NA**	
		SD		**0,13**	**1,70**	**0,69**	**NA**	**1,51**	**0,49**	**NA**	
		p	NA		2,53	1,91	-23,68	NA	6,39	5,92	NA	e
		p	NA		2,63	-0,07	-24,16	NA	4,41	5,43	NA	e
Neottia ovata (L.) Bluff & Fingerh.	dry calcareous grassland	a	31	2,17	-1,25	-28,18	NA	5,89	-1,14	NA	NA
		a	32	2,13	-2,83	-26,48	NA	3,79	1,00	NA	NA
		a	33	1,97	-3,01	-26,29	NA	5,74	1,47	NA	NA
		a	34	1,92	-2,66	-28,47	NA	5,20	0,15	NA	NA
		a	35	2,21	-1,97	-27,44	NA	5,19	-0,33	NA	NA
		mean		**2,08**	**-2,34**	**-27,37**	**NA**	**5,16**	**0,23**	**NA**	
		SD		**0,12**	**0,73**	**0,98**	**NA**	**0,83**	**1,04**	**NA**	
Ophrys insectifera (L.)	beech forest	a	11	2,23	2,01	-29,68	NA	6,43	0,73	NA	NA
		a	12	2,50	1,99	-29,76	NA	7,31	2,31	NA	NA
		a	13	2,32	1,55	-30,44	NA	8,04	1,64	NA	NA
		a	14	2,21	1,70	-30,60	NA	7,08	1,00	NA	NA
		a	15	2,15	1,23	-30,37	NA	5,82	1,11	NA	NA
		mean		**2,28**	**1,69**	**-30,17**	**NA**	**6,93**	**1,36**	**NA**	
		SD		**0,13**	**0,33**	**0,42**	**NA**	**0,85**	**0,63**	**NA**	
		p	NA		4,25	5,17	-25,41	NA	10,41	6,12	NA	r
		p	NA		5,42	3,25	-26,24	NA	8,49	5,29	NA	r
		p	NA		5,41	3,57	-26,05	NA	8,81	5,48	NA	r
		p	NA		4,42	3,57	-26,08	NA	8,81	5,44	NA	r
		p	NA		4,62	4,05	-26,30	NA	9,29	5,23	NA	r
		p	NA		4,94	2,21	-26,49	NA	7,45	5,04	NA	r
		p	NA		3,90	3,68	-25,90	NA	8,92	5,63	NA	r
		mean			**4,71**	**3,64**	**-26,07**	**NA**	**8,88**	**5,46**	**NA**	
		SD			**0,58**	**0,88**	**0,35**	**NA**	**0,88**	**0,35**	**NA**	
		p	NA		3,67	4,08	-26,19	NA	9,33	5,34	NA	s
		p	NA		3,60	4,72	-26,05	NA	9,96	5,48	NA	s
		p	NA		3,43	5,15	-25,80	NA	10,39	5,73	NA	s
		p	NA		3,15	4,47	-25,76	NA	9,71	5,77	NA	s

Table S1 (continued)

Species	Site	adult orchid (a)/ protocorm (p)	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	protocorm part: entire (e), root (r), shoot (s)
Ophrys insectifera (L.)		p	NA	3,39	4,54	-26,00	NA	9,78	5,53	NA	s
(continued)		p	NA	3,54	4,95	-25,64	NA	10,19	5,89	NA	s
		mean		3,46	4,65	-25,91	NA	9,89	5,62	NA	
		SD		0,19	0,38	0,21	NA	0,38	0,21	NA	
		mean		**3,94**	**3,86**	**-24,24**	**NA**	**8,75**	**5,19**	**NA**	
		SD		**1,18**	**1,15**	**6,81**	**NA**	**2,32**	**1,37**	**NA**	
Orchis militaris (L.)	dry calcareous grassland	a	16	1,06	-0,47	-29,05	NA	5,75	-0,18	NA	NA
		a	17	1,18	-0,17	-29,24	NA	5,83	-0,29	NA	NA
		a	18	1,10	-0,04	-28,41	NA	6,78	0,39	NA	NA
		a	19	1,41	0,42	-28,79	NA	6,37	-0,09	NA	NA
		a	20	1,25	-0,39	-27,17	NA	4,99	1,01	NA	NA
		mean		**1,20**	**-0,13**	**-28,53**	**NA**	**5,94**	**0,17**	**NA**	
		SD		**0,14**	**0,35**	**0,82**	**NA**	**0,68**	**0,54**	**NA**	
		p	NA	1,63	-0,91	-26,83	NA	5,16	1,87	NA	e
		p	NA	1,35	-0,84	-26,98	NA	5,23	1,72	NA	e
		p	NA	1,34	-2,91	-27,32	NA	3,16	1,38	NA	e
		p	NA	1,43	-3,65	-27,30	NA	2,43	1,40	NA	e
		p	NA	1,49	-3,44	-26,67	NA	2,64	2,03	NA	e
		p	NA	2,10	-0,55	-25,34	NA	5,53	3,36	NA	e
		p	NA	2,06	-0,29	-26,49	NA	5,78	2,21	NA	e
		p	NA	1,50	-1,80	-26,65	NA	4,28	2,05	NA	e
		p	NA	1,42	-1,64	-27,23	NA	4,44	1,47	NA	e
		p	NA	1,64	-3,22	-26,75	NA	2,85	1,95	NA	e
		mean		**1,60**	**-1,92**	**-26,76**	**NA**	**4,15**	**1,94**	**NA**	
		SD		**0,27**	**1,28**	**0,58**	**NA**	**1,28**	**0,58**	**NA**	
Platanthera bifolia (L.) Rich.	beech forest	a	21	1,02	2,55	-30,37	-97,67	5,75	2,21	12,30	NA
		a	22	1,02	2,01	-30,32	-77,29	5,52	1,83	38,38	NA
		a	23	1,41	-2,12	-31,08	-99,79	0,41	1,38	19,93	NA
		a	24	1,06	-2,70	-30,22	-96,32	-0,39	2,17	20,80	NA
		a	25	1,49	-0,56	-29,68	-95,71	2,79	1,91	11,00	NA
		mean		**1,20**	**-0,16**	**-30,33**	**-93,36**	**2,81**	**1,90**	**20,48**	
		SD		**0,23**	**2,37**	**0,50**	**9,12**	**2,83**	**0,33**	**10,93**	
		p	NA	3,18	4,70	-24,43	NA	7,67	7,80	NA	e
		p	NA	3,51	3,88	-24,70	NA	6,86	7,53	NA	e
		p	NA	4,49	4,91	-23,88	NA	7,89	8,35	NA	e
		p	NA	4,21	5,06	-24,59	NA	8,04	7,65	NA	e
		p	NA	4,37	5,40	-23,83	NA	8,38	8,41	NA	e
		p	NA	3,70	4,81	-24,27	NA	7,79	7,96	NA	e
		p	NA	4,36	5,77	-23,37	NA	8,75	8,86	NA	e
		p	NA	4,15	4,76	-24,62	NA	7,73	7,62	NA	e
		p	NA	5,28	5,59	-24,03	NA	8,57	8,20	NA	e
		p	NA	4,13	5,22	-23,92	NA	8,20	8,32	NA	e
		p	NA	4,41	5,44	-23,28	NA	8,42	8,95	NA	e
		p	NA	4,68	4,00	-24,38	NA	6,98	7,85	NA	e
		p	NA	4,22	4,60	-24,12	NA	7,58	8,12	NA	e
		p	NA	4,63	4,80	-24,13	NA	7,78	8,11	NA	e
		p	NA	4,67	5,15	-23,46	NA	8,13	8,78	NA	e
		p	NA	5,80	5,10	-24,04	NA	8,08	8,20	NA	e
		p	NA	4,06	4,56	-23,70	NA	7,54	8,54	NA	e
		p	NA	4,02	4,82	-23,85	NA	7,80	8,39	NA	e
		p	NA	3,76	5,00	-23,78	NA	7,98	8,46	NA	e
		p	NA	3,66	5,04	-24,07	NA	8,01	8,17	NA	e
		p	NA	3,76	4,26	-25,04	NA	7,24	7,19	NA	e
		p	NA	4,54	4,00	-24,19	NA	6,98	8,04	NA	e
		p	NA	4,40	5,71	-23,13	NA	8,68	9,10	NA	e
		p	NA	3,91	4,76	-23,79	NA	7,73	8,45	NA	e
		p	NA	4,83	2,92	-23,23	NA	5,90	9,00	NA	e
		p	NA	2,55	7,61	-22,87	NA	10,59	9,36	NA	e

Table S1 (continued)

Species	Site	adult orchid (a)/ protocorm (p)	Plot	N conc. [mmol g$_{dw}^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]	protocorm part: entire (e), root (r), shoot (s)
Platanthera bifolia											
(continued)		p	NA	4,06	4,96	-23,08	NA	7,94	9,15	NA	e
			mean	4,20	4,92	-23,92	NA	7,90	8,32	NA	
			SD	0,63	0,82	0,54	NA	0,82	0,54	NA	
		p	NA	3,47	5,71	-25,40	NA	8,68	6,84	NA	s
		p	NA	2,72	7,38	-25,31	NA	10,35	6,92	NA	s
		p	NA	1,84	6,89	-25,49	NA	9,87	6,75	NA	s
		p	NA	2,70	5,94	-25,03	NA	8,92	7,21	NA	s
		p	NA	3,56	7,43	-24,46	NA	10,41	7,78	NA	s
		p	NA	3,07	5,91	-23,71	NA	8,89	8,52	NA	s
		p	NA	2,65	7,35	-24,36	NA	10,33	7,88	NA	s
		p	NA	2,73	5,15	-23,81	NA	8,12	8,42	NA	s
		p	NA	2,65	4,95	-25,97	NA	7,92	6,27	NA	s
		p	NA	2,63	6,38	-23,60	NA	9,35	8,64	NA	s
		p	NA	2,80	6,53	-23,00	NA	9,50	9,24	NA	s
		p	NA	2,54	6,90	-23,30	NA	9,87	8,93	NA	s
		p	NA	3,23	7,31	-23,94	NA	10,29	8,30	NA	s
			mean	2,81	6,45	-24,41	NA	9,42	7,82	NA	
			SD	0,44	0,86	0,94	NA	0,86	0,94	NA	
		p	NA	3,97	2,87	-24,36	NA	5,85	7,87	NA	r
		p	NA	3,80	3,75	-25,09	NA	6,73	7,15	NA	r
		p	NA	2,69	3,30	-24,70	NA	6,28	7,53	NA	r
		p	NA	2,46	4,37	-25,13	NA	7,34	7,10	NA	r
		p	NA	3,30	4,09	-24,55	NA	7,07	7,68	NA	r
		p	NA	3,57	3,12	-24,25	NA	6,10	7,98	NA	r
		p	NA	4,61	3,78	-24,04	NA	6,76	8,20	NA	r
		p	NA	3,11	6,53	-24,30	NA	9,51	7,94	NA	r
		p	NA	4,48	3,67	-23,87	NA	6,65	8,36	NA	r
		p	NA	3,75	4,35	-23,89	NA	7,33	8,35	NA	r
		p	NA	2,95	4,15	-24,18	NA	7,13	8,06	NA	r
		p	NA	2,33	4,44	-24,44	NA	7,42	7,80	NA	r
		p	NA	2,57	4,36	-24,15	NA	7,34	8,09	NA	r
		p	NA	2,18	4,22	-24,27	NA	7,20	7,96	NA	r
		p	NA	3,87	3,23	-24,79	NA	6,21	7,45	NA	r
		p	NA	4,40	4,20	-23,11	NA	7,18	9,12	NA	r
		p	NA	5,14	5,73	-23,08	NA	8,71	9,15	NA	r
		p	NA	3,53	6,31	-22,53	NA	9,28	9,70	NA	r
		p	NA	3,77	4,84	-22,94	NA	7,81	9,30	NA	r
		p	NA	3,90	4,08	-23,90	NA	7,06	8,33	NA	r
		p	NA	4,52	4,64	-23,67	NA	7,62	8,56	NA	r
			mean	3,57	4,29	-24,06	NA	7,26	8,18	NA	
			SD	0,82	0,95	0,69	NA	0,95	0,69	NA	
			mean	**3,58**	**4,91**	**-23,32**	**NA**	**7,80**	**7,94**	**NA**	
			SD	**0,99**	**1,35**	**4,34**	**NA**	**1,69**	**1,45**	**NA**	
		p	NA	NA	NA	NA	-15,20	NA	NA	98,64	e
		p	NA	NA	NA	NA	-20,42	NA	NA	93,42	e
		p	NA	NA	NA	NA	-25,42	NA	NA	88,42	e
		p	NA	NA	NA	NA	-8,99	NA	NA	104,85	e
		p	NA	NA	NA	NA	-20,25	NA	NA	93,59	e
			mean	**NA**	**NA**	**NA**	**-18,06**	**NA**	**NA**	**95,78**	
			SD	**NA**	**NA**	**NA**	**6,22**	**NA**	**NA**	**6,22**	

Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi

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Table S2: Single and mean δ¹⁵N, δ¹³C, δ²H values, enrichment factors ε¹⁵N, ε¹³C, ε²H and total nitrogen concentration data of all 15 autotrophic reference plant species (n = 105).

Family	Species	Plot	N conc. [mmol g_{dw}^{-1}]	δ¹⁵N [‰]	δ¹³C [‰]	δ²H [‰]	ε¹⁵N [‰]	ε¹³C [‰]	ε²H [‰]
Apiaceae	*Silaum silaus* (L.) Schinz. & Thell.	1	1,86	2,48	-29,21	NA	2,22	0,49	NA
		2	1,70	1,87	-30,61	NA	1,71	-0,20	NA
		3	1,87	1,35	-29,56	NA	0,92	0,39	NA
		4	1,81	2,78	-30,41	NA	2,09	-0,22	NA
		5	2,28	2,11	-29,89	NA	1,63	-0,10	NA
Asteraceae	*Leontodon hispidus* (L.)	16	1,27	-7,15	-29,97	NA	-0,93	-1,10	NA
		17	1,10	-5,87	-30,29	NA	0,13	-1,34	NA
		18	1,27	-6,95	-29,70	NA	-0,14	-0,90	NA
		19	1,07	-5,93	-29,84	NA	0,02	-1,14	NA
		20	1,14	-5,63	-29,14	NA	-0,25	-0,96	NA
		31	1,79	-6,58	-29,48	NA	0,56	-2,44	NA
		32	1,47	-7,58	-29,60	NA	-0,97	-2,12	NA
		33	1,75	-8,35	-28,41	NA	0,40	-0,65	NA
		34	1,75	-7,56	-30,42	NA	0,30	-1,80	NA
		35	2,34	-5,78	-27,83	NA	1,38	-0,71	NA
	Taraxacum campylodes G.E.Haglund	6	0,99	-5,33	-30,45	NA	-0,77	-1,21	NA
		7	1,00	-5,72	-29,94	NA	-1,01	-0,77	NA
		8	1,17	-4,78	-30,35	NA	-0,18	-0,84	NA
		9	1,20	-3,83	-30,28	NA	0,19	-0,49	NA
		10	1,12	-4,58	-30,73	NA	-0,07	-0,45	NA
Cupressaceae	*Juniperus communis* L.	31	0,97	-7,30	-25,31	NA	-0,16	1,73	NA
		32	0,97	-7,19	-25,23	NA	-0,58	2,24	NA
		33	0,81	-8,72	-25,80	NA	0,03	1,96	NA
		34	0,69	-8,47	-26,38	NA	-0,61	2,24	NA
		35	0,76	-8,90	-26,23	NA	-1,74	0,88	NA
Cyperaceae	*Carex* sp.	21	1,39	-0,05	-32,39	-110,02	3,14	0,19	-0,05
		22	1,14	-0,58	-31,56	-95,29	2,93	0,59	20,38
		23	1,12	-0,85	-32,40	-101,71	1,67	0,06	18,02
		24	1,41	-1,00	-32,44	-108,80	1,31	-0,05	8,32
		25	1,15	-0,65	-31,55	-91,72	2,70	0,04	14,99
Euphorbiaceae	*Euphorbia cyparissias* (L.)	6	1,31	-5,29	-27,57	NA	-0,73	1,66	NA
		7	1,30	-5,84	-28,06	NA	-1,13	1,11	NA
		8	1,12	-5,37	-28,15	NA	-0,78	1,36	NA
		9	1,30	-5,20	-28,19	NA	-1,17	1,60	NA
		10	1,47	-5,56	-29,34	NA	-1,05	0,94	NA
		16	1,22	-6,99	-28,64	NA	-0,77	0,22	NA
		17	1,29	-6,55	-28,51	NA	-0,55	0,44	NA
		18	1,30	-6,95	-28,11	NA	-0,14	0,70	NA
		19	1,21	-6,69	-28,09	NA	-0,73	0,61	NA
		20	1,33	-6,48	-27,89	NA	-1,10	0,30	NA
		31	1,92	-7,53	-26,34	NA	-0,40	0,71	NA
		32	1,82	-5,06	-27,59	NA	1,55	-0,12	NA
		33	1,78	-9,18	-29,07	NA	-0,43	-1,31	NA
		34	2,18	-7,54	-29,05	NA	0,31	-0,44	NA
		35	2,20	-6,81	-27,28	NA	0,36	-0,17	NA
Fagaceae	*Fagus sylvatica* (L.)	11	1,72	-4,70	-31,36	NA	-0,29	-0,95	NA
		12	1,74	-6,46	-32,57	NA	-1,13	-0,50	NA
		13	1,59	-6,51	-32,30	NA	-0,02	-0,22	NA
		14	1,69	-5,14	-32,18	NA	0,24	-0,58	NA
		15	1,98	-3,46	-31,64	NA	1,14	-0,16	NA
		21	0,95	-3,12	-32,63	-102,11	0,08	-0,05	7,86
		22	1,00	-4,60	-32,42	-125,60	-1,09	-0,26	-9,93
		23	1,44	-4,16	-33,07	-121,39	-1,64	-0,61	-1,67
		24	1,24	-3,76	-32,88	-117,55	-1,45	-0,49	-0,44
		25	1,21	-4,10	-31,47	-115,33	-0,75	0,12	-8,62

