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

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• **Methods** Published and unpublished data were compiled on the N concentrations, C and N stable isotope abundances (δ^{13}C and δ^{15}N) of fully ($n = 18$) and partially ($n = 22$)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

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

INTRODUCTION

The ability of plants to fix atmospheric carbon (C) and convert it into sugars through photosynthesis (autotrophy) sets this kingdom of organisms apart from others and is the key to the earth's primary productivity. However, some plants have completely lost this ability and rely on alternative means of nutrition such as feeding off symbiotic relationships with mycorrhizal fungi. Some plants retain the ability to photosynthesize, but under certain conditions meet a portion of their C demands via associations with mycorrhizal fungi. Both of these intriguing plant adaptations fall under the category of mycoheterotrophy. Mycoheterotrophy entails plants meeting all or a portion of their C and nutrient demands via symbiotic mycorrhizal fungi (Merckx, 2013). The most striking examples of mycoheterotrophic plants are those that have completely lost the ability to photosynthesize, and their above-ground structures serve only for dispersal and reproduction (Merckx, 2013). Over approximately the last decade, the marriage of methods from plant ecophysiology and molecular ecology has led to new revelations about the population ecology of mycoheterotrophs (reviewed in Merckx *et al*., 2009; Selosse and Roy, 2009). However, fundamental questions remain on the ecology and evolution of this unique plant adaptation. Here we address a set of these questions – can nitrogen (N) concentrations and C and N stable isotope abundances of mycoheterotrophs distinguish distantly related plants that are dependent upon the same functional guild of mycorrhizal fungi?

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

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

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

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

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

With a critical mass of data now accumulated on both the identity and diversity of fungi that host species of orchidaceous and ericaceous mycoheterotrophs, we set out to test whether plant family identity is a significant predictor for N concentration and stable isotope abundances. To avoid the effects of fungal functional guild on mycoheterotroph stable isotope values and N concentration, we selected just those species that form symbioses with EM fungi. We compiled data from 22 published

