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Research

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

Franziska E. Zahn[1], Erik Söll[1], Thomas K. Chapin[2], Deyi Wang[3], Sofia I. F. Gomes[4], Nicole A. Hynson[2], Johanna Pausch[5] and Gerhard Gebauer[1]

[1]BayCEER – Laboratory of Isotope Biogeochemistry, University of Bayreuth, Bayreuth 95440, Germany; [2]Pacific Biosciences Research Center, University of Hawai'i at Mānoa, Honolulu, HI 96822, USA; [3]Naturalis Biodiversity Center, Leiden 2332 AA, the Netherlands; [4]Institute of Biology, Leiden University, Leiden 2333 BE, the Netherlands; [5]Department of Agroecology, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, Bayreuth 95440, Germany

Author for correspondence:
Gerhard Gebauer
Email: gerhard.gebauer@uni-bayreuth.de

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Summary

- Stable isotope signatures of fungal sporocarps have been instrumental in identifying carbon gains of chlorophyllous orchids from a fungal source. Yet, not all mycorrhizal fungi produce macroscopic sporocarps and frequently fungi of different taxa occur in parallel in orchid roots.
- To overcome this obstacle, we investigated stable isotope signatures of fungal pelotons extracted from orchid roots and compared these data to the respective orchid and reference plant tissues. *Anoectochilus sandvicensis* and *Epipactis palustris* represented specialized or unspecialized rhizoctonia-associated orchids. *Epipactis atrorubens* and *Epipactis leptochila* are orchids considered ectomycorrhiza-associated with different preferences for Basidio- and Ascomycota.
- ^{13}C enrichment of rhizoctonia pelotons was minor compared with plant tissues and significantly lower than enrichments of pelotons from ectomycorrhizal *Epipactis* species. ^{15}N values of pelotons from *E. leptochila* and *E. atrorubens* showed similar patterns as known for respective sporocarps of ectomycorrhizal Ascomycota and Basidiomycota, however, with an offset towards lower ^{15}N enrichments and nitrogen concentrations.
- Our results suggest an explicit fungal nutrition source of orchids associated with ectomycorrhizal fungi, whereas the low ^{13}C enrichment in rhizoctonia-associated orchids and fungal pelotons hamper the detection of carbon gains from fungal partners. ^{15}N isotopic pattern of orchids further suggests a selective transfer of ^{15}N-enriched protein-nitrogen into orchids.

Introduction

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

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

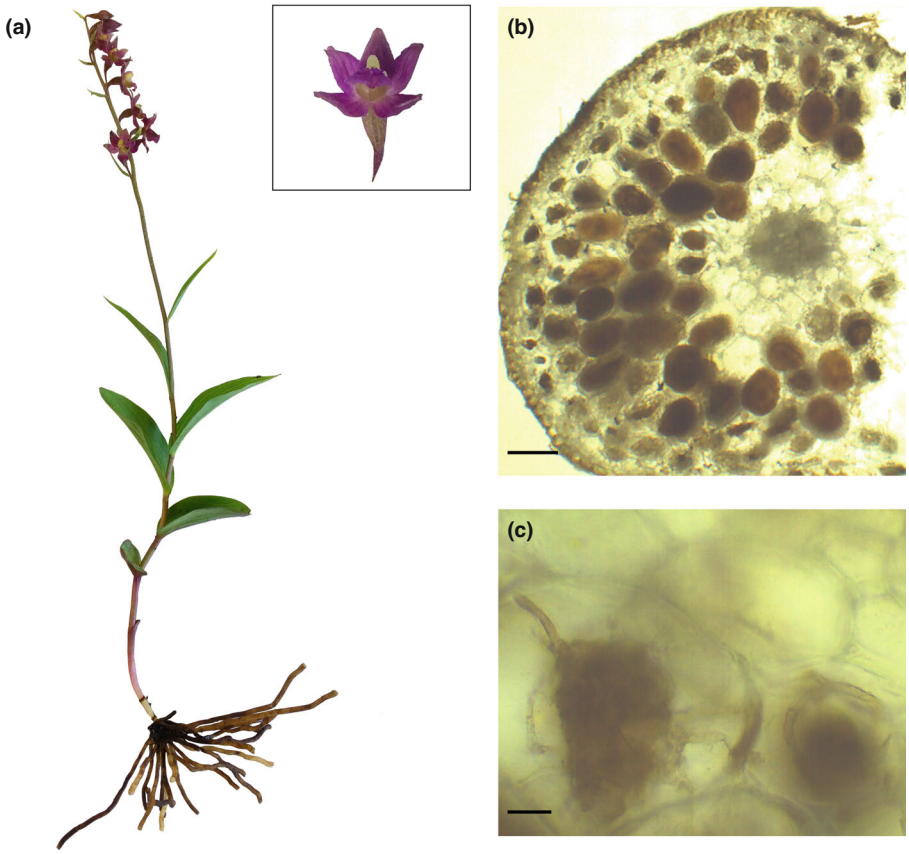

Fig. 1 (a) *Epipactis atrorubens* with magnified flower. (b) Transverse section of an *E. atrorubens* root colonized with intracellular hyphal coils (pelotons). Bar, 100 μm. (c) Fungal pelotons in cortex cells. Bar, 30 μm.

et al. (2023) focused on arbuscular mycorrhizal fungi and orchid mycorrhizal rhizoctonia fungi and thus on gaining stable isotope data of mycorrhizal fungi which do not produce fruit bodies and can only be extracted from plant roots. In this study, we centred specifically upon orchid mycorrhiza. In addition to orchids associated with rhizoctonia fungi, we investigated orchid species that are part of tripartite ectomycorrhizal (ECM) networks with Ascomycota and/or Basidiomycota. In the case of ectomycorrhizal fungi, stable isotope data of respective fungi fruit bodies exist and can be used for comparison with fungal pelotons when originating from the same sampling site (Taylor *et al.*, 2003; Gebauer *et al.*, 2016; Schiebold *et al.*, 2017).