Table S2 (continued)

Family	Species	Plot	N conc. [mmol g$_{dw}$$^{-1}$]	δ^{15}N [‰]	δ^{13}C [‰]	δ^{2}H [‰]	ε^{15}N [‰]	ε^{13}C [‰]	ε^{2}H [‰]
Lamiaceae	*Stachys officinalis* (L.) Trevis.	1	1,57	-2,02	-30,12	NA	-2,28	-0,42	NA
		2	1,32	-3,35	-30,50	NA	-3,51	-0,08	NA
		3	1,45	0,09	-30,65	NA	-0,34	-0,70	NA
		4	1,34	-1,51	-30,50	NA	-2,21	-0,32	NA
		5	1,55	-1,39	-29,69	NA	-1,87	0,10	NA
Rosaceae	*Fragaria vesca* (L.)	11	1,55	-2,96	-29,86	NA	1,45	0,56	NA
		12	1,50	-2,77	-31,08	NA	2,55	0,99	NA
		13	1,81	-5,51	-30,71	NA	0,98	1,37	NA
		14	1,89	-4,06	-30,81	NA	1,32	0,79	NA
		15	1,63	-3,77	-30,78	NA	0,82	0,70	NA
		23	0,80	-2,56	-31,91	-136,07	-0,04	0,55	-16,35
		24	1,04	-2,16	-31,85	-124,99	0,14	0,54	-7,87
	Filipendula ulmaria	26	1,38	-1,60	-29,37	NA	0,42	0,54	NA
		27	1,18	-2,32	-29,45	NA	0,18	0,44	NA
		28	1,27	-1,90	-29,66	NA	0,47	0,56	NA
		29	1,41	-2,27	-29,14	NA	0,14	0,91	NA
		30	1,54	-2,10	-30,33	NA	0,87	0,04	NA
	Potentilla erecta	26	1,34	-2,37	-29,82	NA	-0,36	0,09	NA
		27	1,26	-2,22	-29,89	NA	0,27	0,00	NA
		28	1,27	-2,51	-30,27	NA	-0,13	-0,05	NA
		29	0,93	-2,90	-30,60	NA	-0,49	-0,56	NA
		30	0,90	-2,56	-29,42	NA	0,41	0,95	NA
	Sanguisorba officinalis (L.)	1	1,65	0,31	-29,78	NA	0,05	-0,07	NA
		2	1,55	1,97	-30,14	NA	1,80	0,28	NA
		3	1,65	-0,15	-29,64	NA	-0,58	0,31	NA
		4	1,63	0,81	-29,64	NA	0,12	0,54	NA
		5	1,19	0,71	-29,80	NA	0,23	0,00	NA
Pinaceae	*Picea abies* (L.) H.Karst.	11	1,41	-5,58	-30,02	NA	-1,16	0,39	NA
		12	1,29	-6,75	-32,57	NA	-1,43	-0,49	NA
		13	1,32	-7,45	-33,23	NA	-0,96	-1,15	NA
		14	1,44	-6,95	-31,81	NA	-1,57	-0,21	NA
		15	1,39	-6,55	-32,02	NA	-1,96	-0,54	NA
		21	0,53	-6,42	-32,72	-117,78	-3,22	-0,14	-7,81
		22	0,45	-5,34	-32,48	-126,12	-1,83	-0,33	-10,45
		25	0,55	-5,30	-31,77	-113,09	-1,95	-0,17	-6,37
Plantaginaceae	*Plantago lanceolata* (L.)	6	0,58	-3,06	-29,68	NA	1,50	-0,45	NA
		7	0,60	-2,57	-29,50	NA	2,14	-0,34	NA
		8	0,61	-3,63	-30,04	NA	0,96	-0,53	NA
		9	0,61	-3,04	-30,89	NA	0,99	-1,10	NA
		10	0,84	-3,39	-30,77	NA	1,12	-0,49	NA
		26	1,22	-2,08	-30,55	NA	-0,06	-0,64	NA
		27	1,02	-2,94	-30,33	NA	-0,45	-0,44	NA
		28	0,97	-2,72	-30,74	NA	-0,34	-0,51	NA
		29	0,93	-2,06	-30,40	NA	0,35	-0,36	NA
		30	1,16	-4,25	-31,36	NA	-1,28	-0,99	NA
Poaceae	*Briza media* (L.)	16	0,99	-4,52	-27,99	NA	1,70	0,88	NA
		17	0,93	-5,58	-28,05	NA	0,42	0,90	NA
		18	0,93	-6,54	-28,60	NA	0,28	0,20	NA
		19	0,94	-5,24	-28,16	NA	0,71	0,54	NA
		20	1,29	-4,04	-27,52	NA	1,34	0,66	NA
		mean	**1,31**	**-4,05**	**-29,97**	**-113,84**	**0,00**	**0,00**	**0,00**
		SD	**0,40**	**2,79**	**1,74**	**12,41**	**1,26**	**0,85**	**11,38**

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MANUSCRIPT 5

Light limitation and partial mycoheterotrophy in rhizoctonia-associated orchids

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ABSTRACT

Partially mycoheterotrophic (PMH) plants obtain organic molecules from their mycorrhizal fungi in addition to carbon (C) fixed by photosynthesis. Some PMH orchids associated with ectomycorrhizal fungi have been shown to flexibly adjust the proportion of organic molecules obtained from fungi according to the habitat's light level.

We hypothesise that *Neottia ovata* and *Ophrys insectifera*, two orchids associated with saprotrophic rhizoctonia fungi, are also able to increase uptake of organic molecules from fungi as irradiance levels decrease.

We continuously measured light availability for individuals of *N. ovata* and *O. insectifera* at a grassland and a forest during orchid flowering and fruiting. We repeatedly sampled leaves of *N.* ovata, *O. insectifera* and autotrophic reference species for stable isotope natural abundances ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$) and C and N concentrations.

We found significant ^{13}C enrichment in both orchids relative to autotrophic references at the forest but not the grassland, and significant ^{2}H enrichment at both sites. The ^{13}C enrichment in *O. insectifera* was linearly correlated with habitat irradiance levels.

We conclude that both species can be considered as PMH and at least in *O. insectifera* the degree of partial mycoheterotrophy can be fine-tuned according to light availability. However, exploitation of mycorrhizal fungi appears less flexible in saprotroph-associated orchids than in orchids associated with ectomycorrhizal fungi.

KEY WORDS

INTRODUCTION

The presence of chlorophyll in leaves or stems is not sufficient to classify plants in general as fully autotrophic (Press *et al.* 1987; Schulze *et al.* 1991; Eiler 2006; Smith & Read 2008). Partial mycoheterotrophy is one of these facultative nutritional modes. Partially mycoheterotrophic plants exploit mycorrhizal fungi to supplement photosynthesis (Gebauer & Meyer 2003;). In contrast to fully mycoheterotrophic plants that lack chlorophyll and only have rudimentary scale-like leaves, partially mycoheterotrophic (PMH) plants have green leaves, stems and/or fruits (Merckx 2013). Partial mycoheterotrophy has been discovered in diverse species of the families Orchidaceae, Ericaceae, Burmanniaceae and Gentianaceae (Zimmer *et al.* 2007; Hynson *et al.* 2009, 2016; Cameron & Bolin 2010; Merckx *et al.* 2013; Bolin *et al.* 2015; Schiebold *et al.* 2018). The trophic strategy of partial mycoheterotrophy can exhibit intermediate stages along the continuum between autotrophy and full mycoheterotrophy that can be quantified by stable isotope natural abundance analysis and the application of a simple two-source linear mixing model (Hynson *et al.* 2013; Merckx 2013).

The percentage of organic molecules gained from mycorrhizal fungi by PMH plants has been well investigated for species in the Orchidaceae; carbon (C) gain from fungi in *Cephalanthera damasonium* can range from 33 % in an open forest dominated by Scots pine (*Pinus sylvestris*) to 85 % in a dark forest dominated by the common beech (*Fagus sylvatica*) (Gebauer 2005; Hynson *et al.* 2013). *Cephalanthera damasonium* associates with fungi that simultaneously form ectomycorrhizae with surrounding forest trees. For *C. damasonium* and its sister species *C. rubra*, Preiss *et al.* (2010) have shown partial mycoheterotrophy to be a flexible nutritional mode driven by light availability. Low light levels drive the orchids to exhibit pronounced mycoheterotrophy whereas high light levels enable full autotrophy, e.g. the proportional degree of partial mycoheterotrophy and thus the reliance on fungal carbon obtained by an ectomycorrhizal orchid species can be fine-tuned according to the light level. Consequently, partial mycoheterotrophy can be considered as a backup for the photosynthetic apparatus in times of low light intensity or habitats with low light levels.

Most terrestrial orchid species form mycorrhizae with rhizoctonia fungi, a polyphyletic group of basidiomycetes that are typically saprotrophic and form rather inconspicuous fruiting bodies hard to be found. These orchids have been shown to gain up to 20 % organic molecules from mycorrhizal fungi (Schweiger *et al.* 2018). However, whether the degree of partial mycoheterotrophy in these rhizoctonia-associated orchids is predominantly determined by habitat light availability is unclear. Preiss *et al.* (2010) and Liebel *et al.* (2015) found no evidence for a flexible mode in two rhizoctonia-associated orchids, *Cypripedium calceolus* (geophyte) and *Goodyera repens* (evergreen). Lallemand *et al.* (2018) questioned whether rhizoctonia-associated *Epipactis palustris* is autotrophic, but claimed that, as autotrophic plants, *E. palustris* exhibits decreased δ13C

abundances with decreasing irradiance levels. These three studies monitored orchids under narrow natural light gradients due to the limited habitat preferences of the orchids investigated. For example, a light indicator value (*L*) of 8 was assigned to *E. palustris,* characterising this orchid species as a full-light plant (Ellenberg *et al.* 1991). In contrast, some orchid species associated with rhizoctonia fungi, e.g. the common twayblade *Neottia ovata* (L.) Bluff. & Fingerh. and the fly orchid *Ophrys insectifera* L. occur in a variety of habitats with different irradiance levels such as open meadows and closed-canopy forests. These orchids occurring under wider natural light gradients could be more suitable to examine the ability to adjust the degree of mycoheterotrophic nutrition with a microhabitat's light level.

We hypothesise that some rhizoctonia-associated orchid species able to thrive under different light regimes can regulate exploitation of mycorrhizal fungi dynamically in relation to prevalent light availability. We propose that *N. ovata* and *O. insectifera*, increase their proportional fungal C gain, expressed as ^{13}C enrichment of their leaf tissue in comparison to neighbouring fully autotrophic plants, with decreasing local light availability. We test this hypothesis by continuously measuring light availability next to individuals of *N. ovata* and *O. insectifera* at a grassland site and in a forest during their flowering and fruiting period. In addition, we repeatedly sampled leaves of *N.* ovata, *O. insectifera* and autotrophic reference species for stable isotope natural abundances (δ^{13}C, δ^{15}N, δ^{2}H, δ^{18}O) and C and N concentrations.

MATERIALS AND METHODS

Study locations and sampling scheme

We chose a dry calcareous grassland (50.1°N/11.2°E) and a mixed forest dominated by common beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) H.Karst) (49.7°N/11.4°E) in North-East Bavaria (Germany) with sympatrically growing populations of *Neottia ovata* (L.) Bluff. & Fingerh. and *Ophrys insectifera* L. as study sites. A total of 48 orchid individuals ($n = 12$ per species and site) and accompanying autotrophic non-orchid reference plant species (grassland site: *Juniperus communis* L., *Euphorbia cyparissias* L., *Leontodon hispidus* L.; forest site: *Fagus sylvatica* L., *Picea abies* (L.) H.Karst, *Fragaria vesca* L.) were sampled three times during orchid flowering and fruiting in May and June 2012 for measurements of $\delta^{13}C$ and $\delta^{15}N$ stable isotope abundances and N and C concentrations. Sampling took place on day of year (DOY) 148 = t_1, 167 = t_2 and 174 = t_3 at the grassland site and DOY 149 = t_1, 166 = t_2 and 174 = t_3 at the forest site and followed the plot-wise sampling scheme proposed by Gebauer & Meyer (2003). Unfortunately, six individuals of each orchid species died during the field campaign and could not be resampled at t_2 and/or t_3. Additional samples of *N. ovata* and *O. insectifera* ($n = 5$ per species and site each) and the same set of reference species as during the field campaign in 2012 were collected in the subsequent year for δ^2H and $\delta^{18}O$ stable isotope abundance measurements.

Stable isotope abundances, N and C concentration analysis

Leaf samples of the two orchid species ($n = 151$) and autotrophic references ($n = 513$) were washed with deionised water, dried to constant weight at 105°C, ground to a fine powder in a ball mill (Retsch Schwingmühle MM2, Haan, Germany) and stored in a desiccator fitted with silica gel until analysis. Relative C and N isotope natural abundances of the leaf samples were measured in a dual element analysis mode with an elemental analyser (Carlo Erba Instruments 1108, Milano, Italy) coupled to a continuous flow isotope ratio mass spectrometer (delta S, Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Thermo Fisher Scientific, Bremen, Germany) as described in Bidartondo *et al.* (2004). Relative H and O isotope natural abundances of the leaf samples were measured with thermal conversion through pyrolysis (HTO, HEKAtech, Wegberg, Germany) coupled to a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific) via a ConFlo IV open-split interface (Thermo Fisher Scientific) as described in Gebauer *et al.* (2016). In order to minimise bias of post-sampling H atom exchange between organically-bound hydroxyl groups in our samples and H_2O in ambient air (Yakir 1992), we analysed samples of orchids and their respective reference plants together in identical sample batches. Due to memory bias each sample was analysed three times and the first two sample runs were skipped for reliable H isotope abundance determination. The O isotope abundances

were measured to rule out a transpiration effect as a cause of differences in the H isotope abundance between orchids and non-orchid reference plants (Ziegler 1988). Measured relative isotope abundances are denoted as δ values that were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, δ^2H or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. Standard gases (Riessner, Lichtenfels, Germany) were calibrated with respect to international standards (CO_2 vs. PDB, N_2 vs. N_2 in air, H_2 and CO vs. SMOW) with the reference substances ANU sucrose and NBS19 for the C isotopes, N1 and N2 for the N isotopes, CH7, V–SMOW and SLAP for H isotopes and IAEA601 and IAEA602 for the O isotopes, all provided by the IAEA (International Atomic Energy Agency, Vienna, Austria). Reproducibility and accuracy of the C and N isotope abundance measurements were routinely controlled by measuring the laboratory standard acetanilide (Gebauer & Schulze, 1991). In relative C and N isotope natural abundance analyses, acetanilide was routinely analysed with variable sample weight at least six times within each batch of 50 samples. The maximum variation in $\delta^{13}C$ and $\delta^{15}N$ both within and between batches was always below 0.2 ‰. For relative H and O isotope natural abundance analyses, benzoic acid was routinely analysed with variable sample weight at least six times within each batch of 40 samples. The maximum variation in δ^2H and $\delta^{18}O$ both within and between batches was always below 4 ‰ for δ^2H and 0.6‰ for $\delta^{18}O$. Total N and C concentrations in leaf samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on measurements of the laboratory standard acetanilide with a known nitrogen concentration of 10.36 % and carbon concentration of 71.09 % (Gebauer & Schulze 1991).