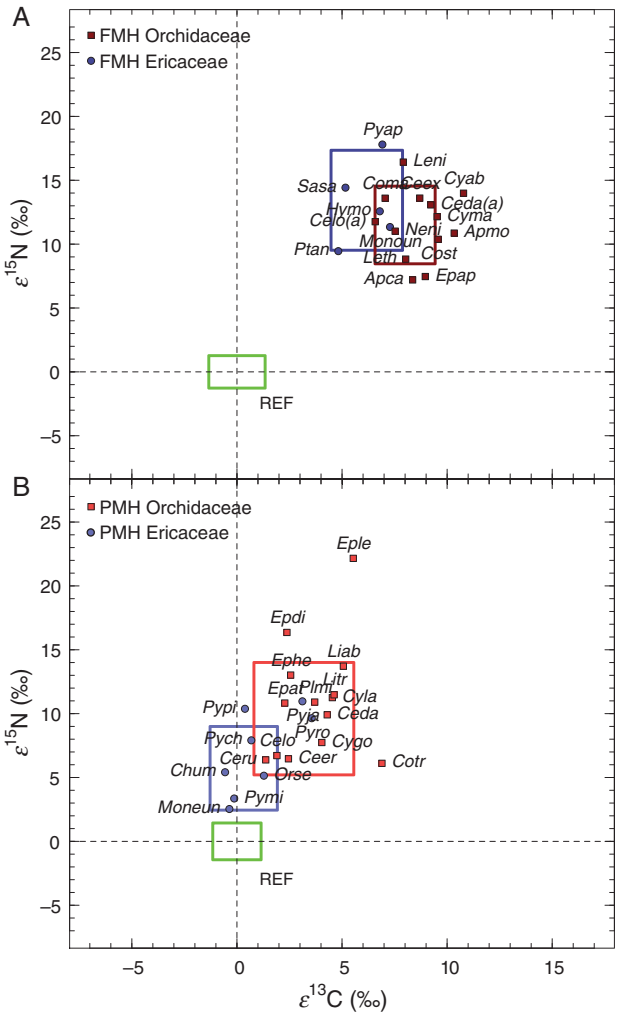

FIG. 1. Mean enrichment factors (ε) for ^{13}C and ^{15}N of (A) fully mycoheterotrophic (FMH) Orchidaceae and Ericaceae and (B) partially mycoheterotrophic (PMH) Orchidaceae and Ericaceae associated with fungi forming ectomycorrhizas. Boxes represent one standard deviation of the mean ε values for the four significantly distinguished groups of (partially) mycoheterotrophic Orchidaceae and Ericaceae and for their respective photosynthetic reference plants (REF, n = 1433. Abbreviations of the species and numbers of replicates (n) are as follows: FMH Orchidaceae (n = 126): *Apca*, *Aphyllorchis caudata*; *Apmo*, *A. montana*; *Ceda(a)*, *Cephalanthera damasonium* albino; *Ceex*, *C. exigua*; *Celo(a)*, *C. longifolia* albino; *Coma*, *Corallorhiza maculata*; *Cost*, *Corallorhiza striata*; *Cyab*, *Cymbidium aberrans*; *Cyma*, *C. macrorhizon*; *Epap*, *Epipogium aphyllum*; *Leni*, *Lecanorchis nigricans*; *Leth*, *Lecanorchis thalassica*; *Neni*, *Neottia nidus-avis*; (REF, n = 628). FMH Ericaceae (n = 134): *Hymo*, *Hypopitys monotropa*; *Monoun*, *Monotropa uniflora*; *Ptan*, *Pterospora andromedea*; *Pyap*, *Pyrola aphylla*; *Sasa*, *Sarcodes sanguinea*; (REF, n = 403). PMH Orchidaceae (n = 189): *Ceda*, *Cephalanthera damasonium*; *Ceer*, *C. erecta*; *Celo*, *C. longifolia*; *Ceru*, *C. rubra*; *Cotr*, *Corallorhiza trifida*; *Cygo*, *Cymbidium goeringii*; *Cyla*, *C. lancifolium*; *Epat*, *Epipactis atrorubens*; *Epdi*, *E. distans*; *Ephe*, *E. helleborine*; *Eple*, *E. leptochila*; *Liab*, *Limodorum abortivum*; *Litr*, *L. trabutianum*; *Plmi*, *Platanthera minor*; (REF, n = 662). PMH Ericaceae (n = 606): *Chum*, *Chimaphila umbellata*; *Moneun*, *Moneses uniflora*; *Orse*, *Orthilia secunda*; *Pych*, *Pyrola chlorantha*; *Pyja*, *P. japonica*; *Pymi*, *P. minor; Pypi*, *P. picta*; *Pyro*, *P. rotundifolia*; (REF, n = 627).

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

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

MATERIALS AND METHODS

Data compilation

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

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

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

investigation of chlorophyll concentration gradients (Stöckel *et al*., 2011) or by investigation of different developmental stages (Roy *et al*., 2013; Gonneau *et al*., 2014). We did include data from mutant achlorophyllous (albino) orchids that are fully mycoheterotrophic. Data on C and N stable isotope natural abundances as well as total N concentrations in leaf or stem tissues of FMH and PMH species in the plant families Ericaceae and Orchidaceae known to partner with EM fungi were either directly extracted from the original publications or were obtained by personal contact with the respective authors. Specifically, unpublished data on plant N concentrations from the investigations by Bidartondo *et al*. (2004), Zimmer *et al*. (2007, 2008), Hynson *et al*. (2009, 2015), Liebel *et al*. (2009), Motomura *et al*. (2010), Preiss *et al*. (2010), Yagame *et al*.