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

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

(**1**) *Partial mycoheterotrophy of green orchids.* ^{13}C enrichment of green orchid leaves relative to reference plant leaves is an indicator for partial mycoheterotrophy, at least for orchids associating with ECM-type fungi. However, this remains challenging for rhizoctonia-associated orchids, because leaves of green orchids with rhizoctonia fungal partners are usually not enriched in ^{13}C relative to autotrophic reference plants (Hynson *et al.*, 2013). Yet, leaves of rhizoctonia-associated orchids tend to be slightly

enriched in ^{15}N, have higher total N concentrations (Bidartondo *et al.*, 2004; Hynson *et al.*, 2013; Stöckel *et al.*, 2014), and a hydrogen isotope (^{2}H) approach provides evidence for a hidden C transfer from fungi to orchids (Gebauer *et al.*, 2016; Schiebold *et al.*, 2018). In this approach, a significant ^{2}H enrichment of orchid leaves serves as an indicator for a fungus-to-plant organic matter (i.e. carbon) transfer based on the finding that secondary heterotrophic organic compounds, that is originating from fungi, are enriched in ^{2}H compared with primary photosynthetic compounds (Yakir, 1992). Interestingly, seedlings of rhizoctonia-associated orchids (so-called protocorms) were found to be enriched in ^{13}C and ^{15}N, but their low ^{13}C enrichment made them hardly distinguishable from their respective adult stages and neighbouring autotrophic plants (Stöckel *et al.*, 2014). Altogether, there is reason to presume that ^{13}C enrichment of rhizoctonia fungi is relatively small resulting in no suspicious enrichment of their orchid associates' leaves (Selosse & Martos, 2014). We, thus, expect rhizoctonia pelotons isolated from *A. sandvicensis* and *E. palustris* to be less ^{13}C enriched than pelotons from orchids in tripartite ECM networks with forest trees (*E. leptochila* and *E. atrorubens*) or even not enriched in ^{13}C at all. (**2**) *Fungal associates and their function*. When utilizing organic matter as N source, fungi become enriched with the heavy isotope ^{15}N in relation to their bulk substrate (Gebauer & Taylor, 1999). Certainly, ^{15}N values of fruit bodies show distinct patterns depending on their fungal guild; for example, *Tuber* species (ECM Ascomycota) display higher $\delta^{15}N$ values than ECM Basidiomycota (Schiebold *et al.*, 2017). Commonly different fungi species have characteristic isotopic signature depending on their ecology and habitat (cf. Selosse & Martos, 2014). Based on the fungal guild a plant associates with, studies have shown predictable patterns in plant isotope enrichment (Hobbie & Colpaert, 2003; Hobbie & Högberg, 2012; Hynson *et al.*, 2013; Schiebold *et al.*, 2017). We thus expect isotope compositions of orchid leaves to reflect the isotopic signature of respective pelotons, and thus to provide predictable patterns of their functional, nutrition-specific fungal associates. For orchids having fungal hosts additional to or other than rhizoctonia (presumably *E. leptochila* and *E. atrorubens*), we predict pelotons to mirror the ^{15}N enrichment of fruit bodies of the respective fungal guilds they associate with.

Materials and Methods

Study species and sampling locations

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

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

Isolation of pelotons and sample preparation

Pelotons were extracted from roots using mechanical approaches. For *Epipactis* spp., 1 cm root segments with and without pelotons were separated based on optical differentiation, which was validated by microscopic observation, and cleaned by sonication (5 min, 35 kHz) in deionized water. Roots without pelotons and a subsample of the colonized root samples with pelotons were kept for isotopic analysis, thus dried at 105°C and stored in a desiccator. The isolation method for colonized root segments of *Epipactis* is based on Klink *et al.* (2020) with adjustments for orchid roots: Colonized root cells were opened by longitudinal sections using a razor blade, followed by another sonication (15 min) in deionized water to release the pelotons from the sliced roots. The solution containing the extracted pelotons was transferred to another centrifuge tube using a pipette. This step was repeated several times by consistently adding deionized water to the root fragments until the solution appeared no longer turbid. Purification steps comprised a combination of stacked 250, 200, 125, 90 and 63 µm sieves (Retsch test sieve, stainless steel, DIN/ISO 3310-1; Retsch, Haan, Germany) to separate pelotons from remaining plant residues. The sieve surfaces were rinsed with deionized water several times, thus remaining contaminates were sifted out and maximal purity of pelotons was achieved depending on mesh size. We observed the largest pelotons and the highest colonization in the roots of *E. leptochila*, followed by *E. atrorubens* and *E. palustris*. Most pelotons were retained by the 90 µm sieve for *E. atrorubens*, and by the 63 µm sieve for *E. palustris* and *E. leptochila*, which were then pipetted into Eppendorf tubes and centrifuged (10 s at 1306 ***g***, Eppendorf Centrifuge 5415 C; Eppendorf AG, Hamburg, Germany). The supernatant liquid was removed because it did not comprise any pelotons and the peloton pellet was dried at 105°C for 72 h and placed in tin (sample weight range of 50–2000 µg) or annealed silver (sample weight range of 1000–2000 µg) capsules. Note that all cleaning steps were monitored by microscopic observation. Pelotons within *A. sandvicensis* roots were extracted from roots

(*c.* 2 cm in length) by maceration in sterile deionized water and pipetting, to generate a concentrated peloton solution, which was directly pipetted into tin capsules and lyophilized (Labconco FreeZone 230V; Labconco, Kansas City, MO, USA) to generate 10 samples of varying weights within a range of 100–500 μg. For both peloton isolation methods applied here, we assume a similar level of purification and thus no large effect of different extraction methods on isotope signatures. All peloton samples were stored in a desiccator until isotope analysis (Table S3).