Irradiance measurements

Irradiance in the flowering and fruiting period of the two orchid species was continuously recorded every 15 minutes for 24 days between DOY 150 and DOY 174 using calibrated silicon photodiodes (BPW 21, Infineon, Neubiberg/Germany) connected to mini data loggers (HOBO H8, Onset, Bourne/USA). The photodiodes were installed on the first day of the field campaign in 2012 and placed right next to each selected orchid individual at a height of approximately 15 cm. The irradiance measurements were started as early as possible, i.e. as soon as the vegetative plant parts could be identified as the respective correct plant species. Thus, biomass produced before the start of the light measurements was marginal compared to the biomass produced after start of the light measurements. Furthermore, all investigated species at the forest site came up after canopy closure of the deciduous trees and thus, the light conditions before and after beginning of the light measurements were equivalent. The measured values were read out, converted into photosynthetic active radiation (PAR) [μmol photons m^{-2} s^{-1}] and averaged as daily means from sunrise to sunset. The sensors were removed after the last sample collection on DOY 174 at both sites.

Fungal DNA analysis

We identified the mycorrhizal fungi of *N. ovata* and *O. insectifera* by molecular methods. Two roots per orchid individual sampled during the additional field campaign in 2013 were cut, rinsed with deionised water, placed in CTAB buffer (cetyltrimethylammonium bromide) and stored at −18°C until further analysis. Root cross-sections were checked for presence and status of fungal pelotons in the cortex cells. Two to six root sections per orchid individual were selected for genomic DNA extraction and purification with the GeneClean III Kit (Q–BioGene, Carlsbad, CA, USA). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primer combinations ITS1F/ITS4 and ITS1/ITS4-Tul (Gardes & Bruns 1993; Taylor & McCormick 2008) as described in Bidartondo and Duckett (2010). All positive PCR products were purified with ExoProStart (GE Healthcare, Buckinghamshire, UK) and sequenced bidirectionally with an ABI3730 Genetic Analyser using the BigDye 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. All DNA sequences were quality-checked (edited and the ends trimmed) and visually aligned with Geneious version 7.4.1 (http://www.geneious.com, Kearse *et al.* 2012) and compared to GenBank using BLAST (http://blast.ncbi.nlm.nih.gov). All unique DNA sequences have been submitted to GenBank (accession numbers: KY630472, KY630474, MH231516).

Calculation and statistics

To enable comparisons of C, N, H and O stable isotope abundances between *N. ovata* and *O. insectifera* between plots and the two different sites we used an isotope enrichment factor approach to normalise the data. Normalised enrichment factors (ε) were calculated from measured δ values as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, δ^2H or $\delta^{18}O$ value of an orchid individual or an autotrophic reference plant and δ_{REF} is the mean value of all autotrophic reference plants by plot (Preiss & Gebauer 2008). We tested for pairwise differences in the isotopic enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, ε^2H and $\varepsilon^{18}O$ and nitrogen concentration between the sampled orchid species and all autotrophic reference plants using a conservative, nonparametric Mann–Whitney *U*-test to accommodate the rather small sample sizes. We furthermore fitted linear models to investigate the relationship between ^{13}C–enrichment and irradiance in *N. ovata* and *O. insectifera*. For statistical analyses we used R version 3.3.2 (R Development Core Team 2016) with a significance level of $\alpha = 0.05$.

RESULTS

Light climate measurements

Irradiance in the flowering and fruiting period of the two orchid species recorded between DOY 150 and DOY 174 was on average about three times lower at the forest site (171.2 µmol m^{-2} s^{-1}) than at the grassland site (517.0 µmol m^{-2} s^{-1}). The gradient in mean diurnal photon flux density ranged between 177.2 µmol m^{-2} s^{-1} for *N. ovata* and 46.2 µmol m^{-2} s^{-1} for *O. insectifera* at the forest site to a maximum of about 630 µmol m^{-2} s^{-1} for both orchid species and their references at the grassland site.

Stable isotope abundances, **and** *N and C concentrations*

At the forest site, pairwise Mann-Whitney *U*-tests showed that both species, *N. ovata* and *O. insectifera,* were significantly enriched in ^{13}C compared to their autotrophic references at $t_1 - t_4$, whereas at the grassland site both species could not be differentiated from their references. *Ophrys insectifera* was significantly enriched in ^{15}N compared to autotrophic references at both sites at all sampling dates, whereas *N. ovata* only showed significant ^{15}N enrichment at the grassland site and not at the forest site (Fig. 1). There were no significant differences in $\varepsilon^{13}C$ or $\varepsilon^{15}N$ among t_1, t_2 and t_3 in any of the two orchid species regardless of the site. Furthermore, $\varepsilon^{13}C$ and $\varepsilon^{15}N$ measured at t_4 were not different from the previous year ($P > 0.172$).

Both species, *N. ovata* and *O. insectifera,* were significantly enriched in ^2H at the grassland and the forest site (Fig. 2). This enrichment is not due to higher evapotranspiration rates of the orchids as then the enrichment in ^{18}O of both orchid species and autotrophic references would be significantly different from each other, which is not the case (Suppl. Fig. 1).

Nitrogen concentration in leaf tissue of both orchid species was significantly higher than in the leaves of autotrophic references at the forest site throughout all sampling dates and at the grassland site at t_1 and t_4 at the beginning of the growing season. At t_2 and t_3 at the grassland site the leaf N concentration of orchids and autotrophs was almost identical (Fig. 1). Leaf N concentration in *N. ovata* and *O. insectifera* significantly decreased over the growing season at both sites (grassland site: *N. ovata*: $r^2_{adj} = 0.67$, $P < 0.001$; *O. insectifera*: $r^2_{adj} = 0.56$, $P < 0.001$; forest site: *N. ovata*: $r^2_{adj} = 0.31$, $P < 0.001$; *O. insectifera*: $r^2_{adj} = 0.32$, $P = 0.002$) (Fig. 1).

Leaf C concentrations of *O. insectifera* and autotrophic references were almost identical throughout the growing season at both sites, whereas leaf C concentrations of *N. ovata* were significantly lower except at t_2 in the grassland site (Suppl. Fig. 2). There was a significant increase in leaf C concentration at both sites in *O. insectifera* (grassland site: $r^2_{adj} = 0.28$, $P = 0.002$; forest site: $r^2_{adj} = 0.42$, $P < 0.001$) and a significant decrease in leaf C concentration at both sites in *N. ovata* (grassland site: $r^2_{adj} = 0.47$, $P < 0.001$; forest site: $r^2_{adj} = 0.27$, $P = 0.002$).
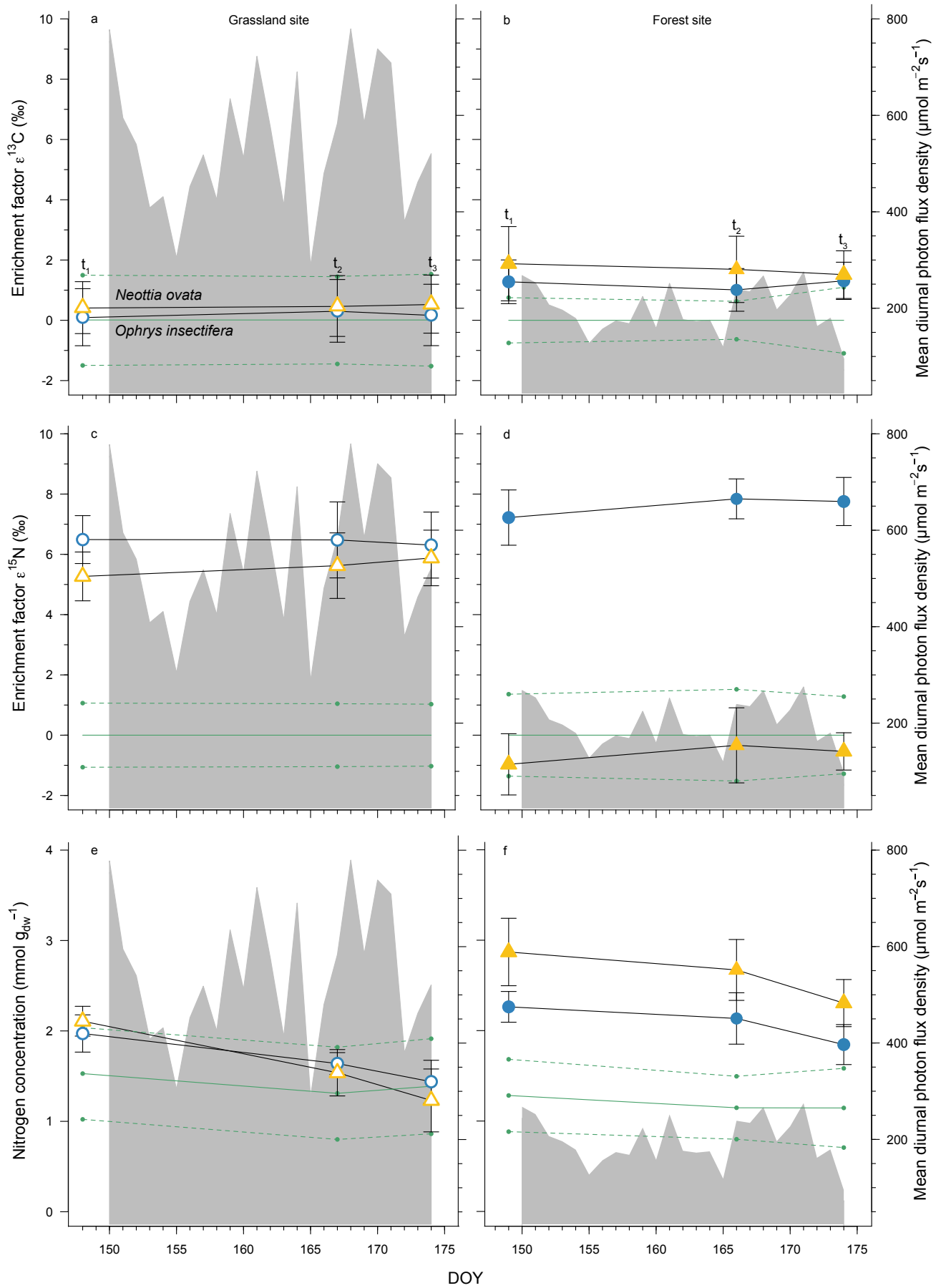
Fig. 1 Mean irradiance at the grassland and the forest site overlaid by (a, b) mean enrichment factor $\varepsilon^{13}C \pm SD$, (c, d), mean enrichment factor $\varepsilon^{15}N \pm SD$ and (e, f) mean nitrogen concentration $\pm$ SD; yellow triangles for *Neottia ovata* and blue circles for *Ophrys insectifera*; unfilled symbols at the grassland site, filled symbols at the forest site; solid green lines represent the mean values of the autotrophic references $\pm$ SD (dashed lines).
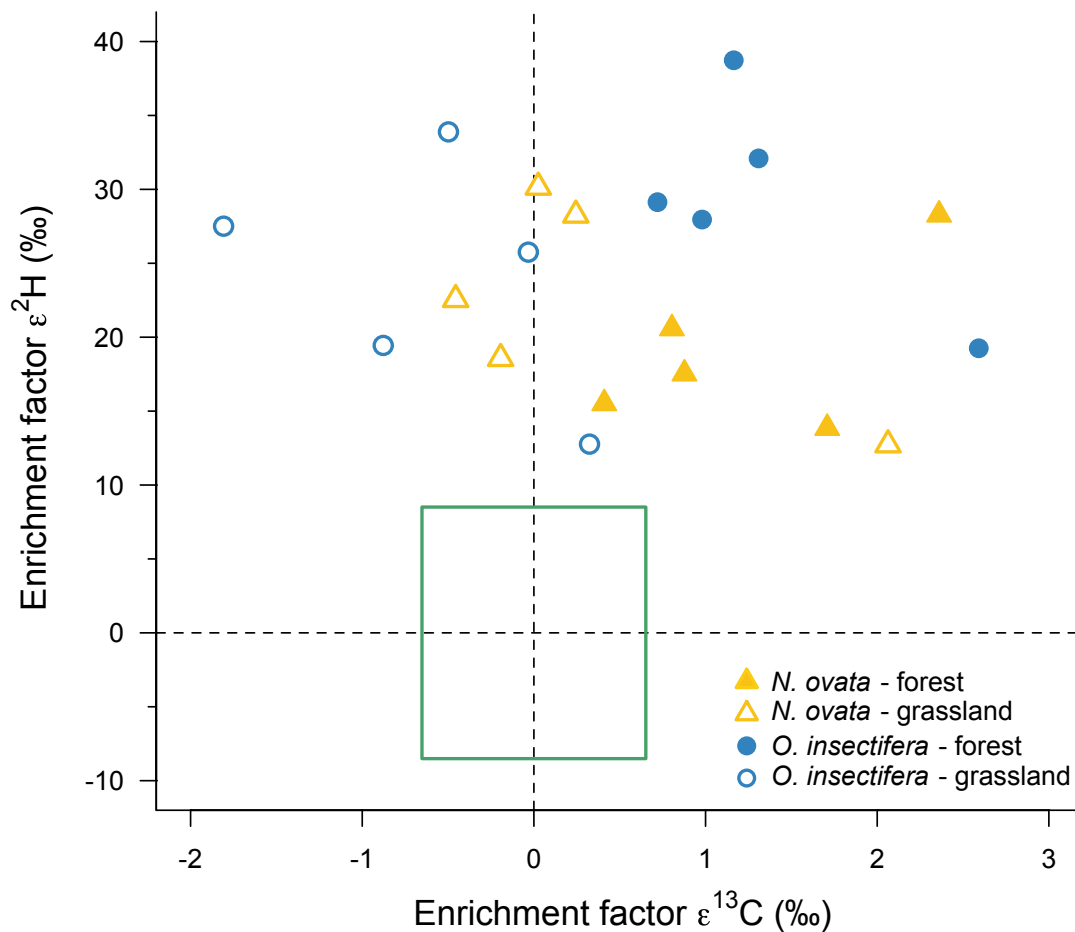
Fig. 2 Enrichment factors $\varepsilon^{2}H$ and $\varepsilon^{13}C$ of *Neottia ovata* (yellow triangles) and *Ophrys insectifera* (blue circles) at the grassland site (unfilled symbols) and the forest site (filled symbols) ($n = 5$ per species and site); the green box represents mean enrichment factors $\pm$ SD for the autotrophic reference plants ($n = 42$) that were sampled together with the two orchid species whereas mean ε values of reference plants are zero by definition.

Relationship between irradiance and ^{13}C enrichment

While a significant relationship between irradiance and ^{13}C enrichment failed marginally for *Neottia ovata* ($r^2 = 0.036$; $P = 0.074$), $\varepsilon^{13}C$ in *Ophrys insectifera* significantly decreased with increased irradiance ($r^2 = 0.252$; $P < 0.001$). The mean enrichment factor $\varepsilon^{13}C$ of the autotrophic references was by definition zero regardless of light climate (Fig. 3).
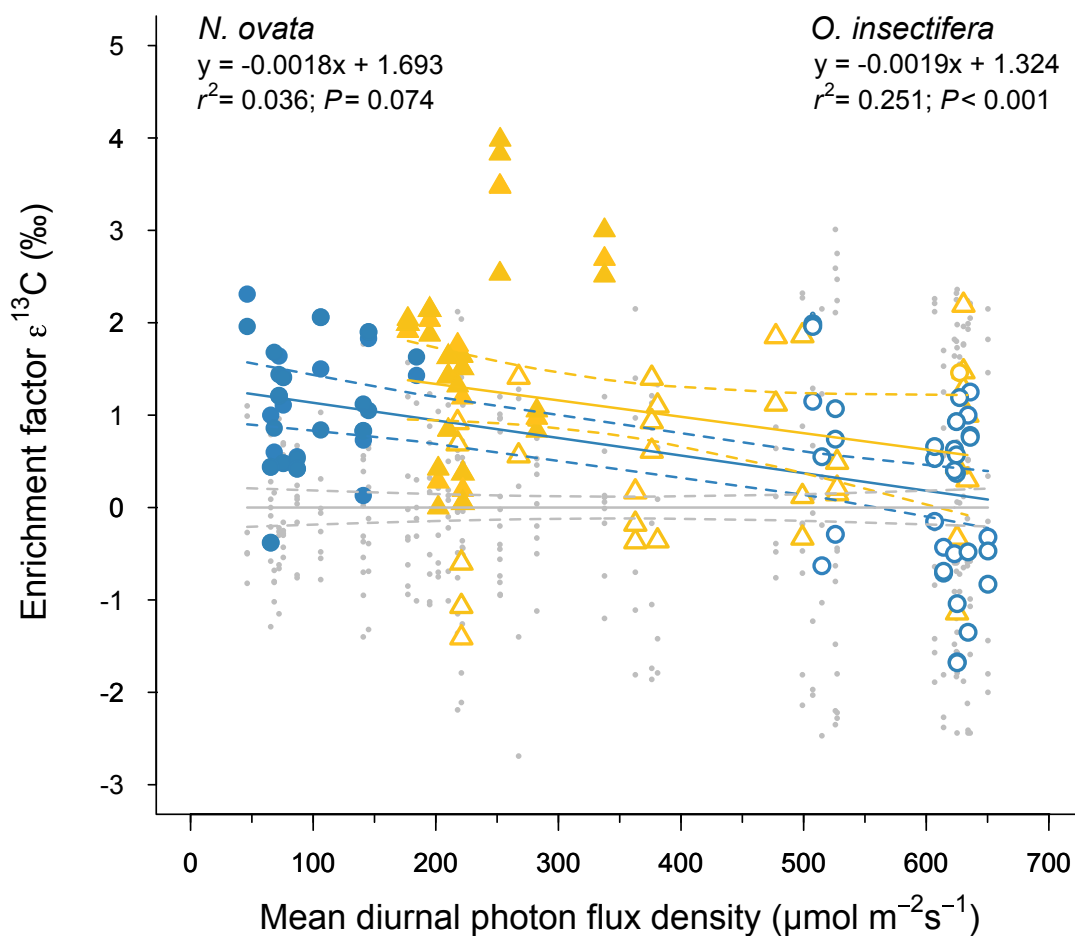
Fig. 3 Relationship between irradiance and ^{13}C enrichment for *Neottia ovata* (yellow triangles) ($n = 66$), *Ophrys insectifera* (blue circles) ($n = 65$) and autotrophic references (grey dots) ($n = 453$); regression lines (solid lines) displayed with 95% confidence intervals (dashed lines); grassland site (unfilled symbols) and forest site (filled symbols). For representation of the sampling times $t_1 - t_3$ please refer to Fig. 1.