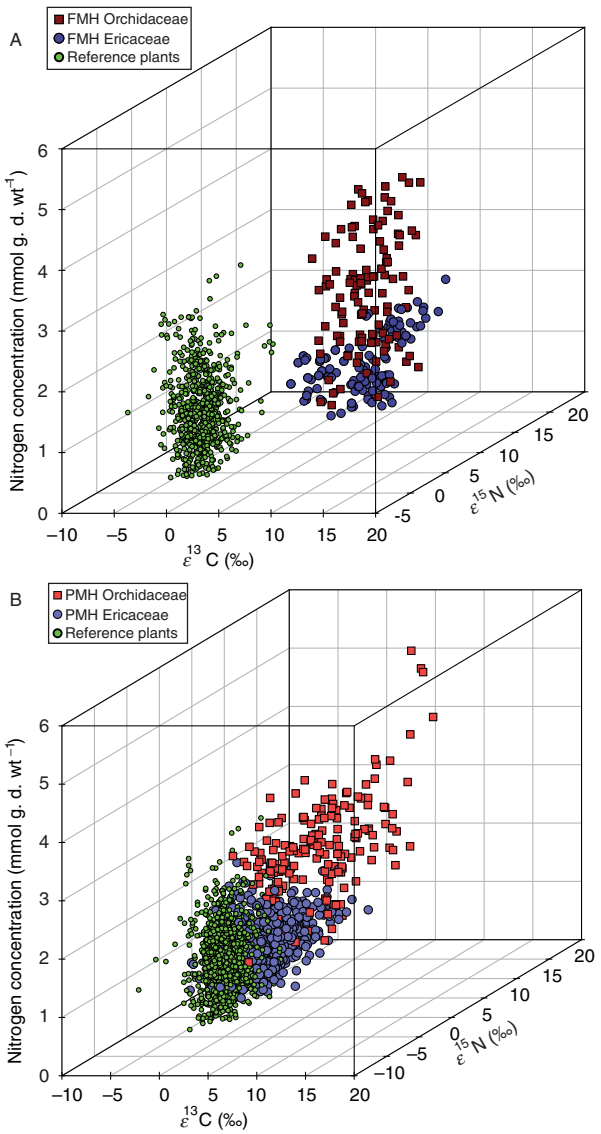

FIG. 2. Single values for enrichment factors ε^{13}C and ε^{15}N and nitrogen concentrations (mmol g d. wt^{-1}) of (A) fully mycoheterotrophic (FMH) Orchidaceae and Ericaceae and the respective photosynthetic reference plants (REF, n = 804) and (B) partially mycoheterotrophic (PMH) Orchidaceaeand Ericaceae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

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

Thus, in total, we compiled C and N stable isotope abundance and N concentration data from 22 studies for a total of 18 FMH species, 22 PMH species and 156 of their neighbouring autotrophic reference species, of which 11 species were non-mycoheterotrophic Ericaceae (Table 1). We did not include any green orchids that partner with rhizoctonia fungi as references because all orchids are initially mycoheterotrophic in their germination stages. Data collection resulted in 260 data points for ^{13}C and ^{15}N abundances for full mycoheterotrophs, 795 data points for ^{13}C and 803 data points for ^{15}N abundances for partial mycoheterotrophs and 1433 data points for ^{13}C and 1461 data points for ^{15}N abundances for neighbouring autotrophic references (Figs 2 and 3). Nitrogen concentration data were only available for a reduced data set of 235 data points for full mycoheterotrophs, 767 for partial mycoheterotrophs and 1355 for autotrophic references (Figs 2 and 3). For non-mycoheterotrophic Ericaceae within the autotrophic reference species, 118 and 126 data points were available for ^{13}C and ^{15}N abundances, respectively, and 111 data points for N concentration data.