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

Stable isotope analyses

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

Isotope compositions ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$) of orchid and reference plant leaves were compared fitting linear mixed models 'lmer' (package LME4, v.1.1.29; Bates *et al.*, 2015). Due to our plot-wise sampling scheme and the data normalization per plot, the model included the 'plot' as a random effect. 95% confidence intervals (CIs) and *P*-values were computed using a Wald *t*-distribution approximation. Analogously, we fitted linear mixed models to compare isotopic compositions of sample types, particularly orchid leaves and pelotons, across orchid species. For *post hoc* pairwise comparison between species, we used the 'emmeans' and 'contrast' functions (package EMMEANS, v.1.7.5; Russell *et al.*, 2022) with Bonferroni–Holm *P*-value adjustment. To test for differences in total N concentration of orchid compartments, pelotons and reference plants, we conducted nonparametric Kruskal–Wallis *H*-tests and *post hoc* Dunn's test with Bonferroni–Holm correction for multiple comparisons (Dinno, 2017). We used a significance level of $\alpha = 0.05$ for the statistical tests. Statistical analyses were conducted in R v.4.2.0 (R Core Team, 2022).

Fungal sequencing

Root fungi of *A. sandvicensis* at our sampling site have already been identified by Swift *et al.* (2019) and confirmed by fungal ITS Sanger sequencing a subset of individual root fragments to be *Ceratobasidium* sp. 1 (*sensu* Swift *et al.*, 2019). For the three *Epipactis* species (*E. leptochila*, *E. atrorubens* and *E. palustris*), five individuals of each species were used for DNA extraction and fungal metabarcoding, which was done as follows. Root pieces were surface sterilized using 1% sodium hypochlorite and rinsed in sterile distilled water. For each sample, five root sections with 1 mm thickness were randomly cut and chosen for DNA extraction using a CTAB method (Doyle & Doyle, 1987). The fungal forward primer fITS7 (GTGARTCATCGAATCTTTG; Ihrmark *et al.*, 2012) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC; White *et al.*, 1990) were used to amplify the nuclear ITS2 region. This primer pair has been shown to amplify the substantial diversity of orchid mycorrhizal fungi and other soil fungal communities (Waud *et al.*, 2014; Li *et al.*, 2021). It is important to note that the primer pair used in this study may not capture all fungal taxa reside in orchid root communities because of the potential primer bias. Particularly for the family Tulasnellaceae, its abundance might be underestimated due to the high variability of the ITS2 region of Tulasnellaceae (Waud *et al.*, 2014; Oja *et al.*, 2015; Vogt-Schilb *et al.*, 2020; Li *et al.*, 2021). Polymerase chain reaction (PCR) reactions were duplicated for each DNA template as PCR replicates were found to reduce PCR stochasticity and increase the detected fungal diversity (Alberdi *et al.*, 2018). PCR replicates were pooled before being tagged. After checking the amplification products by electrophoresis gels, amplicons of the PCR replicates in the first PCR were pooled together, cleaned with magnetic beads and attached with identical Nextera indexes in the second PCR. After quality assessment using Fragment Analyzer 5100 (Agilent Technologies, Santa Clara, CA, USA), amplicons of 15 *Epipactis* samples, together with another 385 samples, were pooled in equimolarity per sample. The end pool was processed to generate pair-end reads of 300 bp using the MiSeq platform within one run (BaseClear, Leiden Bio Science Park, the Netherlands).

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

Results

^{13}C stable isotope signatures

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

Within-site comparison showed that leaf ^{13}C signature differed between sample types (reference vs orchid) only for *E. leptochila* and *E. atrorubens*. Leaf ^{13}C compositions of *E. palustris* and *A. sandvicensis* were not statistically distinct from respective reference plants (Table 2). Compared with other plant compartments (stem and blossom, roots), the orchid leaves tended to be least enriched or even depleted in ^{13}C (*A. sandvicensis*) relative to reference plants (Fig. 2).

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

^{15}N stable isotope signatures

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

We found that leaves of all investigated orchids were significantly enriched in ^{15}N relative to their respective reference plants (Table 2). Enrichment of orchid leaves in ^{15}N was distinct from

Table 1 Pairwise comparisons of orchid species effect (*Epipactis leptochila*, *Epipactis atrorubens*, *Epipactis palustris* and *Anoectochilus sandvicensis*) on $\varepsilon^{13}C$ and e $\varepsilon^{15}N$ of leaves and pelotons, respectively, obtained from linear mixed models' *post hoc* tests comparing differences in means.

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

P-values were adjusted using Bonferroni–Holm correction. Underlying models were: lmer ($\varepsilon^{13}C_{Leaves}$ ~ orchid species + (~1|plot)): $R^2 = 0.78$; lmer ($\varepsilon^{15}N_{Leaves}$ ~ orchid species + (~1|plot)): $R^2 = 0.97$; lmer ($\varepsilon^{13}C_{Pelotons}$ ~ orchid species + (~1|plot)): $R^2 = 0.80$; lmer ($\varepsilon^{15}N_{Pelotons}$ ~ orchid species + (~1|plot)): $R^2 = 0.90$. Significances are highlighted in bold.