Fungal DNA analysis

Approximately 90% of the cortex cells in roots of *N. ovata* at the grassland site were densely colonised with fungal pelotons formed by *Serendipita herbamans* sp. nov. (sister to *Sebacina vermifera*) of the orchid mycorrhizal family Serendipitaceae (placed within Sebacinales clade B) (Riess *et al.* 2014). Roots of *N. ovata* sampled at the forest site during the growing period of this orchid species were not colonised by mycorrhizal fungi. Resampling of *N. ovata* roots in November 2015 yielded a colonisation level of root cortex cells of 50-80 % by *Sebacina epigaea* of the ectomycorrhiza-forming Sebacinales and *Pseudotomentella* sp., and rhizoctonial Ceratobasidiaceae. Cortex cells of the roots of *Ophrys insectifera* at both sites were highly colonised (80-90 %) with fungi identified as *Tulasnella* sp.

DISCUSSION

This is the first study to successfully demonstrate that partial mycoheterotrophy in rhizoctonia-associated orchids is stimulated by light limitation. Both orchid species under study, *N. ovata* and *O. insectifera*, were significantly enriched in ^{13}C relative to their autotrophic references at the forest site with significantly lower irradiance levels. The ϵ^{13}C in *Ophrys insectifera* significantly increased with decreasing irradiance. Increase of ϵ^{13}C in *N. ovata* was not significantly correlated with mean diurnal photon flux density, but a trend is clear. The pattern observed in *N. ovata* might be due to the smaller gradient in light availability under which *N. ovata* was monitored. *Ophrys insectifera* could be sampled under a larger natural irradiance gradient in its natural habitat than *N. ovata* (Fig. 3). Other studies aiming to find a relationship between the degree of rhizoctonia exploitation under different irradiance levels monitored orchids under about half the gradient monitored in this study (Preiss *et al.* 2010; Liebel *et al.* 2015). Preiss *et al.* (2010), Liebel *et al.* (2015) and Lallemand *et al.* (2018) observed that leaf δ^{13}C values of rhizoctonia-associated orchid species (*Goodyera repens*, *Cypripedium calceolus* and *Epipactis palustris*, respectively) responded to changes in light availability in a similar way as the leaf δ^{13}C values of a wide spectrum of autotrophic reference plants, that is a decrease in δ^{13}C values with decreasing light availability. This consistent pattern is based on plant stomatal regulation affecting the intercellular stomatal partial pressure of CO_2 and the C isotope discrimination during C_3 photosynthesis (Farquhar *et al.* 1989; Preiss *et al.* 2010). Preiss *et al.* (2010) also found an increase in ^{13}C enrichment in *Cephalanthera damsonium* and *C. rubra* with decreasing irradiance. This observation suggests more flexible exploitation of mycorrhizal fungi in orchids with ectomycorrhizal fungi than in orchids with rhizoctonia fungi. An explanation for the lack of an increase in ^{13}C enrichment under lower light levels in *G. repens* might be its evergreen appearance and clonal growth form. Carbon fixed during photosynthesis by clones growing under higher irradiance levels could be redistributed and then allocated to rosettes that grow under shadier conditions. The absence of decrease in ^{13}C enrichment under darker conditions in *E. palustris* might be based on the sampling design. Shaded individuals were growing on an open meadow but under *Salix* sp. that did not provide permanent shade like a forest with closed canopy.

Ophrys insectifera is flexible, occurring in habitats with varying light availability; its occurrence in very sunny and especially shady habitats may be facilitated by consistently high colonisation by *Tulasnella*. In *N. ovata*, fungal colonisation was lower or even seasonally absent especially in the forest site; it was not able to thrive under irradiance levels as low as *O. insectifera*. However, both *N. ovata* and *O. insectifera* were significantly enriched in ϵ^2H in grassland and forest and were thus partially mycoheterotrophic regardless of light availability (Fig. 2). So far, it remains unknown

whether δ^2H responds to irradiance levels in a similar way as $\delta^{13}C$ but here we detected no significant differences in ^2H enrichment between sites and / or species (Fig. 2).

Interestingly, the ^{15}N enrichment in *N. ovata* varied greatly (–0.96 ± 1.02 to 5.88 ± 0.92 ‰) between sites confirming a pronounced plasticity in $\varepsilon^{15}N$ as in previous studies (Gebauer & Meyer 2003; Abadie *et al.* 2006; Tedersoo *et al.* 2007; Liebel *et al.* 2010). This pattern might be based on the different mycorrhizal fungi that *N. ovata* associates with at the grassland site (*Serendipita herbamans*) and at the forest site (ectomycorrhizal *Sebacina epigaea* and *Pseudotomentella*, and rhizoctonial Ceratobasidiaceae), and the inconsistent mycorrhization at the forest site. Sebacinales as dominant mycorrhizal fungi of *N. ovata* supplemented by Ceratobasidiaceae and few ectomycorrhizal fungi (Oja *et al.* 2014; Těšitelová *et al.* 2015), and a variability in *N. ovata*'s mycorrhizal fungi, has been reported (Oja *et al.* 2014). However, we detected neither seasonal differences between t_1, t_2 and t_3 in 2012, nor interannual differences with t_4 in the isotopic signal of $\delta^{13}C$ and $\delta^{15}N$. Our findings support a seasonal constancy of stable isotope abundances that has occasionally been challenged in the past (Gonneau *et al.* 2014). Significant decreases in leaf N concentrations in *N. ovata* and *O. insectifera* over the growing season at both sites are due to leaf senescence (Gebauer *et al.* 1987; Gebauer & Schulze 1991). Leaf senescence in both orchids is more pronounced than in the autotrophic reference species due to the orchids' shorter growth cycles. In contrast, leaf C concentrations remained constant.

The response of C exploitation of mycorrhizal fungi in relation to light availability of *N. ovata*, *O. insectifera* or other orchids with rhizoctonia fungi could be further investigated by gas-exchange measurements (Julou *et al.* 2005; Bowler *et al.* 2017) or $^{13}CO_2$ pulse-labelling (Cameron *et al.* 2009) monitoring photosynthetic rates under different irradiance levels. Nevertheless, those methods provide snapshots of current or potential assimilation instead of the complete time-integrating picture provided by stable isotope natural abundance analyses and thus, bear the risk of entirely misleading conclusions (Suetsugu *et al.* 2018). Approaches to assess the increase of fungal C with decreasing irradiance including an experimental shading treatment are generally difficult as C stable isotope abundance in bulk plant leaf tissue is a rather conservative parameter changing only slowly over time. Thus, it may take several growing seasons to make a shading effect in C isotope abundance in bulk plant tissues visible. In an experimental shading approach with a 50 % shading treatment no ^{13}C enrichment was detected in the leaf bulk material of shaded *Pyrola picta* individuals, but ^{13}C enrichment of extracted leaf soluble sugars was observed (Hynson *et al.* 2012). In another study where an experimental PAR reduction of 95 % was achieved, no differences in the C isotope abundances of bulk leaf material between shaded and unshaded individuals of *Epipactis helleborine* were detected (Gonneau *et al.* 2014).

Our data support the hypothesis that the rhizoctonia-associated orchid *O. insectifera* is able to adapt the proportional exploitation of its mycorrhizal fungi dynamically in response to the prevalent light availability, i.e. there is increasing ^{13}C enrichment with decreasing local light availability. *Neottia ovata* exhibits a similar tendency, however, due the smaller gradient in light availability and inconsistent association with different fungi this trend failed marginally statistical significance. Over all, exploitation of mycorrhizal fungi appears less flexible in rhizoctonia-mycorrhizal orchids than in orchids associated with ectomycorrhizal fungi.

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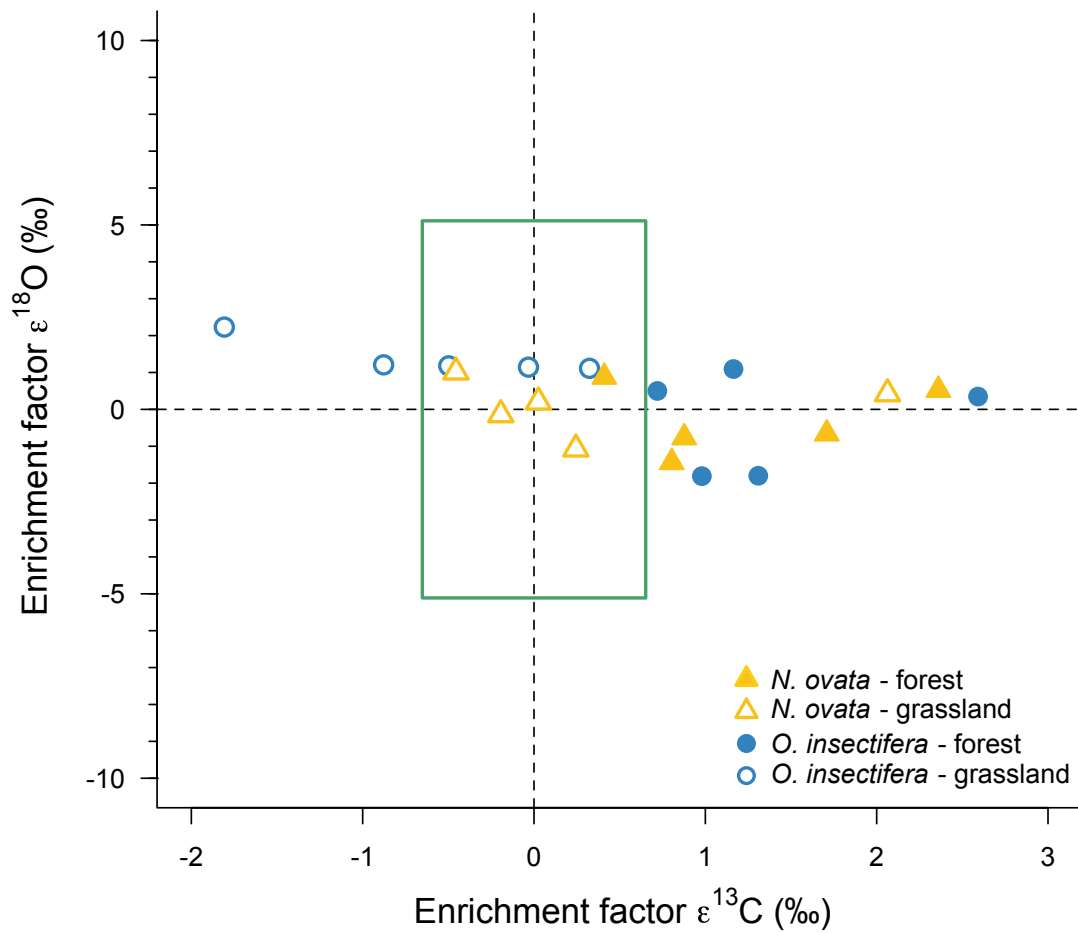
Suppl. Fig. 1 Mean irradiance at (a) the grassland site and (b) the forest site overlaid by the mean carbon concentration ± SD; yellow triangles for *Neottia ovata* and blue circles for *Ophrys insectifera*; unfilled symbols at the grassland site, filled symbols at the forest site; solid green lines represent the mean values of the autotrophic references ± SD (dashed lines).
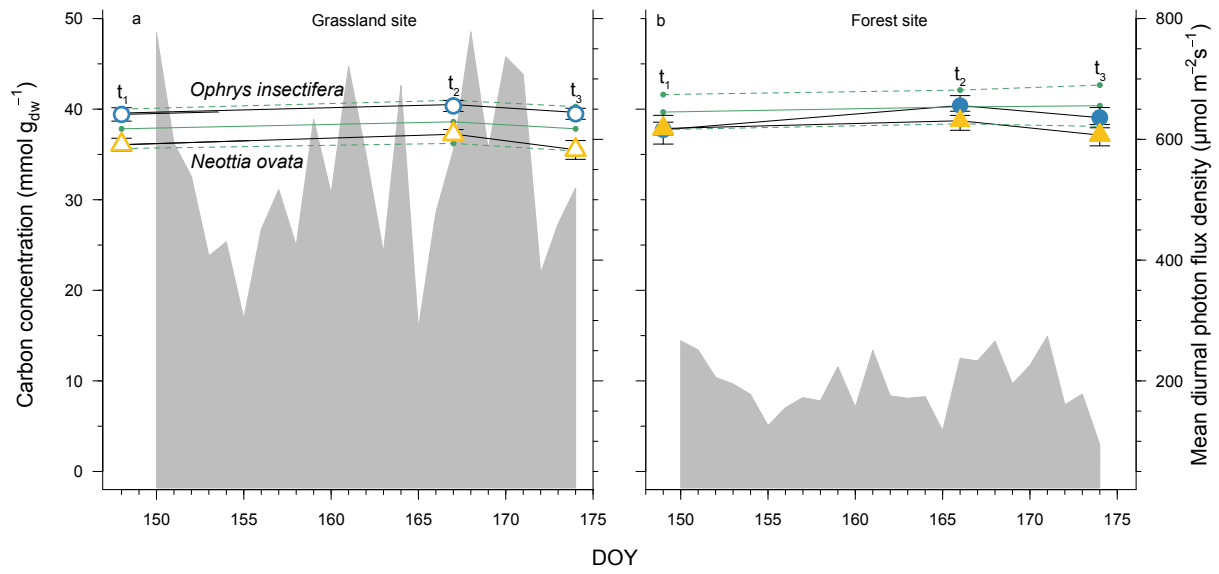
Suppl. Fig. 2 Enrichment factors ε^{18}O and ε^{13}C of *Neottia ovata* (yellow triangles) and *Ophrys insectifera* (blue circles) at the grassland site (unfilled symbols) and the forest site (filled symbols) ($n = 5$ per species and site); the green box represents mean enrichment factors ± SD for the autotrophic reference plants ($n = 42$) that were sampled together with the two orchid species whereas mean ε values of reference plants are zero by definition.

APPENDIX I

Book section **"Stable isotope natural abundance analysis as a tool for understanding orchid mycorrhizal nutrition"**

in the chapter **"Orchid Mycorrhizal Associations"**

by G. Gebauer & **J. M.-I. Schiebold (Schweiger)**

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Case Study 3.1

Stable isotope natural abundance analysis as a tool for understanding orchid mycorrhizal nutrition

by Gerhard Gebauer and Julienne M-I. Schiebold
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Most elements of biological interest are composed of two or more stable isotopes. Isotopes of an element have identical numbers of protons and electrons. However, they are distinguished by the number of neutrons in their nucleus and therefore have different atomic mass units. These differences in atomic properties of isotopes cause slight thermodynamic and kinetic isotope effects that change the isotopic composition of ecosystem compartments in predictable ways as elements cycle through the biosphere. A characteristic feature of these isotope effects is the enrichment of heavy isotopes along food chains (DeNiro and Epstein 1978), which cause fungi as heterotrophic organisms being enriched in heavy isotopes of nitrogen (Gebauer and Dietrich 1993), carbon (Gleixner et al. 1993), and hydrogen (Ziegler 1995), in comparison to accompanying autotrophic plants.