Data treatment and statistical analysis

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

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

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

and

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

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

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

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

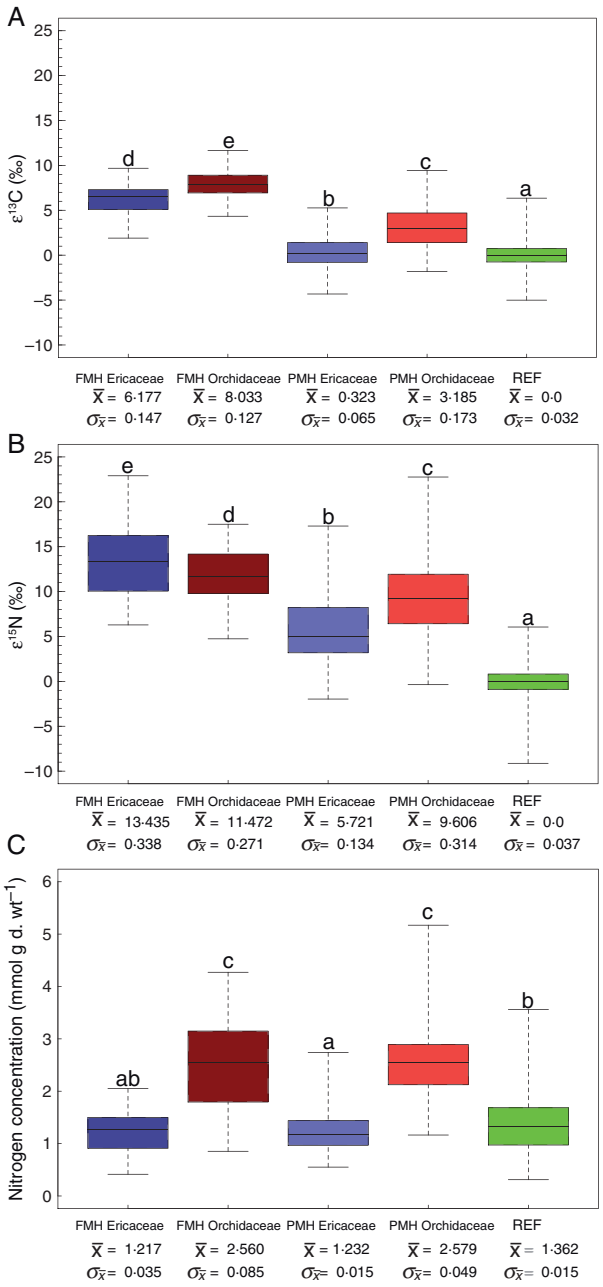

FIG. 3. Box-and-whisker plots and summary statistics for the compiled data sets on FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references in (A) enrichment factor $\varepsilon^{13}C$, (B) enrichment factor $\varepsilon^{15}N$ and (C) nitrogen concentration. The box spans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

We used non-metric multidimensional scaling (NMDS) to visualize the organization of samples in two-dimensional space graphically, whereas their spatial arrangement exactly represents the similarity between the objects. For this, the Bray–Curtis index was used to calculate distance matrices from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data using the function 'metaMDS' with two dimensions and 100 permutations in the R package 'vegan' (Oksanen *et al.*, 2015). Stress values were calculated to evaluate how well the configuration provides a representation of the distance matrices; generally, a stress value <0·05 provides an excellent representation in reduced dimensions. Fitted vectors were calculated to display the response variables $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration in the ordination space and to indicate the differences between the groups in association with these variables (Fig. 4). Each arrow shows the direction of the increasing response variable while its length is proportional to the correlation (R^2) between the variable and the ordination (Fig. 4, Oksanen *et al.*, 2015).

The function 'adonis' in the R package 'vegan' was used to perform a permutational MANOVA (multivariate analysis of variance) to test for significance of differences between group means using the aforementioned calculated distance matrices (Anderson, 2001). *P*-values from multiple pairwise comparisons were adjusted (P_{adj}) using the sequential Bonferroni correction. For statistical analyses, we used the software environment R [version 3.1.2 (Pumpkin Helmet); R Development Core Team, 2014)] supported by the add-on packages 'coin' (version 1.0-24; Hothorn *et al.*, 2006, 2008*a*), 'multcomp' (version 1.3-8; Hothorn *et al.*, 2008*b*), 'scattergrid' (version 1.0; Gassem, 2015), 'scatterplot3d' (version 0.3-35; Ligges and Mächler, 2003) and 'vegan' (version 2.2-1; Oksanen *et al.*, 2015) with a significance level of $\alpha = 0{\cdot}05$.