Table 2 Summaries of linear mixed models (estimated using REML and NLOPTWRAP OPTIMIZER, R function: lmer) predicting $\varepsilon^{13}C$ and $\varepsilon^{15}N$, respectively, with type (orchid vs reference) for the orchid species *Epipactis leptochila*, *Epipactis atrorubens*, *Epipactis palustris* and *Anoechtochilus sandvicensis*.

E. leptochila

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

E. atrorubens

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

E. palustris

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

A. sandvicensis

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

For fixed effects type (Orchid) is the baseline. All models include 'plot' as random effect. Model diagnostic was applied. 95% confidence intervals (CIs) and *P*-values were computed using a Wald *t*-distribution approximation. Significances are highlighted in bold.

each other when comparing all four species pairwise (Table 1), with one exception (*A. sandvicensis* vs *E. palustris*).

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

Total N concentrations

Mean total N concentrations of reference plant leaves were similar across the *Epipactis* sampling sites: 1.35 ± 0.25 mmol g_{DW}^{-1}

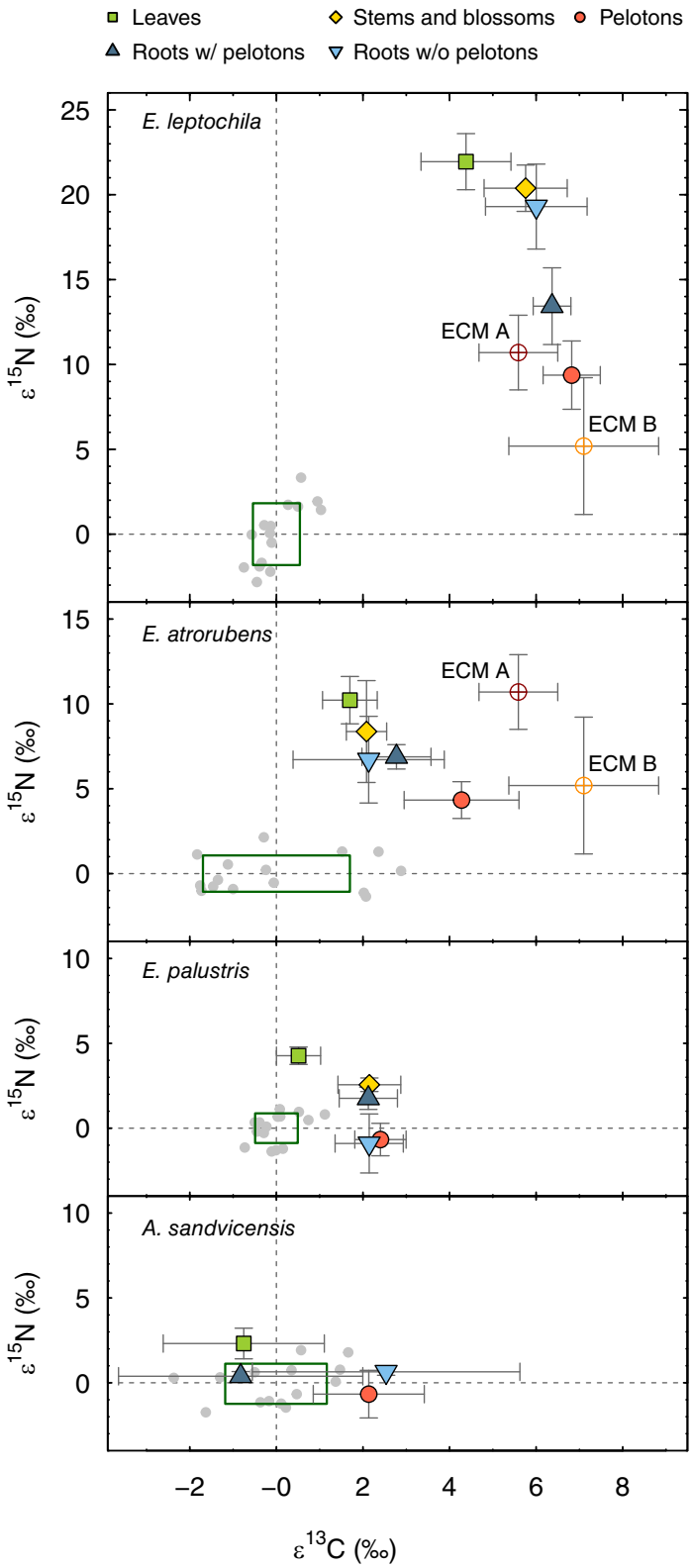

Fig. 2 Carbon and nitrogen stable isotope enrichment factors ε (mean ± SD) of orchid parts ('leaves', 'stems and blossoms', 'roots with pelotons' and 'roots without pelotons') and isolated pelotons for *Epipactis leptochila*, *Epipactis atrorubens*, *Epipactis palustris* and *Anoectochilus sandvicensis*.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

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

Mean total N concentrations of pelotons extracted from *E. leptochila* (2.44 ± 0.12 mmol g_{DW}^{-1}) and *E. atrorubens* (2.36 ± 0.53 mmol g_{DW}^{-1}) were similar to orchid leaf N concentrations (2.51 ± 0.41 mmol g_{DW}^{-1} and 2.03 ± 0.19 mmol g_{DW}^{-1}, respectively). Peloton N concentrations extract from *E. palustris* (1.25 ± 0.35 mmol g_{DW}^{-1}) and *A. sandvicensis* (1.19 ± 0.26 mmol g_{DW}^{-1}) were in the range of those of reference plant leaves (Tables S2, S3). For *A. sandvicensis*, extracted pelotons had significantly smaller total N concentrations than orchid leaves (Fig. 3; Table S3).