Due to their tiny, endospermless seeds, all orchids rely for germination and development in the early seedling (protocorm) phase on full nutritional support by suited fungi and are, like fungi, enriched in heavy isotopes. The degree of protocorm heavy isotope enrichment depends on the identity of their fungal hosts. Orchids germinating with fungi that simultaneously form ectomycorrhizas with forest trees are more enriched in the heavy carbon and nitrogen isotopes, ^{13}C and ^{15}N, than orchids germinating with fungi of the polyphyletic rhizoctonia group (Stöckel et al. 2014). Later on, the majority of orchids develop green leaves and become putatively autotrophic, yet remain in various states of mycorrhizal dependency. However, about 1% of all orchid species never develop green leaves and depend on fungal carbon and mineral nutrient support throughout their life cycle. These orchids, known as full mycoheterotrophs (Leake 1994), have been identified as heavily enriched in ^{13}C and ^{15}N in comparison to accompanying autotrophic reference plants (Gebauer and Meyer 2003; Trudell et al. 2003). Later research found that different patterns in ^{13}C and ^{15}N enrichment of fully mycoheterotrophic orchids depended upon the type of fungal hosts (Lee et al. 2015b; Ogura-Tsujita et al. 2009). These different patterns enable distinction between fully mycoheterotrophic orchids associated with

ectomycorrhizal fungi or saprotrophic wood- or litter-decaying fungi (Figure 3.1a). Examples of fully mycoheterotrophic orchids are *Epipogium aphyllum*, *Gastrodia fontinalis*, and *Galeola falconeri* (Figure 3.2a–c).
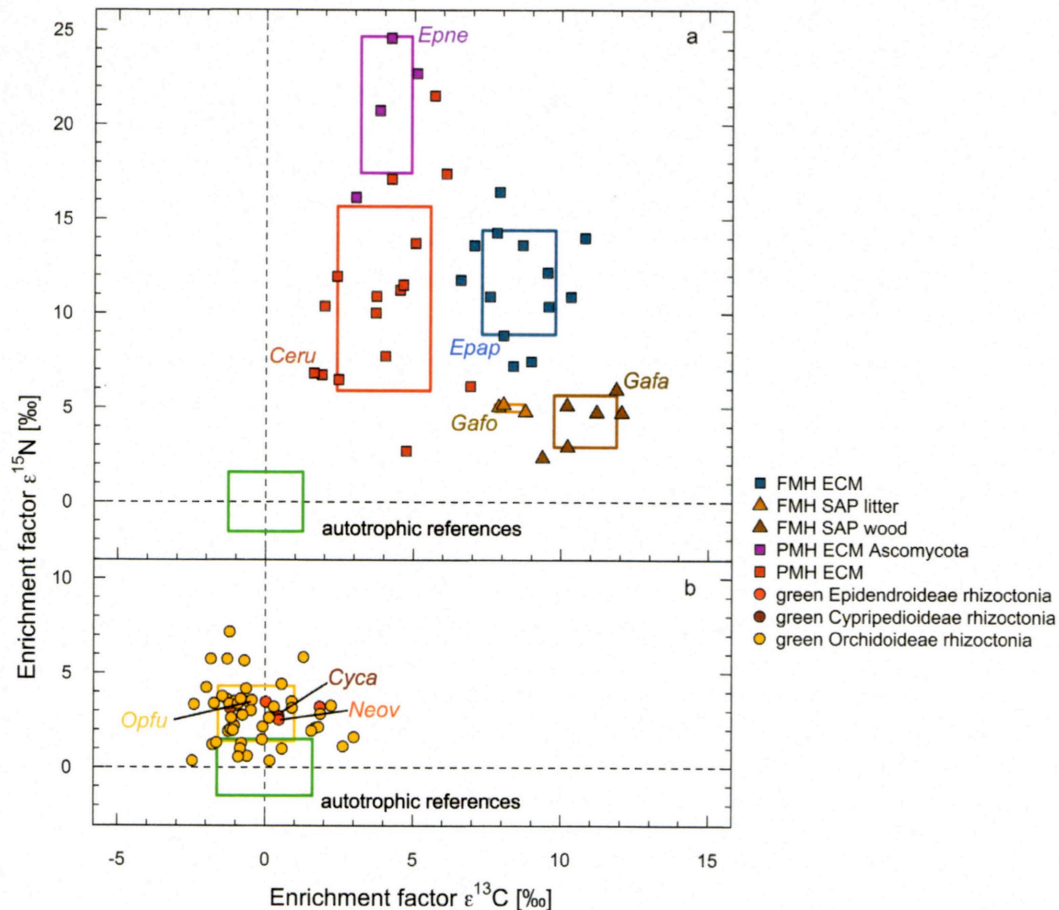
Figure 3.1   Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ of fully (FMH) and partially (PMH) mycoheterotrophic Orchidaceae species (for definition of the enrichment factor ε see Preiss and Gebauer (2008)). The boxes represent ± 1 SD of the mean ε values for the different groups of PMH, FMH, and green Orchidaceae species and autotrophic references. (a) FMH orchid species associated with ectomycorrhizal fungi (FMH ECM) ($n = 13$; 135 data points with $n = 3$-62 per species), FMH orchid species associated with saprotrophic litter-decomposing fungi (FMH SAP litter) ($n = 3$; 15 data points with $n = 5$ per species), FMH orchid species associated with saprotrophic wood-decomposing fungi (FMH SAP wood) ($n = 6$; 31 data points with $n = 1$-10 per species), PMH Orchidaceae associated with ectomycorrhizal Ascomycota (PMH ECM Ascomycota) ($n = 4$, 28 data points with $n = 5$-9 per species), PMH Orchidaceae associated with ectomycorrhizal fungi (PMH ECM) ($n = 16$, 285 data points with $n = 2$-72 per species) and autotrophic reference species ($n = 197$, 1459 data points). (b) Green-leaved Orchidaceae species from the subfamilies Epidendroideae ($n = 4$, 42 data points with $n = 5$-30 per species), Cypripedioideae ($n = 1$; *Cypripedium calceolus* with $n = 5$) and Orchidoideae ($n = 45$; 405 data points with $n = 4$-49 per species) and autotrophic reference species ($n = 162$, 1302 data points). One orchid species is illustrative for each of the eight groups; photographs are shown in Fig. 3.3. Abbreviations are as follows: *Epap = Epipogium aphyllum*, *Gafo = Gastrodia fontinalis*, *Gafa = Galeola falconeri*, *Epne = Epipactis neglecta*, *Ceru = Cephalanthera rubra*, *Neov = Neottia ovata*, *Cyca = Cypripedium calceolus*, *Opfu = Ophrys fuciflora*. Data extracted from Yagame et al. (2012), Hynson et al. (2013b), Roy et al. (2013), Johansson et al. (2015), Stöckel et al. (2014), Ercole et al. (2015), Lee et al. (2015a), Liebel et al. (2015), Gebauer et al. (2016), Hynson et al. (2016).

Figure 3.2   (a) *Epipogium aphyllum*, Norway spruce (*Picea abies*) forest, Nord-Trøndelag, Central Norway. (b) *Gastrodia fontinalis*, dense bamboo (*Phyllostachys edulis*) forest, Nantou County, Taiwan. (c) *Galeola falcaneri*, broadleaf forest dominated by *Phoebe formosana* and *Machilus japonica* (Lauraceae), Nantou County, Taiwan. (d) *Epipactis neglecta*, broad-leaf forest dominated by beech (*Fagus sylvatica*), NE Bavaria, Germany. (e) *Cephalanthera rubra*, mixed deciduous and coniferous forest dominated by Norway spruce (*Picea abies*), Vorarlberg, Austria. (f) *Neottia ovata*, coniferous forest dominated by Norway spruce (*Picea abies*), S Bavaria, Germany. (g) *Cypripedium calceolus*, broad-leaf forest dominated by beech (*Fagus sylvatica*), NE Bavaria, Germany. (h) *Ophrys fuciflora*, open grassland, N Italy (photos: Heiko Liebel (a) & (h), Yung-I Lee (b) & (c), Florian Fraaß (d), Gerhard Gebauer (e) and Julienne Schiebold (f) & (g)).

In adult green orchids, some species have been identified as being positioned between autotrophic reference plants and fully mycoheterotrophic plants in their carbon isotope composition (Gebauer and Meyer 2003) and associate with ectomycorrhizal fungi (Bidartondo et al. 2004). These orchids, known as partial mycoheterotrophs, gain carbon from two sources—photosynthesis and fungal hosts. Recent investigations elucidated a further separation of these partially mycoheterotrophic orchids into two groups based on their nitrogen isotope composition. Partially mycoheterotrophic orchids, such as *Epipactis neglecta* (Figure 3.2d), exclusively associate with ectomycorrhizal Ascomycota and are significantly more enriched in ^{15}N than partially mycoheterotrophic orchids associated with ectomycorrhizal Basidiomycota (Figure 3.1a), like *Cephalanthera rubra* (Figure 3.2e). Full and partial mycoheterotrophy allows orchid's thriving in the deepest shade of forest grounds or even below ground (like the Western Australian *Rhizanthella gardneri*).

In contrast, the isotopic composition of the large group of green orchids mycorrhizal with fungi of the polyphyletic rhizoctonia group remained puzzling. Irrespective of whether belonging to the orchid subfamilies Epidendroideae (like *Neottia ovata*, Figure 3.2f), Cypripedioideae

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(e.g., *Cypripedium calceolus*, Figure 3.2g), or Orchidoideae (e.g., *Ophrys fuciflora*, Figure 3.2h), these orchids as a group are not enriched in ^{13}C, but are significantly enriched in ^{15}N, in comparison to autotrophic reference plants (Figure 3.1b). Hynson et al. (2013a) speculated whether a kind of *cryptic partial mycoheterotrophy* might account for the isotope abundance pattern of rhizoctonia mycorrhizal orchids. In a recent investigation by Gebauer et al. (2016), adding hydrogen stable isotope abundance to the pattern of carbon and nitrogen isotope abundance confirms this assumption. This study found that seven green forest orchid species, irrespective of whether associated with ectomycorrhizal or rhizoctonia fungi and irrespective of whether
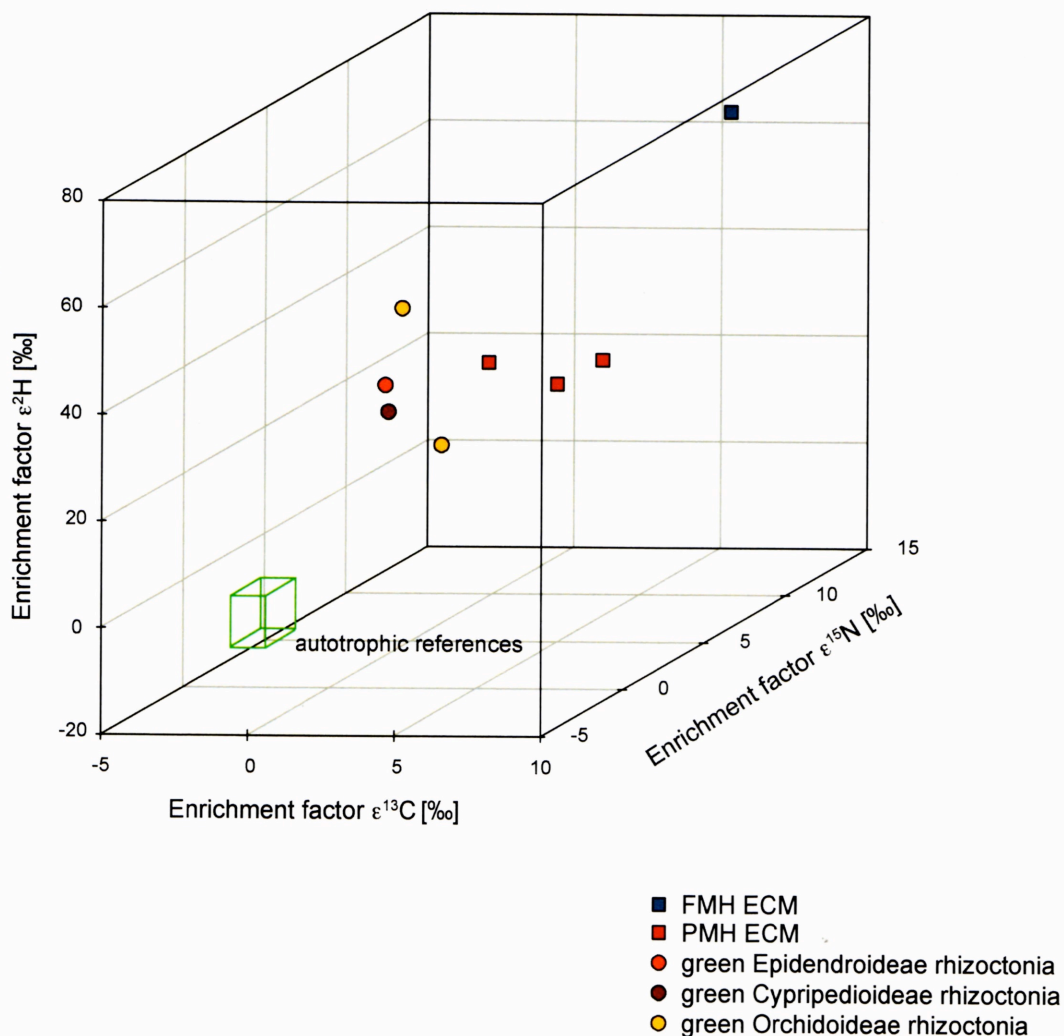
Figure 3.3   Mean enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, and $\varepsilon^{2}H$ of fully mycoheterotrophic Orchidaceae species associated with ectomycorrhizal fungi (FMH ECM) ($n = 1$; *Neottia nidus-avis* with $n = 5$), partially mycoheterotrophic Orchidaceae species associated with ectomycorrhizal fungi (PMH ECM) ($n = 3$; *Cephalanthera damasonium* and *C. rubra, Epipactis atrorubens*; 16 data points with $n = 5$-6 per species), green-leaved Orchidaceae species from the subfamilies Epidendroideae ($n = 1$; *Neottia ovata* with $n = 5$), Cypripedioideae ($n = 1$; *Cypripedium calceolus* with $n = 5$), and Orchidoideae ($n = 2$; *Ophrys insectifera* and *Platanthera bifolia* with $n = 5$ per species) and autotrophic reference species ($n = 10$, 146 data points) sampled in a broad-leaf forest dominated by beech (*Fagus sylvatica*), NE Bavaria, Germany. The box represents ± 1 SD of the mean ε values for the autotrophic references. Data extracted from Gebauer et al. (2016).

enriched in ^{13}C or not, were significantly enriched in the heavy hydrogen isotope ^{2}H and there-fore, were positioned between autotrophic references and a fully mycoheterotrophic orchid (Figure 3.3). This finding supports the conclusion that it is likely that many more orchids than previously assumed follow the partially mycoheterotrophic mode of nutrition.

APPENDIX II

The giant mycoheterotrophic orchid *Erythrorchis altissima* is associated mainly with a divergent set of wood-decaying fungi

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ORIGINAL ARTICLE

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The giant mycoheterotrophic orchid *Erythrorchis altissima* is associated mainly with a divergent set of wood-decaying fungi

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Abstract

The climbing orchid *Erythrorchis altissima* is the largest mycoheterotroph in the world. Although previous in vitro work suggests that *E. altissima* has a unique symbiosis with wood-decaying fungi, little is known about how this giant orchid meets its carbon and nutrient demands exclusively via mycorrhizal fungi. In this study, the mycorrhizal fungi of *E. altissima* were molecularly identified using root samples from 26 individuals. Furthermore, in vitro symbiotic germination with five fungi and stable isotope compositions in five *E. altissima* at one site were examined. In total, 37 fungal operational taxonomic units (OTUs) belonging to nine orders in Basidiomycota were identified from the orchid roots. Most of the fungal OTUs were wood-decaying fungi, but underground roots had ectomycorrhizal *Russula*. Two fungal isolates from mycorrhizal roots induced seed germination and subsequent seedling development in vitro. Measurement of carbon and nitrogen stable isotope abundances revealed that *E. altissima* is a full mycoheterotroph whose carbon originates mainly from wood-decaying fungi. All of the results show that *E. altissima* is associated with a wide range of wood- and soil-inhabiting fungi, the majority of which are wood-decaying taxa. This generalist association enables *E. altissima* to access a large carbon pool in woody debris and has been key to the evolution of such a large mycoheterotroph.