RESULTS

Based on comparisons of FMH and PMH Orchidaceae and Ericaceae, significant patterns have emerged (Fig. 3; Supplementary Data Table S2). Differences among our groups in ^{13}C enrichment (ε) found support for all three of our hypotheses (Fig. 3A; Table S2). In support of hypotheses (1) and (2), FMH and PMH Orchidaceae were on average significantly more enriched in ^{13}C than FMH and PMH Ericaceae (Fig. 3A; Table 2). In support of hypothesis (3) and as anticipated from previous plant population studies, autotrophic reference species were less enriched in ^{13}C relative to PMH species from both families, which were less enriched in ^{13}C than all FMH species (Fig. 3A; Table 2). A comparison of FMH and PMH Ericaceae with autotrophic Ericaceae from the reference plant group confirms these findings. Autotrophic Ericaceae were significantly depleted in ^{13}C compared with FMH and PMH Ericeaceae and even more depleted in ^{13}C than the remaining reference plants (Supplementary Data Fig. S1A; Table S1). Congruent with our non-parametric comparisons, effect sizes for $\varepsilon^{13}C$ among groups were high (Table 3), especially so for FMH Orchidaceae and Ericaceae relative to autotrophic references (d = 5·979 and 4·463, respectively). Interestingly, $\varepsilon^{13}C$ values of PMH Orchidaceae had a higher scatter than all other groups (Figs 2B and 3A) despite this variation in the data, *P*-values and effect sizes between this group, autotrophic references, FMH species and PMH Ericaceae were significant and high. The more tightly clustered $\varepsilon^{13}C$ values of PMH Ericaceae varied little based on effect size from references (d = 0·229), while being statistically distinguishable from references based on our non-parametric test ($P_{adj} < 0{\cdot}001$).