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

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

Root fungi isolated from *Epipactis* species

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

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

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

Discussion

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

Obtaining stable isotope data of orchid mycorrhiza pelotons, we intended to investigate both, orchids associated with rhizoctonia

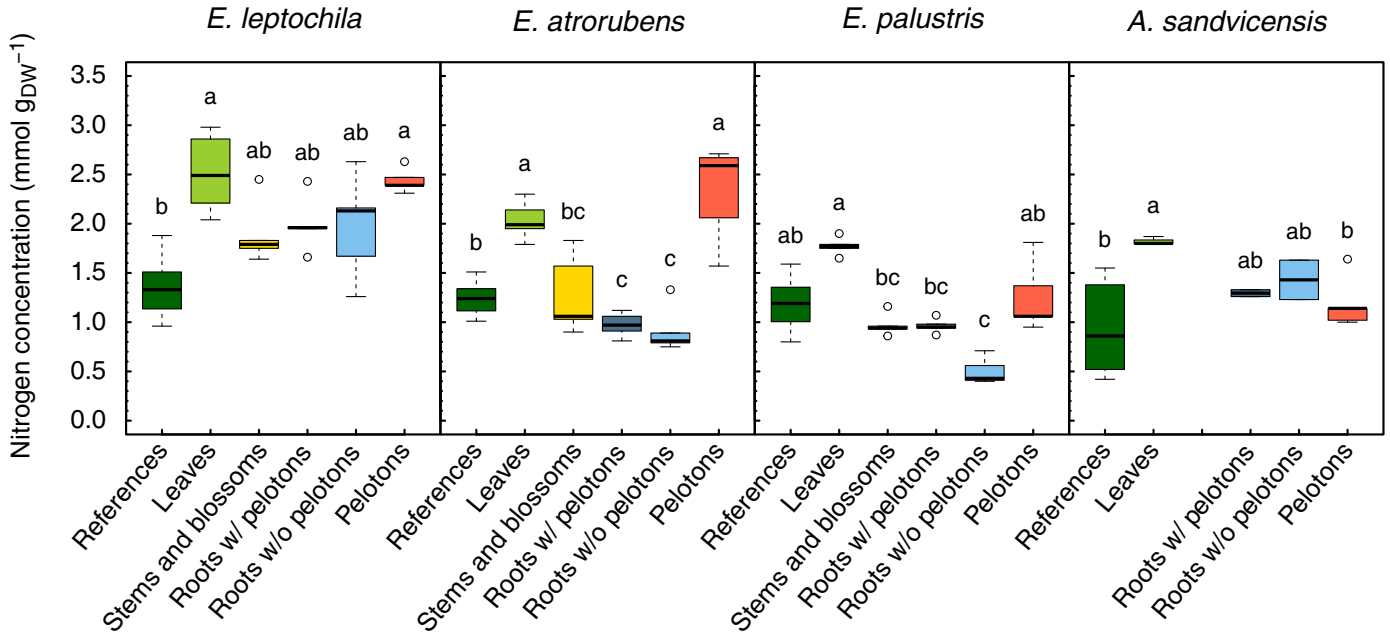

Fig. 3 Total nitrogen concentration of orchid parts ('leaves', 'stems and blossoms', 'roots with pelotons' and 'roots without pelotons') for *Epipactis leptochila* ($n = 5$), *Epipactis atrorubens* ($n = 5$), *Epipactis palustris* ($n = 5$), *Anoectochilus sandvicensis* ($n = 6$) and extracted pelotons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

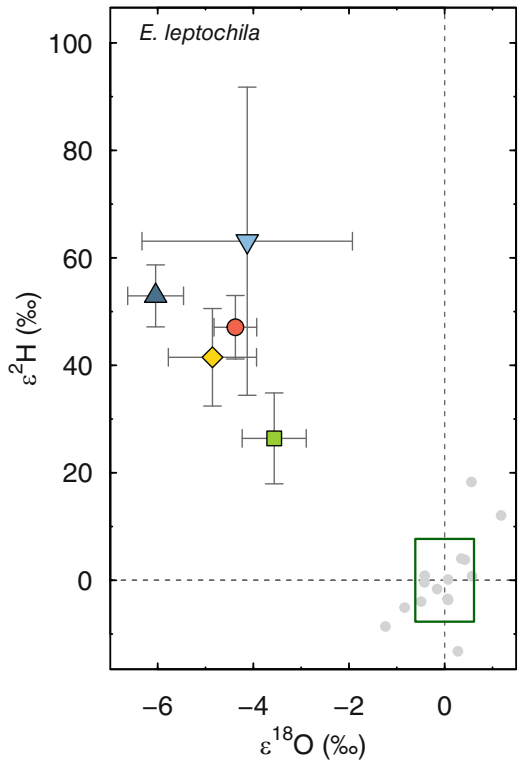

Fig. 4 Hydrogen and oxygen stable isotope enrichment factors ε (mean ± SD) of orchid parts 'leaves' (square, $n = 5$), 'stems and blossoms' (diamond, $n = 5$), 'roots with pelotons' (triangle upwards, $n = 5$), 'roots without pelotons' (triangle downwards, $n = 5$) and isolated pelotons (circle, $n =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