KEYWORDS
mycoheterotrophy, mycorrhiza, orchid, stable isotope, symbiotic germination, wood-decaying fungi

1 | INTRODUCTION

Mycorrhizas are an ancient, widespread association between fungi and land plants. They are based on a mutualistic symbiosis in which the fungus provides water and nutrients to the plant in return for fixed carbon from the plant (Smith & Read, 2008). Although these mutualistic associations are widespread among the majority of photosynthetic plants, mycoheterotrophic (MH) plants, which have evolved independently in 17 plant families (Merckx et al., 2013), have completely lost their photosynthetic ability and obtain all of their carbon through mycorrhizal associations (Leake, 1994). In most cases, MH plants rely on the two dominant mycorrhizal symbioses, the arbuscular mycorrhizal association and ectomycorrhizal (ECM) association, which allow MH plants to obtain carbon from surrounding autotrophic plants via shared mycorrhizal mycelia (Merckx, 2013). Whereas such tripartite systems provide access to the common mycorrhizal network of arbuscular mycorrhizal and ECM fungi linking the autotrophic plants (Bidartondo, 2005), associations with free-living litter- or wood-decaying (WD) fungi have been shown in several MH orchids. Early studies based on the isolation technique found this association in several MH orchids, such as *Gastrodia elata* (Kusano, 1911) and *Cyrtosia septentrionalis* (as *Galeola septentrionalis*; Hamada, 1939) associating with the plant pathogenic WD fungus *Armillaria*, *Gastrodia javanica* associating with the WD polypore *Xerotus javanicus*, and *Didymoplexis minor* associating with the litter-decaying fungus *Marasmius coniatus* (Burgeff, 1932). Recent molecular work has also confirmed the association of tropical or warm-temperate MH orchids with WD fungal linages, such as *Epipogium roseum* with Psathyrellaceae (Yamato, Yagame, Suzuki, & Iwase, 2005), *Eulophia zollingeri* with *Psathyrella candolleana* (Ogura-Tsujita & Yukawa, 2008), *Gastrodia similis* with *Resinicium* (Martos et al., 2009), and *Cyrtosia* and *Galeola* species with Meripilaceae (Lee, Yang, & Gebauer, 2015; Umata, Ota, Yamada, Watanabe, & Gale, 2013). Furthermore, litter-decaying Mycenaceae and Marasmiaceae have been found to associate with MH orchids, such as *Wullschlaegelia aphylla* (Martos et al., 2009) and *Gastrodia* species (Kinoshita et al., 2016; Lee et al., 2015; Ogura-Tsujita, Gebauer, Hashimoto, Umata, & Yukawa, 2009; see Selosse et al., 2010 for more detail). Decomposition of woody debris and leaf litter by saprotrophic fungi plays a key role in regulating the carbon (C) and nutrient cycles of all terrestrial ecosystems (Berg & McClaugherty, 2003). Woody debris is a major component of forest biomass, and this large C store represents up to 20% of the total aboveground biomass (Bradford et al., 2009; Laiho & Prescott, 1999). MH plants that are associated with saprotrophic fungi likely depend on the forest C cycle from plant debris, but understanding of mycorrhizal associations with litter- or wood-decaying fungi is still limited.

The giant mycoheterotroph *Erythrorchis altissima* (Blume) Blume (as *Galeola altissima* and *Erythrorchis ochobiensis*) is expected to have a unique symbiosis with WD fungi, which could act as a new model for understanding mycorrhizal diversity and specificity in MH plants. This species is the largest mycoheterotroph. It is a climbing, perennial hemi-epiphytic orchid species without foliage leaves, with both an aerial and subterranean root system, and with a distribution ranging from warm-temperate to tropical regions in East to South-East Asia (Comber, 1990; Figure 1). Its stems climb over dead wood or living trees and often reach a length of 10 m (Averyanov, 2011). Despite such remarkable characteristics of *E. altissima*, the fundamental basis of how it meets its C and nutrient demands exclusively via mycorrhizal fungi is unknown. Early research by Hamada and Nakamura (1963) and previous in vitro studies (Umata, 1995, 1997a, b, 1998a,b, 1999; Umata, Fujimoto, & Arai, 2000; see more details in Table S1) have shown that 19 basidiomycete species, most of them WD fungi that were never previously shown to be mycorrhizal fungi, had mycorrhizal association with *E. altissima*. These studies indicate that *E. altissima* is a mycorrhizal generalist, targeting a wide phylogenetic range of WD basidiomycetes, which has not been demonstrated for any other plant.

An association with ECM fungi has also been suggested, as shown by successful germination with the ECM fungus *Lyophyllum shimeji* (Umata, 1997b). In fact, both saprotrophic *Gymnopus* and the ECM fungus *Russula* have been identified from underground roots in *Erythrorchis cassythoides* (Dearnaley, 2006), which is the sister species of *E. altissima* and is also a climbing mycoheterotrophic orchid in Australia (Jones, 2006). Based on these studies, *E. altissima* is assumed to lack fungal specificity, targeting a range of wood-inhabiting fungi in addition to ECM fungal associations, which indicates a mixed C gain from WD and ECM fungi. Stable isotope natural abundance can be used to assess a plant's nutritional mode and is particularly useful in MH plants that fully depend on fungal-derived C and nitrogen (N) as they are heavily enriched in ^{13}C and ^{15}N (Gebauer & Meyer, 2003). This approach has been applied to a number of MH species associated with ECM fungi (Abadie et al., 2006; Bidartondo, Burghardt, Gebauer, Bruns, & Read, 2004; Liebel et al., 2010), arbuscular mycorrhizal fungi (Bolin, Tennakoon, Majid, & Cameron, 2017;
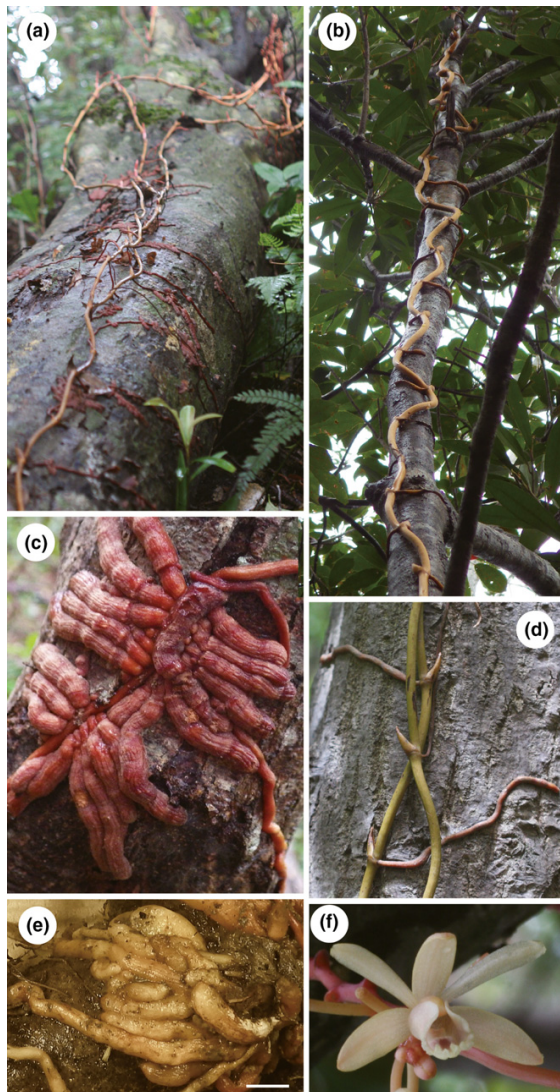
FIGURE 1 Stem, root and flower morphology of *Erythrorchis altissima*. Stems climbing on fallen dead wood (a) or on standing living trees (b). A thick and densely branched root clump (c) and thin and elongated roots (d). (e) Underground root clump (bar = 1 cm). (f) Flower of *E. altissima* [Colour figure can be viewed at wileyonlinelibrary.com]

Merckx, Stöckel, Fleischmann, Bruns, & Gebauer, 2010) and also saprotrophic fungi (Lee et al., 2015; Martos et al., 2009; Ogura-Tsujita et al., 2009). The difference in isotopic signatures between WD and ECM fungi can distinguish which fungal group covers the majority of the C and N demand of *E. altissima* (Hobbie, Sánchez, & Rygie-wicz, 2012; Kohzu et al., 1999).

This study is the first to investigate the mycoheterotrophy of *E. altissima* comprehensively by combining molecular, in vitro culture and mass-spectrometric approaches. To reveal its mycorrhizal fungal diversity and specificity, we first analysed 26 individuals from six sites using molecular identification. Second, to confirm the

mycorrhizal potential of identified fungi, we isolated five mycorrhizal fungal strains from root tissues and used them for coculture with seeds in conjunction with a decay test to compare the wood-decay ability of these isolates. Third, natural stable isotope abundances of C and N were analysed to confirm the mycoheterotrophy and reveal the pathways for nutrient acquisition in *E. altissima*.

2 | MATERIALS AND METHODS

2.1 | Field sites and sample collection

Plant and fungal materials were collected from six sites of warm-temperate (S1–S3) or subtropical (S4–S6) regions in Japan from 2013 to 2016 (Table 1, Figure S1). The habitats of *E. altissima* were shaded to semi-open places in evergreen broadleaf forests dominated by *Castanopsis sieboldii*. Most of the individuals found in this study were hemi-epiphytes with stems climbing on fallen or standing dead trunks and living trees from underground (Figure 1a,b); however, a few individuals were creeping on the ground without host trees. The average length of aboveground stems among 29 individuals was 3.9 m, ranging from 1.5 to 7.0 m at site S6. The most common host tree species was *C. sieboldii* at all sites, but *Distylium racemosum*, *Elaeocarpus japonicus*, *Elaeocarpus zollingeri*, *Myrsine seguinii*, *Syzygium buxifolium* and *Cinnamomum daphnoides* were also found (Tables 2 and 3). The level of decay of host trees was surveyed according to Fukasawa, Osono, and Takeda (2009) and assigned to five classes: (i) wood, hard; (ii) wood, somewhat hard, a knife penetrates <1 cm into the wood; (iii) wood, distinctly softened, a knife penetrates ~1–4 cm into the wood, bark partly lost; (iv) wood, strongly decayed, a knife penetrates ~5–10 cm into the wood, bark lost in most places; and (v) wood, very decayed, a knife penetrates more than 10 cm into the wood, original log circumference not recognizable or hardly recognizable.

Root morphology was categorized into two groups: thick and densely branched root clumps (Figure 1c,e) and thin and elongate roots (Figure 1d). Both types appeared in aerial (Figure 1c,d) and underground (Figure 1e) plant stems. Mycorrhizal colonization was confirmed with a light microscope using free-hand sections of all collected roots. Our preliminary observation showed that mycorrhizal fungi mainly colonized densely branched roots (Figure 2) while elongated roots were scarcely colonized. Thus, the former roots were used mainly for the following microscopy observations and molecular identification.

As mycorrhizal association with WD fungi has been suggested by previous studies (Hamada & Nakamura, 1963; Umata, 1995, 1997a,b, 1998a,b, 1999; Umata et al., 2000), sporocarps of WD fungi were also collected from host trees of *E. altissima* and identified at the species level by morphology or molecular identification. Voucher specimens of *E. altissima* and sporocarps were deposited in the Herbarium of the National Museum of Nature and Science, Tokyo (TNS8501221, 8505147, 8505854–8505857 for *E. altissima*, and TNS-F-80541, 80542 for *Trichaptum* cf. *durum*) and in the Tottori University Mycological Herbarium (TUMH62765 for *Coniophorafomes matsuzawae*).

TABLE 1 Samples of *Erythrorchis altissima* used for fungal identification. Location, sampling year, number of individuals and roots, and voucher number at each sampling site are listed

Site	Location	Sampling year	No. of individuals	No. of roots	Voucher
Warm-temperate area					
S1	Tanegashima Is., Kagoshima, Japan	2013, 2014, 2015	9	91	TNS8505855
S2	Tanegashima Is., Kagoshima, Japan	2005	1	5	TNS8505147
S3	Kuchinoerabu Is., Kagoshima, Japan	2013	1	2	–
Subtropical area					
S4	Kunigami, Okinawa, Japan	2007	1	1	TNS8501221
S5	Kunigami, Okinawa, Japan	2013	2	10	–
S6	Okinawa city, Okinawa, Japan	2015, 2016	12	41	TNS8505854

Is., Island.

2.2 | Microscopy observation

For assessment of mycorrhizal colonization in root tissues, collected mycorrhizal roots were fixed in 50% ethanol/formaldehyde/acetic acid, 90:5:5 for microscopy observation. Root pieces were dehydrated in a graded ethanol series, embedded in paraffin, cut transversely into 10-μm-thick sections and stained with safranin-O/fast green. The sections were dehydrated through an alcohol-xylene series, mounted with Bioleit (Oken Shoji, Tokyo, Japan), and fungal colonization was observed under a light microscope.

2.3 | Molecular identification of mycorrhizal fungi

In total, 150 roots from 26 individuals were collected from six sites for molecular identification of mycorrhizal fungi (Table 1). One to 14 root pieces were collected from each individual, and when the individuals had several root clumps on the host tree, root tips were collected from each clump because our preliminary observation showed that if there are several independent rooting zones, each root clump establishes mycorrhizas separately. To check the annual change in mycorrhizal associations, the roots were collected each year from the same individual (individuals Ea3 and Ea4) for 3 years (Table 2). Collected roots were washed in water and sectioned with a razor blade, and fungal colonization was confirmed with a light microscope. To avoid detection of surface-inhabiting nonmycorrhizal fungi, the root epidermis was removed from mycorrhizal root tissues and the colonized cortex layer was excised under a stereomicroscope. For sporocarps, a piece of tissue was excised from collected sporocarps and used for molecular identification. The excised mycorrhizal roots and sporocarps were washed in sterilized water and stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at −20°C before use.

DNA was extracted from the samples of mycorrhizal roots and sporocarps using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. PCR and sequencing were performed as described by Ogura-Tsujita and Yukawa (2008). The fungal internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was amplified with ITS1F/ITS4 or ITS1F/ITS4B primer combinations (Gardes & Bruns, 1993; White, Bruns, Lee, & Taylor, 1990). To avoid overlooking Tulasnellaceae, a typical orchid symbiont, due to primer mismatch, all root samples were also amplified using the ITS1/ITS4-Tul primer combination (Taylor & McCormick, 2008). The partial large subunit (LSU) nrDNA sequences were additionally amplified using LR0R/LR5 primers (Moncalvo, Lutzoni, Rehner, Johnson, & Vilgalys, 2000) when the ITS sequence had low resolution in a homology search of the GenBank database. Additional internal primers, ITS2 and ITS3 (White et al., 1990) for the ITS region and LR3 (Vilgalys & Hester, 1990) and LR3R (Hopple & Vilgalys, 1999) for the LSU region, were used for sequencing. The PCR products were purified using a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and sequenced using a BIGDYE TERMINATOR v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products that were difficult to sequence directly were cloned using a pGEM-T Vector System II (Promega, Madison, WI, USA). Five colonies were sequenced in each cloned sample. Obtained sequences were grouped into operational taxonomic units (OTUs) at 99% similarity, and taxonomic affiliations for each fungal OTU were assigned based on the closest match to sequences available in GENBANK using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences determined in this study were deposited in the DDBJ/EMBL/GENBANK databases. The accession nos. are listed in Tables S2 and S3.

2.4 | Symbiotic germination

To test whether the mycorrhizal fungi identified in this study induce symbiotic germination of *E. altissima*, mycorrhizal fungi were isolated from roots collected at site S1 in 2013 by the single peloton isolation method (Rasmussen, 1995). Colonized cortex layers of mycorrhizal roots were excised under a stereomicroscope, rinsed three times with sterile water and cut open under sterile water to release the fungal pelotons. Sterile water mixed with pelotons was dropped onto 2% malt extract agar (MA) plates and incubated at 25°C in the dark. After 3 days, fungal hyphae growth from coiled pelotons was checked under a light microscope and hyphal tips were transferred to fresh MA plates for subculture and purification. DNA was

TABLE 2 Occurrence of fungal OTUs in each individual of *Erythrorchis altissima* at warm-temperate sites

Putative taxonomic identity	Individual ID →	Y159	Ea3 2013	Ea3 2014	Ea3 2015	Y161	Y162	D113	Ea4 2013	Ea4 2014	Ea4 2015	Ea4D	Ea6	Ea10	Y05-10	C396
Site		S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S2	S3
Year collected			2013	2014	2015				2013	2014	2015					
Host tree (stage)		Cs (F)	Dr (F)	Dr (F)	Dr (F)	Dr (L)	None	Cs (L)	Dr (F)	Dr (F)	Dr (F)	None	Ej (S)	Cs (S)	Ez (S)	Cs (L)
Decay class		N	3	3	N	1	–	N	2–3	3	3–4	–	3	N	N	1~3
Sebacinales	*Serendipitaceae* sp.1														1	
Trechisporales	*Sistotremastrum* sp.1														2	
	Hyphodontia sp.1												2			
	Trichaptum cf. *durum*		11	2	2				11	2	3					
Polyporales	*Ceriporia* sp.1	2					8					4				
	Ischnoderma sp.1														1	
	Phanerochaete sp.2							2								
	Phlebia sp.1															2
	Phlebia sp.2				2								2			
	Phlebia sp.3													3		
	Phlebia sp.4										1					
Corticiales	*Vuilleminia* sp.1		2													
Russulales	*Russula* sp.1					5										
	Russula sp.2	2														
	Scytinostroma sp.1													3		
	Coniophorafomes matsuzawae							2								
Agaricales	*Gymnopus* sp.1					4			2	1						
	Hypholoma sp.1													8		
	Mycena sp.1							2								
Atheliales	*Athelia* sp.1														1	
	Not detected					1							2			

Numbers in brackets indicate the number of root samples in which the respective fungus was detected. The root samples collected from underground are shown in bold. Host tree species (Cs = *Castanopsis sieboldii*, Dr = *Distylium racemosum*, Ej = *Elaeocarpus japonicus*, Ez = *Elaeocarpus zollingeri*) and the stage of the trees (F = fallen dead trunk, S = standing dead trunk, L = living tree) are shown. The stems of Y162 and Ea4D were creeping on the ground without the host tree. The root samples of Ea3 and Ea4 were collected annually between 2013 and 2015. The level of decay of host trees was categorized into five classes as described by Fukasawa et al. (2009). N means that no data were available. The root samples from which we failed to obtain PCR products are shown as "Not detected".