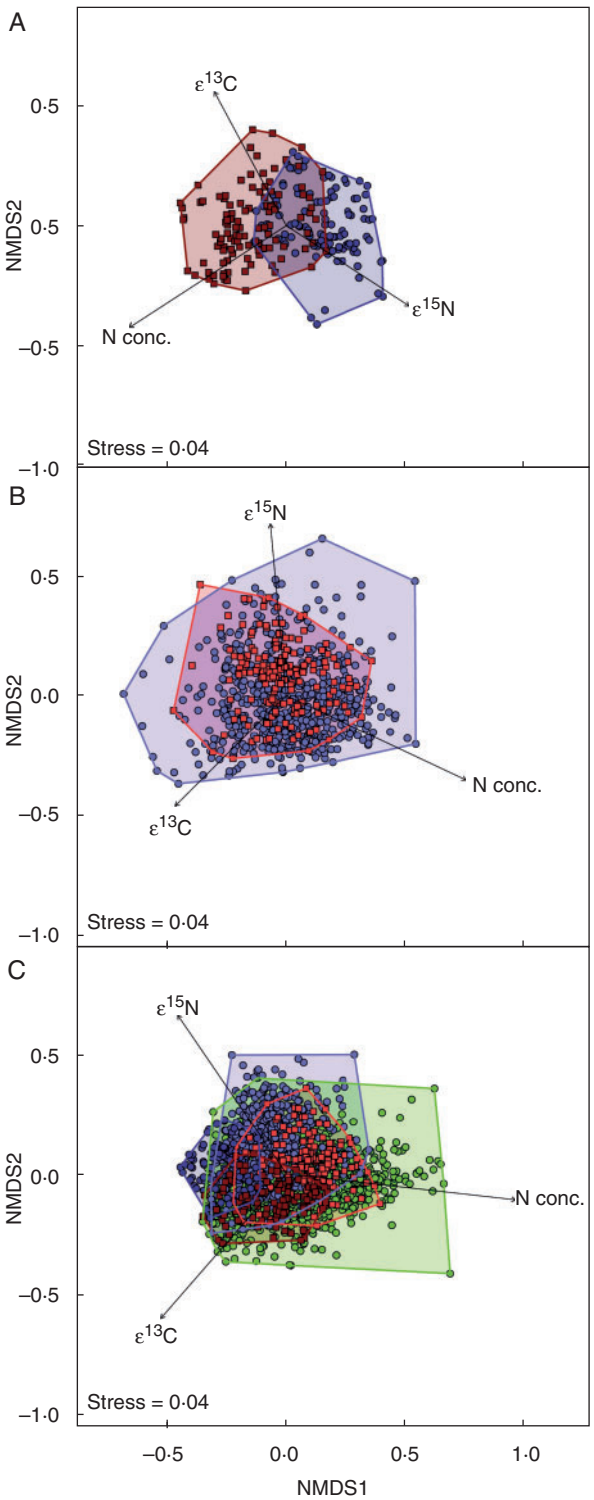

FIG. 4. NMDS plots visualize Bray–Curtis dissimilarity matrices calculated from enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and nitrogen concentration data in two-dimensional space. Fitted vectors display the response variables $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration in the ordination space and indicate the differences between the groups in association with these variables. (A) FMH Ericaceae and Orchidaceae, stress = 0·04, 100 permutations; MANOVA $R^2 = 0{\cdot}185$, $P < 0{\cdot}001$; (B) PMH Ericaceae and Orchidaceae, stress = 0·04, 100 permutations; MANOVA $R^2 = 0{\cdot}22$, $P < 0{\cdot}001$; and (C) FMH Ericaceae, FMH Orchidaceae, PMH Ericaceae, PMH Orchidaceae and respective autotrophic references, stress = 0·04, 100 permutations; MANOVA $R^2 = 0{\cdot}678$, $P_{adj} < 0{\cdot}001$.

From comparisons of ^{15}N enrichment among our study groups, a similar pattern emerges where we found support for all three of our hypotheses (Fig. 3B; Table S2). However, in contrast to patterns of ^{13}C enrichment, FMH Ericaceae were significantly more enriched in ^{15}N relative to FMH Orchidacae ($P_{adj} = 0{\cdot}005$; Fig. 3B; Table 2). Similar to ^{13}C, PMH Orchidaceae were significantly more enriched in ^{15}N relative to PMH Ericaceae ($P_{adj} < 0{\cdot}001$; Fig. 3B; Table 2). All groups were significantly more enriched in ^{15}N relative to references, and full mycoheterotrophs were significantly more enriched in ^{15}N than partial mycoheterotrophs (Figs 2 and 3B; Table 2). Effect sizes among all groups were also high (Table 3), especially so for FMH Orchidacae and Ericaceae vs. references ($d = 4{\cdot}866$ and $4{\cdot}711$, respectively). However, based on our non-parametric test, FMH Orchidaceae were significantly more enriched in ^{15}N relative to PMH Orchidaceae ($P_{adj} < 0{\cdot}001$), effect size between these two groups was medium ($d = 0{\cdot}480$). Autotrophic Ericaceae were significantly depleted in ^{15}N compared with FMH and PMH Ericaceae and only slightly enriched in ^{15}N compared with the remaining reference plants (Supplementary Data Fig. S1B; Table S1).

While N concentration data werenot available for all species for which we had stable isotope profiles (Fig. 3C; Table S2), we were still able to detect significant differences among groups. We found support for our first hypothesis where FMH Ericaceae had, on average, lower N concentrations relative to FMH Orchidaceae ($P_{adj} < 0{\cdot}001$; Fig. 3C; Table 2). We also found support for hypothesis (2) where PMH Orchidaceae had significantly higher N concentrations relative to PMH Ericaceae ($P_{adj} < 0{\cdot}001$; Fig. 3C; Table 2). However, we did not find significant differences between the N concentrations of FMH and PMH Ericaceae ($P_{adj} = 1{\cdot}0$) or FMH and PMH Orchidaceae ($P_{adj} = 1{\cdot}0$) (Fig. 3C; Table 2). The effect sizes for differences in N concentration lend further support to hypotheses (1) and (2) where comparisons between FMH Ericaceae and Orchidaceae and partial mycoheterotrophs in both families were high (Table 3). In general, mean N concentrations in FMH and PMH Orchidaceae were about twice as high as in reference plants (Fig. 3C; Table 2) while mean N concentrations in FMH and PMH Ericaceae were, on average, lower than in reference plants (Fig. 3C; Table 2). Furthermore, N concentrations of autotrophic Ericaceae among the reference plants which included both arbutoid and ericoid mycorrhizal species were lower than those found for FMH and PMH Ericaceae (Supplementary Data Fig. S1C; Table S1).