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

We expected the smallest enrichment in ^{13}C for pelotons extracted from *A. sandvicensis* and *E. palustris* because leaf material of rhizoctonia-associated orchids is usually not enriched in ^{13}C (Hynson *et al.*, 2013). Indeed, as hypothesized, enrichment in ^{13}C of pelotons extracted from *A. sandvicensis* and *E. palustris* was similar irrespective of fungal specificity and considerably smaller than ε^{13}C of pelotons isolated from the forest-dwelling, ECM-associated *E. leptochila* and *E. atrorubens*. Although pelotons of all investigated orchid species (including *Ophrys insectifera*

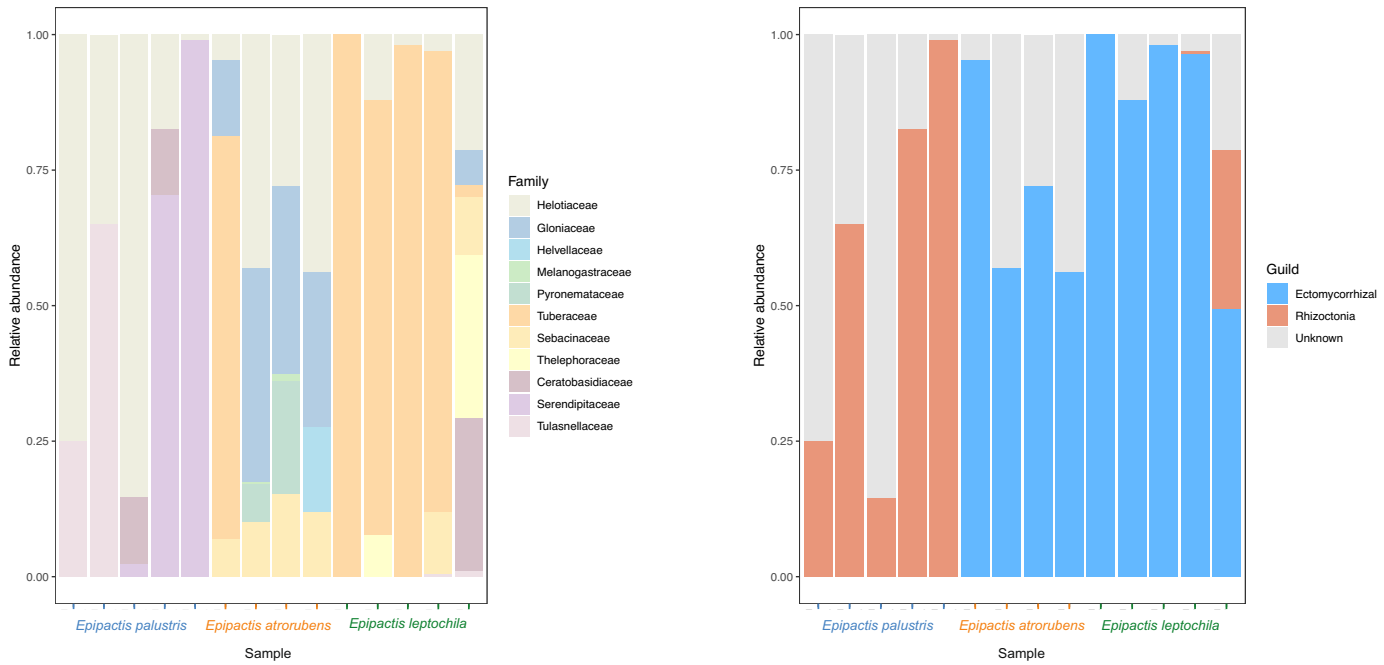

Fig. 5 Fungal diversity detected in the roots of individual plants of the orchid species: *Epipactis leptochila* ($n = 5$), *Epipactis atrorubens* ($n = 4$), *Epipactis palustris* ($n = 5$) represented per fungal family (left panel) and fungal guild (right panel). The ecological role of Helotiaceae in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

and *Orchis militaris*, Gomes *et al.*, 2023) displayed by *c.* 3‰ higher mean $\varepsilon^{13}C$ values than leaves of respective reference plants, the relatively small ^{13}C enrichment of rhizoctonia pelotons extracted from *A. sandvicensis* and *E. palustris* may explain the inconspicuous ^{13}C enrichment of rhizoctonia-associated orchids. Yet, the small $\varepsilon^{13}C$ of rhizoctonia pelotons (< 3‰) limits the suitability as a nutrition indicator for mycoheterotrophic C gain of rhizoctonia-associated orchids. Nonetheless, enrichment in ^{15}N and higher N concentrations relative to reference plant leaves hint at the capability of *A. sandvicensis* and *E. palustris* for a cryptic manifestation of partial mycoheterotrophy as previously suggested (Schiebold *et al.*, 2017; Lallemand *et al.*, 2018; Swift *et al.*, 2019). Yet, autotrophy remains as a possible nutrition mode of *A. sandvicensis* and *E. palustris*. Pursuing the ^{2}H approach according to Gebauer *et al.* (2016), future ^{2}H and ^{18}O analysis of extracted pelotons and leaves could contribute substantially to resolving the degree of mycoheterotrophic nutrition for rhizoctonia-associated orchids, like from *A. sandvicensis* and *E. palustris*. So far, we were able to get the first ^{2}H and ^{18}O data merely of pelotons extracted from *E. leptochila* because isolating sufficient material remains challenging.

