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

Julienne M.-I. Schiebold[1], Martin I. Bidartondo[2,3], Peter Karasch[4], Barbara Gravendeel[5] and Gerhard Gebauer[1,*]

[1]Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany, [2]Department of Life Sciences, Imperial College London, London SW7 2AZ, UK, [3]Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK, [4]Deutsche Gesellschaft für Mykologie (German Mycological Society), Kirchl 78, 94545 Hohenau, Germany and [5]Naturalis Biodiversity Center, Leiden, the Netherlands
**For correspondence. E-mail Gerhard.Gebauer@uni-bayreuth.de*

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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 $\delta^{13}C$ 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.

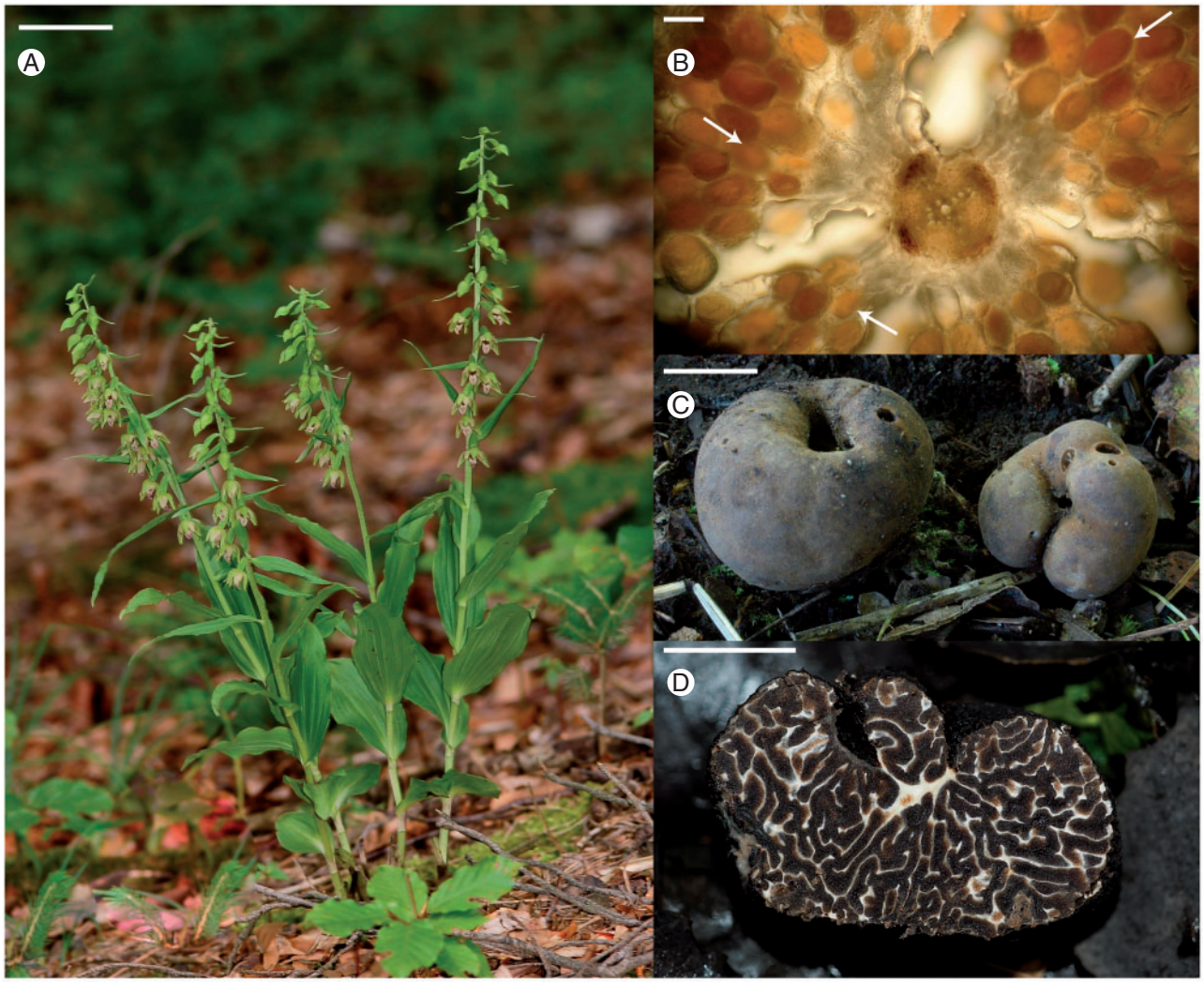

FIG. 1. (A) *Epipactis neglecta* at forest site 9 in the Nördliche Frankenalb in July 2009. Scale bar = 5 cm. Image courtesy of Florian Fraaß. (B) Light micrograph showing a transverse section of a root of *Epipactis neglecta*. Fungal colonization is visible as exodermal, outer and inner cortex cells 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 $MgCl_2$ (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 $\alpha = 0{\cdot}05$.