TABLE 3 Occurrence of fungal OTUs in each individual of *Erythrorchis altissima* at subtropical sites

Putative taxonomic identity	Individual ID	Y07-18	K58-1	K58-2	Ea12	Ea21	Ea23	Ea24	Ea28	Ea29	Ea30	Ea31	Ea35	Ea37	Ea45	Ea63
Site		S4	S5							S6						
	Host tree (stage)	N	Cs (S)	Cs (S)	Cs (S)	Ms (S)	Sb (S)	Cs (L)	Cs (F)	None	Cs (L)	Cs (S)	Cd (F)	Cs (L)	N	Cs (F)
	Decay class	N	N	N	1	N	N	N	N	–	N	N	3	N	–	N
Cantharellales	Tulasnella sp.1		2													
	Ceratobasidiaceae sp.1												2	2		
Trechisporales	Trechispora sp.1	1														
	Trechisporales sp.1		4													
	Trechisporales sp.2								1							
Hymenochaetales	Fuscoporia sp.1			4												
	Hymenochaetaceae sp.1					2										
Polyporales	Phanerochaete sp.1		2													
	Phanerochaete sp.3									2					1	
	Phanerochaetaceae sp.1															2
	Phlebia sp.2				4								5		3	
	Phlebia sp.5						2									
	Phlebiopsis sp.1							2								
	Stereum sp.1											1				
	Microporus sp.1										1			1		
	Hyphoderma sp.1										1					
Russulales	Asterostroma sp.1									3						
Agaricales	Neonothopanus sp.1														1	
Not detected								1	1		1	2	1		1	

Numbers in brackets indicate the number of root samples in which the respective fungus was detected. Host tree species (Cs = *Castanopsis sieboldii*, Ms = *Myrsine seguinii*, Sb = *Syzygium buxifolium*, Cd = *Cinnamomum daphnoides*) and the stage of the trees (F = fallen dead trunk, S = standing dead trunk, L = living tree) are shown. The stems of Ea29 were creeping on the ground without the host tree. The level of decay of host trees was categorized into five classes as described by Fukasawa et al. (2009). N means that no data were available. The root samples from which we failed to obtain PCR products are shown as "Not detected." Two root samples from K58-1 generated two fugal OTUs from each sample.
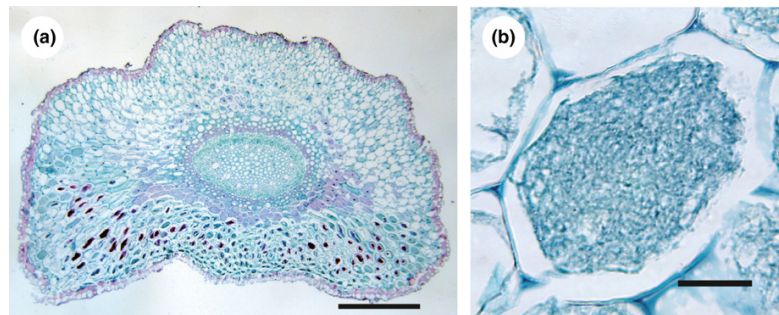
FIGURE 2 Histology of the mycorrhizal root of *E. altissima*. (a) Cross section of the entire mycorrhizal root, bar = 1 mm. (b) Enlarged figure of cells colonized by mycorrhizal fungi, bar = 0.05 mm [Colour figure can be viewed at wileyonlinelibrary.com]

Isolate	Putative taxonomic identity	NBRC No.	Weight loss of sawdust (%)	No. of individuals Stage 1	No. of individuals Stage 2
T-13	*Trichaptum* cf. *durum*	110364	41.3 ± 2.0	2.7 ± 3.2a	0.3 ± 0.7a
T-22	*Gymnopus* sp.1	110366	18.1 ± 3.6	0	0
T-31	*Hyphodontia* sp.1	110368	43.5 ± 1.5	0	0
T-36	*Vuilleminia* sp.1	110369	24.6 ± 4.5	16.8 ± 12.3b	6.7 ± 7.9b
T-40	*Ceriporia* sp.1	110370	4.1 ± 0.5	0	0

TABLE 4 Results of coculture of *Erythrorchis altissima* seeds with fungal isolates. Information about fungal isolates used for culture, percentage weight loss of sawdust exposed for each fungal isolate and the number of individuals germinated by the coculture are shown. Fungal isolate numbers, putative taxonomic identity and NBRC numbers are listed. All isolates were extracted from *E. altissima* roots collected from site S1 in 2013

Values are shown as means ± SD. Different letters indicate significant differences between the inoculated fungal isolates in each stage ($p < .05$, Wilcoxon rank-sum test). The ITS sequences of all isolates completely matched those directly amplified from root samples listed in Table 2. All isolates were deposited to the NITE Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation of Japan. Seed germination was recorded 2 months after sowing and assigned to two germination stages: stage 1 involved rupture of the testa by the enlarged embryo and included protocorms less than 3 mm in diameter; stage 2 included nonrooted protocorms above 3 mm in diameter or rooted protocorms.

extracted from fungal isolates as described by Izumitsu et al. (2012), and fungal OTUs were molecularly identified. In total, five fungal isolates that shared 100% ITS sequence homology with the mycorrhizal fungi directly sequenced from colonized roots were used for the coculture of seeds (Table 4). These isolates were deposited in NITE Biological Resource Center (NBRC110364–110370; Table 4).

A mature fruit of *E. altissima* was collected from site S1 in October 2013. Seeds were stored at 5°C with silica gel until use. Coculture of seeds and fungi was performed as described by Umata (1997a). Sawdust medium containing 80 ml of *Fagus crenata* sawdust and 40 ml of culture solution (water, 1% glucose, 1% yeast powder) was prepared in a 200-ml conical flask and autoclaved twice at 98°C for 2 hr followed by 210°C for 1 hr. The seeds were sterilized with a 10% calcium hypochlorite solution as described by Umata (1997a), and ~100 seeds were sprinkled in a sterilized bamboo stick. Each seed stick was incubated for 2 weeks on potato dextrose agar medium to check for contamination of the seeds, and contaminated sticks were removed. Four seed sticks were transferred to sawdust medium, and four flasks were prepared for each fungal isolate. A 3×3-mm^2 block of fungal culture was inoculated on the surface of the sawdust medium and cultured for 2 months at 25°C in the dark. The experiment was repeated three times with four flasks per

replicate, and in total, 12 flasks were prepared for each isolate. Seed germination was recorded 2 months after sowing and assigned to two germination stages: stage 1 involved rupture of the testa by the enlarged embryo and included protocorms <3 mm in diameter; stage 2 included nonrooted protocorms above 3 mm in diameter or rooted protocorms (Figure 3a). For further development under symbiotic condition, obtained seedlings by culturing with the two isolates (T-13 and T-36) that induced seed germination were transferred to fresh sawdust medium (Figure 3b). As the fungal isolates were colonized in seedling roots, the isolates were also transferred to the medium together with the seedlings. Mycorrhizal roots were collected from a plantlet, and colonizing fungus was molecularly identified to confirm whether the root-colonizing fungus in a plantlet was consistent with the original isolates.

2.5 | Decay test

It seems likely that a WD fungus with strong decay ability may supply carbon stably to the orchid and *E. altissima* could prefer such fungus. To evaluate how the fungal decay ability affects orchid seed germination, five isolates used for coculture were employed for comparison of wood-decay ability based on sawdust weight loss.

FIGURE 3 Seedlings and plantlet formation of *E. altissima* by symbiotic germination with fungal isolates. (a) Stages in development of seedlings. Stage 1: protocorms with 1–3 mm diameter. Stage 2: protocorms >3 mm or with root development, bar = 1 cm. (b) Plantlet after 240 days of culture with fungal isolate *Trichaptum* cf. *durum* (T-13) [Colour figure can be viewed at wileyonlinelibrary.com]

Approximately 1 g of oven-dried sawdust from *C. sieboldii*, which is a common *E. altissima* host tree, was packed in a mesh bag and weighed prior to fungal inoculation. The bags were autoclaved at 121°C for 20 min and transferred to plates containing 20 ml of 2% agar medium. A 4-mm plug of fungal culture was inoculated on the agar plates and incubated at 25°C in the dark. After 5 months of culture, the bags were oven-dried at 70°C for 1 week and weighed. The weight lost from the sawdust was determined as a percentage of the initial mass. Three replicates were prepared in each isolate, and three noninoculated plates served as a control.

2.6 | Isotopic analysis

Plant and fungal samples for stable isotope natural abundance analysis were collected at site S1 in July 2015. Flower stalk (peduncle and rachis), flower, mycorrhizal and/or nonmycorrhizal root(s) were sampled from five individuals of *E. altissima* (individual IDs Ea3, Ea4, Ea10, D113 and D114; Figure 4, Table S4) which were all flowering individuals in this site. The individuals labelled Ea3 and Ea4 grew on fallen dead trunks of *D. racemosum*, while the other three individuals grew on standing dead trunks or living trees of *C. sieboldii* whose heartwood and main branches were partially decayed. Mycorrhizal roots for molecular identification were collected from these individuals (Table 2) except for one individual (D114) that had no root clump aboveground. Collection of underground roots from any of the five individuals would have required major disturbances and was avoided for conservation reasons. Current-year leaves and stems of autotrophic reference plants, *C. sieboldii*, *D. racemosum*, *Psychotria serpens*, *Damnacanthus indicus* and *M. seguinii*, were collected within 1 m of each orchid individual (Table S4). Dead stem-wood material, which was expected to be the main substrate for WD fungi, was sampled from each host tree. In total, five sporocarps, *T.* cf. *durum* from host trees of Ea3 and Ea4, a WD fungus *Microporus* sp. from neighbouring *C. sieboldii* and ECM *Amanita* and *Ramaria* species within 10 m of *E. altissima* individuals, were also collected. All sporocarps were identified by morphology or molecular identification and deposited as dried herbarium specimens (TNS-F-80541–80544, 80568). Samples were dried at 105°C, ground to a fine powder and stored in a desiccator with silica gel until use.

The relative N and C isotope abundances of the samples were measured using the dual-element analysis mode of an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo et al. (2004). Relative isotope abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1,000[‰]$, where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope in the samples and the respective standard. Standard gases (nitrogen and carbon dioxide) were calibrated with respect to international standards using the reference substances N1 and N2 for N isotopes and ANU sucrose and NBS 19 for C isotopes, provided by the International Atomic Energy Agency (Vienna, Austria).

δ values were normalized following the procedure of Preiss and Gebauer (2008) for our comparisons of plant C and N isotope abundances with reference data. Enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) were calculated using δ values for *E. altissima*, the reference plants, and sporocarps as follows: $\varepsilon_{Sx} = \delta_{Sx} - \delta_{REFx}$, where *S* is a single $\delta^{13}C$ or $\delta^{15}N$ value for each sample, *x* is a sampling plot within a certain study site, and δ_{REF} is the mean value of all reference plants. Differences between $\varepsilon^{13}C$ and $\varepsilon^{15}N$ values of *E. altissima* and each reference plant, and between the stem and leaf of each reference plant, were determined using a Mann–Whitney *U* test. A Kruskal–Wallis nonparametric test was used for differences among flower stalks, flowers and roots of *E. altissima*.

Nonmetric multidimensional scaling (NMDS) was used to detect meaningful underlying dimensions and to graphically visualize similarities and dissimilarities between the samples of *E. altissima* and WD fungi as well as decayed wood samples collected from *D. racerosum* and *C. sieboldii* in two-dimensional space. For this, the Bray–Curtis index was used to calculate a distance matrix from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data using the function "metaMDS" with two dimensions and 100 permutations in the R package "VEGAN" (Oksanen et al., 2017). The stress value was calculated to evaluate how well the configuration provided a representation of the distance matrix; generally, a stress value of <0.05 provides an excellent representation in reduced dimensions. Fitted vectors were calculated to display the $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentrations in the ordination space and to indicate the differences between the groups in association with these
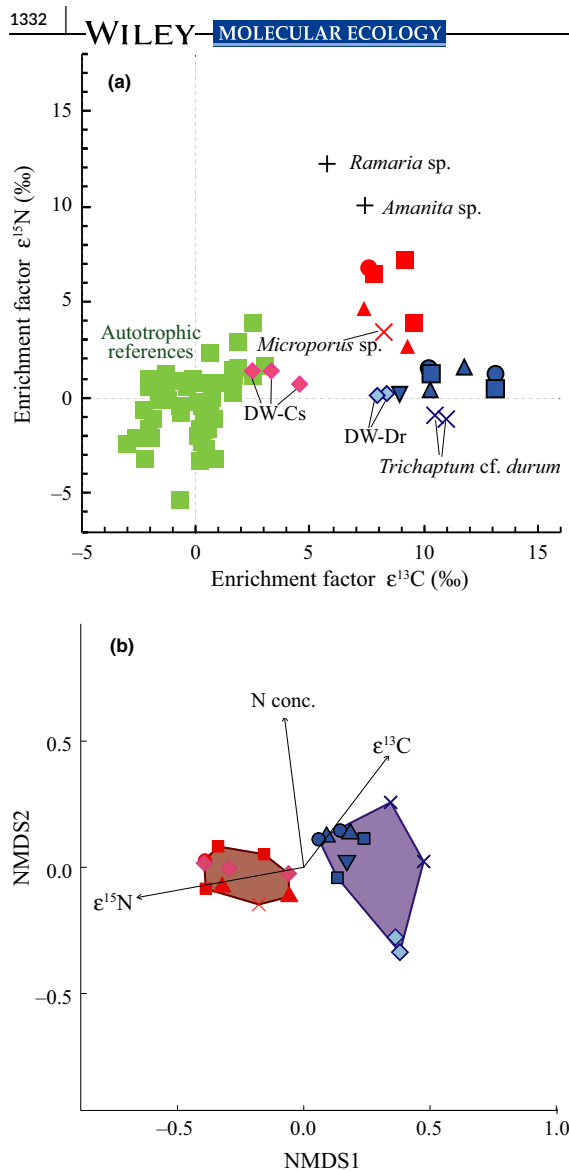
FIGURE 4 (a) Enrichment factors ε^{13}C and ε^{15}N as calculated for five individuals of *E. altissima* (flower stalk: square, flower: circle, nonmycorrhizal root: triangle, mycorrhizal root: inverted triangle), sporocarps of wood-decay fungi (cross) and ectomycorrhizal fungi (plus), decayed wood of *Distylium racemosum* (DW-Dr) and *Castanopsis sieboldii* (DW-Cs) (diamond) and stems of photosynthetic reference plants (Ref, $n = 25$, green square) collected from site S1. *Erythrorchis altissima*, sporocarps and decayed wood collected from *D. racemosum* and *C. sieboldii* are shown in blue with black margin and red, respectively. Decayed wood samples were collected from host trees of each *E. altissima* individual. (b) Nonmetric multidimensional scaling (NMDS) plot based on the Bray–Curtis dissimilarity matrix calculated from enrichment factors ε^{13}C and ε^{15}N and N concentration data for samples collected from *D. racemosum* (blue-coloured) and *C. sieboldii* (red-coloured) ($n = 21$). Fitted vectors display the response variables ε^{13}C, ε^{15}N and N concentration in the ordination space and indicate the differences between the groups in association with these variables. Stress = 0.02, 100 permutations; MANOVA R^2 = .343, p = .001 [Colour figure can be viewed at wileyonlinelibrary.com]

variables. Each arrow shows the direction of the increasing response variable while its length is proportional to the correlation (R^2) between the variable and the ordination (Oksanen et al., 2017). The function "adonis" in the R package "VEGAN" was used to perform a permutational multivariate analysis of variance (MANOVA) to test for significance of differences between group means using the afore-mentioned calculated distance matrix (Anderson, 2001).