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

Isotopic signature reveals nutrient exchange function of root fungi

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

As suspected, ^{15}N values of pelotons extracted from *E. leptochila* and *E. atrorubens* roots showed the same array as fruit bodies of ECM Ascomycota and ECM Basidiomycota, their dominant mycorrhizal root fungi (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017). However, pelotons of rhizoctonia-associated orchids (*A. sandvicensis* and *E. palustris*) were least enriched in ^{15}N, a pattern being reflected in the ^{15}N isotopic signature of the orchid leaves and also reported for rhizoctonia-associated *O. insectifera* L. and *O. militaris* L. (Gomes *et al.*, 2023).

Focusing on the ECM-associated orchids, we identified a difference in isotopic signatures coming along with variation in fungal partners. The higher $\varepsilon^{15}N$ values found for extracted pelotons and orchid tissues of *E. leptochila* (9.37 ± 2.01‰) relative to *E. atrorubens* (4.33 ± 1.09‰) can be explained by the dominance of *Tuber* species in *E. leptochila* roots as *Tuber* species (Ascomycota) have been shown to have higher ^{15}N enrichment than ECM Basidiomycota (10.70 ± 2.20‰ vs 5.19 ± 4.03‰) collected at identical locations (Schiebold *et al.*, 2017). Also, *E. atrorubens* roots were mainly colonized by ECM Ascomycota fungi, with a relatively high share of the *Cenococcum* species (Gloniaceae, Ascomycota), a widespread ectomycorrhizal fungi clade in temperate forest ecosystems (Spatafora *et al.*, 2012). One *E. atrorubens* roots sample even revealed *Tuber* as the dominant root fungi. However, besides ECM Ascomycota we isolated ECM Basidiomycota from *E. atrorubens* roots most likely causing lower ^{15}N natural values of orchid tissues and respective pelotons compared with *E. leptochila*. Remarkably, $\varepsilon^{15}N$ values of pelotons from *E. leptochila* (9.37 ± 2.01‰) and *E. atrorubens* (4.33 ± 1.09‰) showed the same range as fruit bodies of ECM Ascomycota (10.74 ± 2.18‰) and ECM Basidiomycota (5.19 ± 4.04‰), respectively (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017) and therefore mirror the ^{15}N enrichment of sporocarps of the respective fungal phyla they associate with (Fig. 2). While results of fungi DNA analysis within one orchid species can vary substantially across root samples (e.g. *E. leptochila* and *E. atrorubens*, Fig. 5), it is essential to consider that only a small cut-out of the roots is being analysed. Stable isotope analyses of bulk samples, however, enable interferences of plants' physiological processes integrated over space and time (Dawson *et al.*, 2002). We provide evidence that pelotons display distinct ^{15}N patterns depending on the present fungal guild like previously proposed for fruit bodies (Schiebold *et al.*, 2017). Considering that pelotons act as the orchids' direct fungal source and that the difference in isotopy of pelotons is reflected in ^{15}N stable isotope patterns of orchid leaves, we further argue that the isotopic signature of orchid leaves reveals the functional purpose of the present fungi regarding the fungus-to-plant nutrient transfer. Other than Jacquemyn *et al.* (2021), our findings support a linkage between 'functional' mycorrhizal partners and isotope signatures as suggested by Schiebold *et al.* (2017). Furthermore, we claim that a high prevalence of fungal taxa in orchid roots may not be directly indicative of playing a major role in their nutrition. As ^{15}N of mycorrhizal fungi is related to the N sources used (organic N, NH_4^+ and NO_3^-), the depth of soil at which the mycelium occurs and metabolic fractionations (Taylor *et al.*, 1997), the variety of fungi present in orchid roots can be narrowed to a selection of fungi with relevance for the orchids nutrition. Stable isotope analysis thus considerably complements the information about the presence of root fungi with evidence

about their functional role in the plants' nutrition. Nonetheless, more data are needed to substantiate if there is a distinct difference in orchid leaf ^{15}N signature depending on Asco- vs Basidiomycota mycobionts or on a smaller taxonomic level (e.g. fungal family). To this end, the future extraction of larger quantities of fungal pelotons from orchid roots in order to utilize peloton samples for both, stable isotope analysis and fungal sequencing, would allow the isotope data from the pelotons to be attributed directly to the fungal taxa that form them.

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

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

The isotopic pattern of pelotons could be induced by a selective transport of certain compounds, as previous studies have indicated a nutrient transfer from fungus to orchid across the mycorrhizal interface or after a degeneration and lysis of pelotons in the orchid roots cells (e.g. Kristiansen *et al.*, 2001; Selosse *et al.*, 2004; Cameron *et al.*, 2006; Chang & Chou, 2007; Hobbie & Högberg, 2012; Bougoure *et al.*, 2014; Kuga *et al.*, 2014; Suetsugu *et al.*, 2017; Favre-Godal *et al.*, 2020). Aside from soluble carbohydrates (Cameron *et al.*, 2006; Ponert *et al.*, 2021), amino acids, playing a significant role in N transfer, have been recognized to participate in C transfer in orchid mycorrhiza (Cameron *et al.*, 2006, 2008; Dearnaley & Cameron, 2017; Fochi *et al.*, 2017; Favre-Godal *et al.*, 2020; Valadares *et al.*, 2021). Notably, fungal proteins and amino acids are enriched in ^{15}N by *c.* 10‰ relative to fungal chitin, irrespective of the part of the fungus examined (Taylor *et al.*, 1997; Hobbie & Colpaert, 2003). Remarkably, pelotons of *E. leptochila* and *E. atrorubens* were by *c.* 10‰ less enriched in ^{15}N compared with respective orchid leaves. Smaller mean ^{15}N enrichments of pelotons relative to fruit bodies and orchid symbionts could reflect preferential incorporation of ^{15}N-enriched, protein-derived N, that is hyphal cell content, in the plant, while ^{15}N-depleted fungal cell wall chitin-N remains as the extracted pelotons. This is further supported by similar or smaller total N concentrations of pelotons relative to fungal fruit bodies and orchid leaves. Similar reports exist for mycoheterotrophic plants (Trudell *et al.*, 2003; Hobbie & Högberg, 2012) and Ericaceae (Tedersoo *et al.*, 2007). Yet, the possibility of bidirectional transport (Cameron *et al.*, 2008; Suetsugu *et al.*, 2017) and the degree of peloton lysis (Kuga *et al.*, 2014) can cause variations in isotopic patterns of pelotons. Anyhow, a fungal nitrogen source other than mycorrhizal pelotons remains a possible option to be involved in the nutrition of orchids (e.g. other endophytic fungi, bacteria and nonmycorrhizal pelotons). For instance, Helotiaceae were abundant in the investigated orchid roots and DSEs being nonmycorrhizal fungi can also form peloton-like structures in orchids (Liu *et al.*, 2022) and have been shown to affect isotope N signatures of plants (Giesemann *et al.*, 2020). Though unlikely, we cannot entirely exclude extraction artefacts, that is selective loss of parts of the fungal cell content, to have additionally influenced isotopic signatures found for the pelotons.