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 $2{\cdot}07 \pm 0{\cdot}89$ ‰ (*E. helleborine* subsp. *neerlandica*) and $6{\cdot}11 \pm 0{\cdot}91$ ‰ (*E. purpurata*) in ^{13}C and between $7{\cdot}98 \pm 2{\cdot}46$ ‰ (*E. helleborine* subsp. *neerlandica*) and $24{\cdot}60 \pm 1{\cdot}57$ ‰ (*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* ($\mu = 2{\cdot}38 \pm 0{\cdot}44$ mmol g d. wt^{-1}) had significantly higher N concentrations than their respective autotrophic references ($\mu = 1{\cdot}42 \pm 0{\cdot}32$ mmol g d. wt^{-1}). N concentrations in the leaves of *E. microphylla* ($1{\cdot}51 \pm 0{\cdot}32$ mmol g d. wt^{-1}) were only slightly but not significantly higher than the species' references ($1{\cdot}34 \pm 0{\cdot}25$ mmol g d. wt^{-1}) ($U = 48$; $P = 0{\cdot}395$) (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 ($U = 48$; $P = 0{\cdot}001$) but not for ^{13}C ($U = 26$; $P = 0{\cdot}862$). *Epipactis gigantea* was significantly depleted in ^{13}C ($U = 14$; $P = 0{\cdot}017$) and enriched in ^{15}N ($U = 93{\cdot}5$; $P = 0{\cdot}003$) relative to autotrophic references. Enrichment of the *Epipactis* species compiled from the literature varied between $-1{\cdot}19 \pm 0{\cdot}66$ ‰ (*E. gigantea*) and $4{\cdot}25 \pm 1{\cdot}77$ ‰ (*E. fibri*) in ^{13}C and between $3{\cdot}15 \pm 0{\cdot}75$ ‰ (*E. gigantea*) and $22{\cdot}16 \pm 0{\cdot}49$ ‰ (*E. leptochila*) in ^{15}N (Table S2). The N concentrations of all *Epipactis* species extracted from the literature ($\mu = 2{\cdot}70 \pm 0{\cdot}69$ mmol g d. wt^{-1}) were significantly higher than of leaves of their autotrophic reference plant species ($\mu = 1{\cdot}38 \pm 0{\cdot}72$ 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: $\bar{x} = 2·90 \pm 0·38$ mmol g d. wt^{-1}; ECM B: $\bar{x} = 2·81 \pm 0·95$ mmol g d. wt^{-1}; SAP: $\bar{x} = 4·783 \pm 1·854$ mmol g d. wt^{-1}) had significantly higher N concentrations than their autotrophic reference plant species ($\bar{x} = 1·54 \pm 0·40$ 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