3 | RESULTS

3.1 | Molecular identification of mycorrhizal fungi

In total, 150 root samples taken from 26 *E. altissima* individuals from six sites were examined using molecular identification, and fungal sequences were successfully obtained from 141 root samples (Table 1). Basidiomycete sequences were grouped into 37 fungal OTUs based on 99% ITS sequence identity, belonging to nine fungal orders (Table S2). The sequences from two fungal OTUs, *Trichaptum* cf. *durum* and *Coniophorafomes matsuzawae*, completely matched those from adjacent sporocarps. Most of the fungal OTUs were WD basidiomycetes, and ECM fungus Russulaceae and orchid mycorrhizal Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae were additionally identified from the roots (Table S2). Ascomycete lineages, such as *Ilyonectria* and *Trichosporon*, which are hyphal endophytes, were also detected at low frequency (Table S3).

No common fungal OTU was found among the six sites, except that *Phlebia* sp.2 was detected at both warm-temperate site S1 and subtropical site S6 (Tables 2 and 3). The detected fungal OTUs differed for each individual in most cases, although an identical fungal OTU was detected from different individuals within site S1 (*T.* cf. *durum*, *Ceriporia* sp.1, *Phlebia* sp.2 and *Gymnopus* sp.1) and site S6 (Ceratobasidiaceae sp.1, *Phanerochaete* sp.3, *Phlebia* sp.2 and *Microporus* sp.1). *Erythrorchis altissima* was present at various tree stages, but no correlation was found between the tree stage and the fungal species detected. The WD basidiomycete *T.* cf. *durum* dominated *E. altissima* roots on fallen dead wood of *D. racemosum* and was the most common through all years of the study period. *Erythrorchis altissima* frequently appeared on the tree trunk at decay class 3. The fungi detected from underground roots belonged to diverse fungal lineages including both WD and ECM basidiomycetes. Simultaneous association with both fungal groups within a single individual was found in two individuals: Y159 and Y161 (Table 2). The underground roots without aboveground host trees were associated with WD fungus *Ceriporia* sp.1 (Y162 and Ea4D; Table 2). This fungal OTU was detected in both aboveground and underground roots (Table 2).

3.2 | Symbiotic germination and decay test

Five fungal isolates with ITS sequences that were identical to the mycorrhizal fungi directly sequenced from colonized roots were successfully obtained from four individuals at site S1 (Table 4). Two isolates, *T.* cf. *durum* and *Vuilleminia* sp.1, induced seed germination

(Figure 3a), and the number of germinated individuals that inoculated *Vuilleminia* sp.1 was significantly higher than *T.* cf. *durum* (Table 4). The seedlings developed into plantlets with these isolates after being transplanted into fresh medium (Figure 3b). The wood-decay ability of the five isolates was compared using the sawdust weight loss. The average weight losses ranged from 4.1% to 43.5%, with the highest weight losses in *Hyphodontia* sp.1 (43.5%) and *T.* cf. *durum* (41.3%), and the lowest in *Ceriporia* sp.1 (4.1%).

3.3 | Stable isotope abundances

Among five individuals analysed from site S1, Ea3 and Ea4 grew on fallen dead trunks of *D. racemosum*, whereas the other three individuals (Ea10, EaD113 and EaD114) grew on standing dead trunks or living trees of *C. sieboldii*. The former two individuals were associated mainly with the wood-decaying *T.* cf. *durum*, and the latter were mycorrhizal with several WD fungi, such as *Hypholoma*, *Phlebia* and *Phanerochaete* (Table 2). No significant differences in δ^{13}C or δ^{15}N were found among orchid flower stalks, flowers and roots (Kruskal–Wallis test, $p = .77$ for δ^{13}C and .81 for δ^{15}N), or between leaves and stems of each reference plant species (Mann–Whitney U test, $p < .05$), except for δ^{15}N values of *D. racemosum* (Table S5). The enrichment factor (ε) based on the stems of reference plants (Figure 4) showed a similar pattern to the ε for the leaves (Figure S2). Thus, the ε^{13}C and ε^{15}N values based on the stems are shown as the main data because the stem is the organ equivalent to the flower stalk and was the only material collected from all five *E. altissima* individuals (Table S4).

The δ^{13}C values of *E. altissima* were significantly higher compared to those of all reference plant species (Mann–Whitney U test, $p < .01$; Table S5). Based on the enrichment factors, all individuals of *E. altissima* were highly enriched in ^{13}C compared to the reference plants, but varied extremely in ^{15}N, ranging from 0.38‰ to 7.12‰ in ε^{15}N values (Figure 4a). The individuals growing on *D. racemosum* did not differ from reference plants in ^{15}N (ε^{15}N: 0.38‰–1.60‰), whereas those growing on *C. sieboldii* were highly enriched (ε^{15}N: 2.69‰–7.12‰). Furthermore, the enrichment of ^{13}C and ^{15}N in the two former individuals was the closest to those of *T.* cf. *durum* that dominated the mycorrhizal roots of these individuals, while the latter was close to a WD *Microporus* collected from *C. sieboldii* although the individuals EaD113 and EaD114 (ε^{15}N: 4.70‰–7.12‰) were more enriched in ^{15}N than Ea10 (ε^{15}N: 2.69‰–3.89‰). The ^{13}C and ^{15}N enrichments for dead-wood material were also quite different between the two tree species of *D. racemosum* and *C. sieboldii* (Figure 4a).

Ordination of a Bray–Curtis dissimilarity matrix calculated from ε^{13}C, ε^{15}N and N concentration data of *E. altissima* and WD fungi as well as decayed wood samples collected from *C. sieboldii* and *D. racemosum* ($n = 21$) with NMDS elucidated a significant segregation of the two groups in the ordination space (Figure 4b), and a MANOVA showed that the group had a significant effect on the ordination ($R^2 = .343$, $p = .001$). Fitted vectors in the ordination of *E. altissima* collected from *C. sieboldii* and *D. racemosum* were

maximally correlated with ε^{15}N ($R^2 = .683$, $p < .001$), N concentration ($R^2 = .550$, $p < .001$) and ε^{13}C ($R^2 = .470$, $p = .006$). Generally, the stress value of the ordination (stress = 0.02) provided an excellent representation in reduced dimensions (Figure 4b). Thus, the different C and N isotope compositions and N concentrations of the two host tree species *C. sieboldii* and *D. racemosum* turned out as drivers for the C and N isotope compositions and N concentrations not only of the wood-decay fungi living on these two tree species, but also for the C and N isotope compositions and N concentrations of the mycoheterotrophic *E. altissima* individuals collected from the two tree species.

4 | DISCUSSION

4.1 | Mycorrhizal associations

This study provides clear evidence that *E. altissima* is associated with a wide phylogenetic range of fungi inhabiting wood and soil. The fungi detected in this study belong to nine fungal orders, which include different functional guilds, mainly including WD fungi but also ECM and typical orchid mycorrhizal fungi (Table S2), although the fungi occurring at low frequency will need further confirmation. Most of the WD fungi detected from *E. altissima* roots were first found to be mycorrhizal fungi on plant roots in this study, with the exception of the leaf litter or WD fungi *Gymnopus* and *Mycena*, which are associated with several MH orchids, such as *Gastrodia* species (Kinoshita et al., 2016; Martos et al., 2009; Xu & Guo, 2000) and *E. cassythoides* (Dearnaley, 2006). The ECM genus *Russula* was found on underground roots of *E. altissima*, as shown in *E. cassythoides* (Dearnaley, 2006). *Russula* is a common mycorrhizal partner in MH plants, such as temperate orchids, *Corallorhiza* (Taylor & Bruns, 1997, 1999), *Limodorum* (Girlanda et al., 2006) and monotropoid species of Ericaceae (Bidartondo & Bruns, 2001). The *Russula* sequences from *E. altissima* roots share high sequence similarity with those from ECM root tips (Table S2), indicating that some *E. altissima* individuals partially obtain C from ECM fungi. The typical orchid mycorrhizal fungi, such as Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae, were also found in *E. altissima* roots. The ITS sequence of Serendipitaceae sp.1 from roots on decayed wood shared 96% homology with that from *E. cassythoides*, indicating that this fungal group works as a mycorrhizal fungus in *Erythrorchis*. A series of previous studies demonstrated that 19 fungal species induced seed germination by coculture in vitro (Table S1), but we could not detect these fungi from *E. altissima* roots, except for *Microporus* sp.1, which shared 99% sequence homology with *Microporus affinis* and was found in two individuals (Table 3). These results suggest that more fungal species could be associated with *E. altissima* than those found in this study. Ascomycete fungi were also detected from *E. altissima* roots (Table S3), but most of them are common root endophytes or plant root pathogens (Chaverri, Salgado, Hirooka, Rossman, & Samuels, 2011); thus, these fungi are probably nonmycorrhizal on *E. altissima* roots.

This study also provides clear evidence of a WD-associated mycoheterotroph that lacks mycorrhizal specificity. Previous studies showed that WD-associated MH orchids have mycorrhizal specificity towards single fungal orders, genera or even species groups (Ogura-Tsujita & Yukawa, 2008; Yamato et al., 2005), whereas multiple fungal orders including saprotrophic and ECM fungi were detected in *E. cassythoides* (Dearnaley, 2006) and *Gastrodia nipponica* (Kinoshita et al., 2016). A lack of fungal specificity has been shown in some MH plants, such as the ericaceous mycoheterotroph *Pyrola aphylla*, which is associated with a broad range of ECM fungi (Hynson & Bruns, 2009), and species of the MH orchid *Aphyllorchis* with multiple ECM families (Roy et al., 2009). While the generalist association of *P. aphylla* may be an ancestral trait because a partially mycoheterotrophic *Pyrola* is also a generalist (Hynson and Bruns, 2009; Tedersoo, Pellet, Koljalg, & Selosse, 2007), it is notable that the lack of fungal specificity in *E. altissima* has probably evolved from a photosynthetic orchid with a specialized mycorrhizal association. One of the photosynthetic relatives of *E. altissima* within Vanilloideae is the climbing orchid genus *Vanilla* (Cameron, 2009), which is associated mainly with a particular fungal lineage of Ceratobasidiaceae and Tulasnellaceae (Porras-Alfaro & Bayman, 2007).

The few common fungal OTUs among the six sites indicate that the differences in fungal OTUs associated with *E. altissima* may reflect differences in the local community of WD fungi, which are attributed to climate, vegetation and other environmental factors, although randomness of fungal occurrence and contingency should also be considered. Host tree species and their decay class may also affect which fungal OTU associates with *E. altissima*. *Erythrorchis altissima* on fallen decayed wood of *D. racemosum* was frequently associated with *T.* cf. *durum* in this study (Table 2). Wood in decay class 3 was the most common among the dead host trees of *E. altissima* (Tables 2 and 3). In early to mid-stages, WD fungal flora, especially corticoids and polypores, are very species rich (Renvall, 1995; Stockland et al., 2012) and WD basidiomycetes are metabolically active in decayed wood (Rajala, Peltoniemi, Hantula, Mäkipää, & Pennanen, 2011), which may provide the opportunity for *E. altissima* to find fungal partners.

Underground roots were associated with ECM *Russula*, similar to *E. cassythoides* (Dearnaley, 2006), in addition to WD fungal groups (Table 2). The simultaneous association with both fungal groups within a single individual (Y159 and Y161; Table 2) showed mixed C gain from decayed woods and neighbouring ECM-associated autotrophs. Such double association was also found in *Gastrodia nipponica*, which was associated mainly with litter-decomposing Mycenaceae and Marasmiaceae with additional association with Russulaceae (Kinoshita et al., 2016). The WD fungus *Ceriporia* sp.1 was found from the underground roots of the individuals without a host tree (Y162 and Ea4D; Table 2), suggesting that *E. altissima* can survive without an aboveground host tree by utilizing underground woody debris as a nutrient.

Annual root sampling from particular individuals revealed that two individuals (Ea3 and Ea4) retained the dominant association with the same fungal OTU, *T.* cf. *durum*, for 3 years, although other fungal OTUs were partially associated (Table 2). Mycorrhizal roots

collected from four to five root clumps within 1.5 m were exclusively associated with *T.* cf. *durum* in both individuals, and sporocarps of *T.* cf. *durum* were abundant on host logs throughout the study period. These results indicate that this fungal OTU was probably a dominant WD species within these host trunks and continuously supplied nutrients to *E. altissima* for at least 3 years.

4.2 | Symbiotic germination

Among the five isolates, *T.* cf. *durum* and *Vuilleminia* sp.1 induced seed germination and subsequent plantlet formation (Table 4), showing that these two fungal groups that were isolated from adult plants are efficient for seed germination in vitro as well as mycorrhizal association in adulthood. Assessment of decay ability showed that the fungal isolates that were efficient for seed germination do not require a high-decay ability. As the most effective at seed germination, *Vuilleminia* sp.1 showed low weight loss in vitro (24.6%), while *Hyphodontia* sp.1, which did not induce germination, had the highest weight loss (43.5%). No seed germination was observed in three fungal isolates, even though *Ceriporia* sp.1 was one of the most frequent fungal OTUs at site S1. It is possible that fungal specificity is higher in the germination stage than in adulthood, but deviation from optimal culture conditions for some fungal isolates could be one of the possibilities for noninduction of seed germination.

4.3 | Stable isotope abundance

Erythrorchis altissima had C isotope signatures typical of a fully mycoheterotrophic orchid. The $\epsilon^{13}C$ values of *E. altissima* ranged from 7.39‰ to 13.27‰ with an average of 9.97‰, which is similar to the two MH orchids, *Cyrtosia javanica* and *Galeola falconeri*, both of which are closely related to *E. altissima* (Cameron, 2009) and are also associated with WD Polyporales (11.20 ± 0.68‰ and 11.87 ± 0.56‰, respectively; Lee et al., 2015) and ECM-associated orchids reviewed by Hynson, Schiebold, and Gebauer (2016) including 13 MH orchid species (from 6.58 ± 0.24‰ to 10.78 ± 0.62‰). In addition to ^{13}C enrichment, *E. altissima* was highly variable in its ^{15}N enrichment, ranging from 0.38‰ to 7.12‰ in the $\epsilon^{15}N$ values, which is likely due to the difference in host tree species and/or mycorrhizal fungi (Figure 4). An ordination of a Bray–Curtis dissimilarity matrix calculated from $\epsilon^{13}C$, $\epsilon^{15}N$ and N concentration data supports the conclusion that the host tree species may affect ^{13}C and ^{15}N enrichment of *E. altissima*, WD fungi and decayed wood, and might be responsible for the significantly segregated groups.

Although different functional guilds of fungi were associated with *E. altissima*, the comparison of ^{13}C and ^{15}N enrichments with fungal sporocarps showed that *E. altissima* gains C mainly from WD fungi of its host tree. ^{13}C and ^{15}N enrichments of two individuals on *D. racemosum* were similar to the WD fungus *T.* cf. *durum*, which was the main fungal partner of these individuals (Figure 4, Table 2). The enrichments of other individuals on *C. sieboldii* were close to the WD fungus *Microporus* that was collected from *C. sieboldii*. The individuals, EaD114 and EaD113, were more enriched in ^{15}N and

seemed to have intermediate values between *Microporus* and ECM *Amanita*. Because ECM-associated mycoheterotrophs are highly enriched in ^{15}N due to high ^{15}N enrichment in associated fungal tissues (Hynson et al., 2016), it seems likely that the high ^{15}N enrichment of these individuals was due to simultaneous association with ECM and WD fungi, but more replicates are required to evaluate the mixed C gain of *E. altissima*.

5 | CONCLUSION

This study is the first to demonstrate that the largest mycoheterotroph, *E. altissima*, is associated with a wide range of wood- and soil-inhabiting fungi, the majority of which are WD taxa. Additional associations with ECM and orchid mycorrhizal fungi imply a lack of fungal specificity in *E. altissima*, and this study provides clear evidence of a mycorrhizal generalist that targets diverse lineages of WD fungi. Although most of the WD fungi detected in this study have never been found from plant roots as mycorrhizal fungi previously, the successful symbiotic germination in vitro confirms their mycorrhizal ability in this orchid. The measurement of C and N stable isotope natural abundances showed that *E. altissima* is a full mycoheterotroph whose C originates mainly from WD fungi rather than ECM fungi. Woody debris is a large store of C in forest biomass, and WD fungi play a crucial role in the C cycling involved in such woody resources (Stockland, Siitonen, & Jonsson, 2012). By associating with a diverse range of WD fungi, *E. altissima* can access this large C pool, which has probably been important for the evolution of such a large mycoheterotrophic plant.

DATA ACCESSIBILITY

DNA sequences—GenBank Accession nos. LC327023–LC327047, LC322331–LC322337.

AUTHOR CONTRIBUTIONS

Y.O.-T. designed the research. Y.O.-T., H.X., M.K., M.M. and S.I. contributed to molecular experiments. K.T., M.K., T.Y., Y.O.-T. and Y.F.

conducted field work and sample collection. G.G. and J.M.-I.S. performed isotopic analysis and analysed the data. H.U. performed in vitro works. Y.F. and H.X. conducted decay test. N.M. and S.Y. contributed to fungal identification. Y.O.-T., G.G., J.M.-I.S. and T.Y. wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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