Stable isotope signature of different orchid compartments

Comparing stable isotope signature and total N concentrations of different orchid compartments across all here investigated orchid species ^{13}C and ^{15}N patterns resembled and can be attributed to their tissue composition – a pattern similarly found for partially mycoheterotrophic *Pyrola japonica* (Matsuda *et al.*, 2020). Namely, compartments with no or lower photosynthetic capacity, such as 'stems and blossoms' and 'roots' mainly consist of cellulose and hemicellulose, which usually have higher $\delta^{13}C$ values (Gebauer & Schulze, 1991; Gleixner *et al.*, 1993). Photosynthetic leaf tissue, however, has a higher share of secondary metabolites like lipids and proteins with lower $\delta^{13}C$ values (Winkler *et al.*, 1978; Tieszen & Boutton, 1989) and higher total N concentration compared with other orchid compartments (Field & Mooney, 1986; Gebauer *et al.*, 1988; Evans, 1989). Varying differences in N concentration between orchid and respective reference plant leaves among the studied orchid species are likely the result of variation in N surplus and deposition in orchid leaf tissue linked to their degree of nutrient gain from fungi. That we did not detect a clear pattern for ^{15}N and ^{13}C comparing roots with and without pelotons may be due to our separation method based on a rough optical assessment. Both types of root samples may exhibit different levels of purity. Particularly for highly colonized orchid roots, for example for *E. leptochila* and *E. atrorubens*, the presence of pelotons in 'roots without' samples cannot be fully ruled out. Roots without pelotons could also be colonized to a significant extent by endophytic hyphae, which do not form pelotons, but which contribute in some way to the nutrition of the orchid. ^{2}H enrichment of all *E. leptochila* orchid compartments and pelotons matched its mycoheterotrophic nutrition. Differences in transpiration between *E. leptochila* and reference plants, indicated by ^{18}O depletion of orchid leaves, could have even minimized the effect of mycoheterotrophy on ^{2}H enrichment (Ziegler, 1989; Cernusak *et al.*, 2004).

Conclusions and future directions

With this investigation, we present the first natural abundance of ^{15}N and ^{13}C data of fungal pelotons isolated from rhizoctonia-associated orchids (contemporaneous with Gomes *et al.*, 2023) and of pelotons extracted from orchids entangled in ectomycorrhizal networks with Ascomycota and/or Basidiomycota. Because small ^{13}C enrichment of rhizoctonia pelotons is obviously not

suited as a nutrition indicator of rhizoctonia-associated orchids, we suggest refining the ^{2}H approach with regards to pelotons. This could contribute to elucidate the relevance of partial myco-heterotrophy among rhizoctonia-associated orchid species. Yet, isolating sufficient material proves to be difficult but we were able to make a start by providing novel ^{2}H and ^{18}O data of *E. leptochila* pelotons. Though currently limited to C isotopes, within-cell spatial microanalysis of natural stable isotope abundance using ablation-isotope ratio mass spectrometry following Rodionov *et al.* (2019) could be a further approach to solve this issue in the future.

Extracting intracellular hyphal material from plant roots contributes to understanding the complex relationship between plants and their fungal symbionts because it helps to recognize predictable stable isotope patterns of orchid leaves depending on their functional, nutrition-specific fungal symbionts. Extracted pelotons have an advantage over fungal fruit bodies in terms of availability and the possibility to directly obtain the isotopic signature of the abundant multiple-root endophytic fungi and thus provide straightforward insights into the fungi's functional role. Lysis of pelotons in the orchid roots followed by a selective transfer of the fungal cell content, that is rather ^{15}N-enriched protein-N, into orchids is the most likely explanation for our results.

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

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Competing interests

None declared.

Author contributions

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

ORCID

Thomas K. Chapin https://orcid.org/0000-0002-8198-558X
Gerhard Gebauer https://orcid.org/0000-0003-1577-7501
Sofia I. F. Gomes https://orcid.org/0000-0001-7218-1531
Nicole A. Hynson https://orcid.org/0000-0001-7098-9641
Johanna Pausch https://orcid.org/0000-0002-7102-4793
Deyi Wang https://orcid.org/0000-0003-2140-2482

Data availability

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

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

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

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

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

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

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

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

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

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