Ordination of a Bray–Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data of FMH Orchidaceae and Ericaceae with NMDS supports hypothesis (1) as the groups are segregated in ordination space (Fig. 4A), and a MANOVA showed a significant effect of group on the ordination ($R^2 = 0{\cdot}185$, $P = 0{\cdot}001$). Fitted vectors in the ordination of FMH Orchidaceae and Ericaceae were maximally correlated with N concentration ($R^2 = 0{\cdot}821$, $P < 0{\cdot}001$), $\varepsilon^{15}N$ ($R^2 = 0{\cdot}500$, $P < 0{\cdot}001$) and $\varepsilon^{13}C$ ($R^2 = 0{\cdot}493$, $P < 0{\cdot}001$). NMDS for PMH Orchidaceae and Ericaceae (Fig. 4B) also supports hypothesis (2) as a MANOVA revealed a significant effect of group on the ordination ($R^2 = 0{\cdot}220$, $P = 0{\cdot}001$). Here, fitted vectors in the ordination of PMH

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

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

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

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

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

Orchidaceae and Ericaceae were maximally correlated with $\varepsilon^{15}N$ ($R^2 = 0{\cdot}424$, $P < 0{\cdot}001$), N concentration ($R^2 = 0{\cdot}313$, $P < 0{\cdot}001$) and $\varepsilon^{13}C$ ($R^2 = 0{\cdot}261$, $P < 0{\cdot}001$). An ordination of a Bray–Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$ and N concentration data of FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references with NMDS (Fig. 4C) supports hypothesis (3) through the distinct clustering of the groups (PMH, FMH and autotrophic species) in the ordination space, and a MANOVA showed a significant effect of group on the ordination ($R^2 = 0{\cdot}678$, $P_{adj} = 0{\cdot}001$). Here, fitted vectors in the ordination of FMH Orchidaceae and Ericaceae, PMH Orchidaceae and Ericaceae and autotrophic references were maximally correlated with N concentration ($R^2 = 0{\cdot}442$, $P < 0{\cdot}001$), $\varepsilon^{15}N$ ($R^2 = 0{\cdot}306$, $P < 0{\cdot}001$) and $\varepsilon^{13}C$ ($R^2 = 0{\cdot}301$, $P < 0{\cdot}001$). Generally, the stress values of all ordinations provide an excellent representation in reduced dimensions (Fig. 4).

DISCUSSION

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

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

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

Drivers of nitrogen concentrations among plant families and trophic groups

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

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

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

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

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

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

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

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

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

Future directions

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

Adding N concentration as an additional explanatory variable will aid future research in distinguishing differences among full and partial mycoheterotrophs and autotrophs. When N concentrations along with $\varepsilon^{15}N$ and $\varepsilon^{13}C$ values were incorporated into an ordination of a Bray–Curtis dissimilarity matrix with NMDS, MANOVA showed a significant effect of group on the ordination ($P_{adj} = 0{\cdot}001$) and these three factors combined explain approx. 68% of the variation in the data set (Fig. 4). Similar models that segregated FMH Orchidaceae from Ericaceae and did the same for partial mycoheterotrophs for each family found significant differences between plant families, but the three factors only explained about 19 and 22 % of the variation in the data sets, respectively (Fig. 4A, B). Therefore, future investigations should consider measurements of additional explanatory response variables. For instance, analysis of concentrations and stable isotope abundances of additional elements involved in organic matter exchange such as hydrogen, oxygen or sulphur, may prove informative for teasing apart the dependency of mycoheterotrophic plants on fungal-derived organic matter (Gebauer *et al*., 2016). Also, studies that identify the C and N compounds and transfer pathways among different types of mycoheterotrophic plants are urgently needed.

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

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

Conclusions

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

SUPPLEMENTARY DATA

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

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