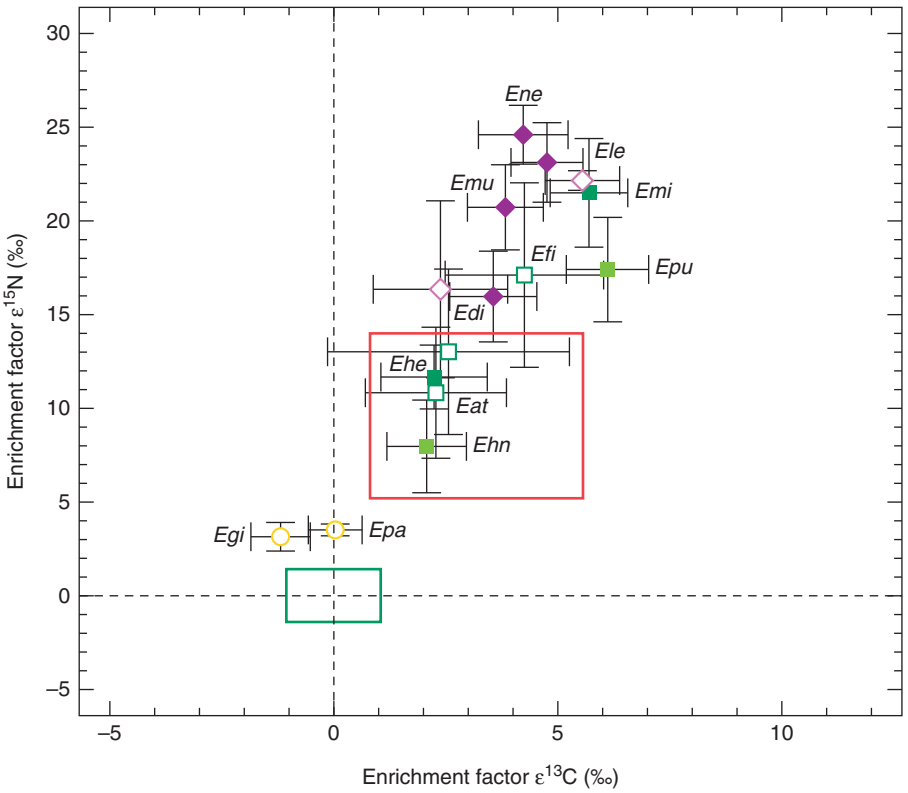

FIG. 2. Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N \pm 1$ s.d. of two *Epipactis* species associated with rhizoctonia fungi (yellow circles; *Egi* = *E. gigantea*, *Epa* = *E. palustris*), two *Epipactis* species associated with ECM basidiomycetes (light green squares; *Ehn* = *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 ±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 ±1 s.d. of all partially mycoheterotrophic orchid species associated with ectomycorrhizal fungi ($\varepsilon^{13}C_{mean} = 3{\cdot}18 \pm 2{\cdot}38$ and $\varepsilon^{15}N_{mean} = 9{\cdot}61 \pm 4{\cdot}40$) published since 2003 that were available from the literature (Hynson *et al.*, 2016).

TABLE 3. *Results from pairwise comparisons for enrichment factors* $\varepsilon^{15}N$, $\varepsilon^{13}C$ *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	$\varepsilon^{15}N$		$\varepsilon^{13}C$		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 ($P = 0{\cdot}199$). The N concentrations of sporocarps of SAP were significantly higher than in ECM A ($P = 0{\cdot}042$) and ECM B ($P = 0{\cdot}006$).

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 ($8{\cdot}03 \pm 0{\cdot}13$ ‰ 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 rhizoctonias 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 ‰ $\delta^{15}N$ 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 of *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

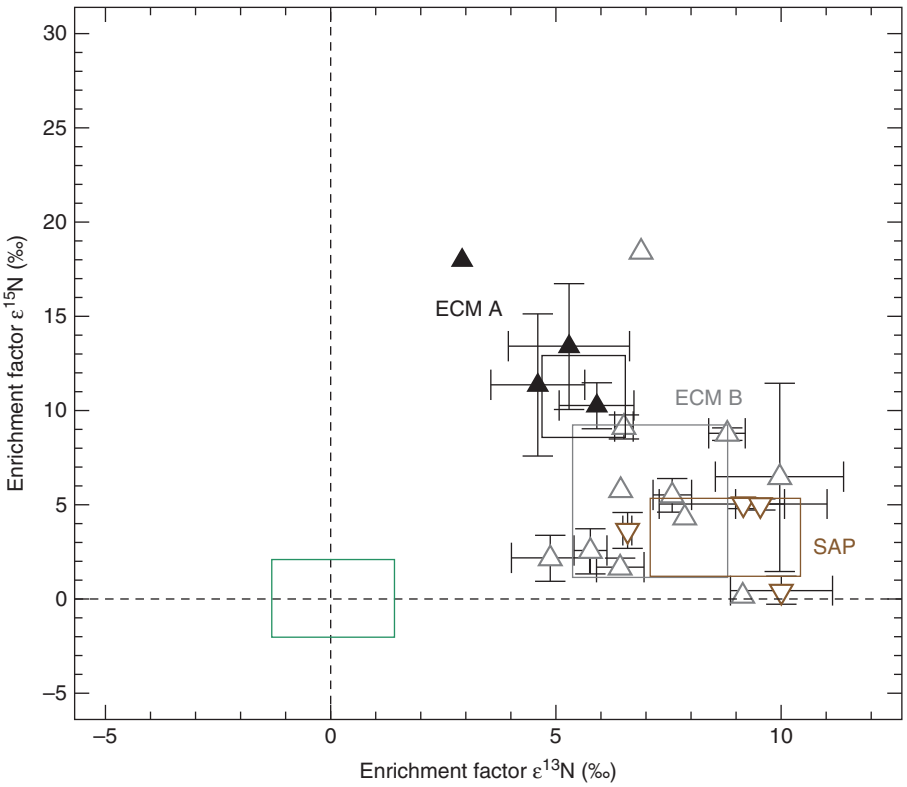

FIG. 3. Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N \pm 1$ s.d. as calculated for sporocarps of four ECM ascomycete *Tuber* species (filled black upward triangles), 11 ECM basidiomycete species (open grey upward triangles) and four saprotrophic basidiomycete species (open brown 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 mycohe-terotrophic protocorms and partially mycohe terotrophic 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 $\delta^{15}N$ and $\delta^{13}C$ values, single and mean enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{13}C$, and single and mean total nitrogen concentration data of all original plant and fungal samples in this study. Table S2: single and mean $\delta^{15}N$ and $\delta^{13}C$ values, single and mean enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{13}C$, 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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