New Phytologist

Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae)

Nicole A. Hynson[1], Katja Preiss[2], Gerhard Gebauer[2] and Thomas D. Bruns[3]

[1]University of California Berkeley, Department of Environmental Science, Policy & Management, Berkeley, CA, USA; [2]Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, Bayreuth, Germany; [3]University of California Berkeley, Department of Plant & Microbial Biology, Berkeley, CA, USA

Author for correspondence:
Nicole A. Hynson
Tel: +1 510 643 5483
Email: nhynson@nature.berkeley.edu

Received: *28 October 2008*
Accepted: *23 December 2008*

New Phytologist (2009) **182**: 719–726
doi: 10.1111/j.1469-8137.2009.02781.x

Key words: ^{15}N and ^{13}C, Ericaceae, mixotrophy, myco-heterotrophy, mycorrhiza, Pyroleae, *Pyrola*, pyroloids.

Summary

- Botanists and mycologists have long debated the potential for full myco-heterotrophy in the achlorophyllous *Pyrola aphylla* (Ericaceae). Here we address the ecophysiology of this putative myco-heterotroph and two other closely related green species in the tribe Pyroleae (*Pyrola picta* and *Chimaphila umbellata*).
- The stable isotopes of carbon and nitrogen (δ^{13}C and δ^{15}N) were analysed from 10 populations of Pyroleae species in California and Oregon, USA. For all populations isotope signatures were tested for significant differences between *P. aphylla*, green pyroloids, surrounding autotrophs and obligate myco-heterotrophs.
- Throughout all populations *P. aphylla* was most similar to myco-heterotrophs that associate with ectomycorrhizal fungi in its ^{13}C signature (average enrichment $\varepsilon^{13}\text{C} = 6.9 \pm 0.9‰$) and even more enriched in ^{15}N than many previously recorded myco-heterotrophic species (average enrichment $\varepsilon^{15}\text{N} = 18.0 \pm 2.2‰$). The two green Pyroleae species were not enriched in ^{13}C compared with the autotrophic understory (*C. umbellata* average enrichment $\varepsilon^{13}\text{C} = -0.5 \pm 1.0‰$ and *P. picta* average $\varepsilon^{13}\text{C} = 0.3 \pm 1.4‰$) and their ^{15}N signatures were similar to myco-heterotrophs that associate with ectomycorrhizal fungi (*C. umbellata* average enrichment $\varepsilon^{15}\text{N} = 10.6 \pm 1.6‰$ and *P. picta* average $\varepsilon^{15}\text{N} = 10.6 \pm 1.9‰$).
- This is the first study to analyse the isotope signatures of *P. aphylla* from a wide geographic region and our results confirm the variable trophic strategies of adult plants within the Pyroleae and the myco-heterotrophic status of *P. aphylla*.

Introduction

The physiology and taxonomy of pyroloids (species within the tribe Pyroleae, family Ericaceae) has confounded researchers for over 200 years (de Jussieu, 1789; Holm, 1898; Henderson, 1919; Camp, 1940; Haber, 1987). The debate over the taxonomy of pyroloids has been partly fueled by the occurrence of leafless forms of plants within the genus *Pyrola* that are potentially myco-heterotrophic. In particular the leafless form of *Pyrola picta* Sm. referred to here as *Pyrola aphylla* Sm. (Fig. 1) is thought by some researchers to be an extreme morphological variant of *P. picta* that receives nutrition through parasitizing its mycorrhizal associates (Camp, 1940). Conversely, Haber (1987) considered *P. aphylla* flower stalks to be connected via a rhizome to *P. picta* rosettes that are were responsible for photosynthesis for the entire plant, while Smith (1814) considered them discrete individuals and therefore physiologically independent. Smith's 1814 determination of *P. picta* and *P. aphylla* as separate species is supported by the existence of *P. aphylla* populations in the absence of *P. picta* plants (Haber, 1987; own pers. obs.). This observation also supports the potential for myco-heterotrophy in *P. aphylla*. Obligate myco-heterotrophy entails a complete dependence on organic nutrient gains via a symbiosis with a fungus (Leake, 1994). In many cases these plants are 'epiparasites' that receive the majority of their carbon indirectly from surrounding autotrophic plants through a shared mycorrhizal fungus (Taylor *et al.*, 2002), but even in these cases nitrogen is received directly from the fungus (Leake, 1994).

Recently Freudenstein (1999) and Kron *et al.* (2002) used phylogenetic methods to support the placement of pyroloids in their own tribe: the Pyroleae, which is one of three tribes within the subfamily Monotropoideae. However, the evolutionary relatedness of the tribes in Monotropoideae and the phylogenetic

Fig. 1 Photographs of *Pyrola aphylla*, its rare 'leafy' form, and *Pyrola picta*. From left to right, flowering stalks of *Pyrola aphylla* (inset, close-up of flowers), *P. aphylla* with small leaves (arrow) and a rosette of *P. picta*.

delimitation species in the *P. picta*/*P. aphylla* complex has yet to be determined. Despite their unresolved taxonomy pyroloids are of particular interest to those who study the ecology and evolution of myco-heterotrophy as the tribe contains closely related taxa that are all myco-heterotrophic in their early stages of development (Leake, 1994), but upon reaching adulthood appear to occupy the full spectrum of trophic habits, from autotrophy to mixotrophy (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007) to potentially full myco-heterotrophy in *P. aphylla*. From an evolutionary perspective the variety of trophic abilities in the Pyroleae is intriguing as the tribe's two closest relatives the Monotropeae and the Pterosporeae contain only obligate myco-heterotrophic species (Kron & Johnson, 1997; Freudenstein, 1999). The ecological factor(s) driving the variability in photosynthetic abilities between closely related Pyroleae species remain elusive, but it has been proposed that both limited light availability and the presence of particular mycobionts may be responsible (Bidartondo *et al.*, 2004; Julou *et al.*, 2005).

In this study, rather than using a phylogenetic approach to examine evolutionary relationships between pyroloids (this has been done to some extent by Freudenstein, 1999) we chose to address the ecophysiology of these plants through the analysis of the natural abundances of the stable isotopes of carbon ($^{13}C : ^{12}C$) and nitrogen ($^{15}N : ^{14}N$) of pyroloids, surrounding autotrophs and obligate myco-heterotrophs. The analysis of the natural abundances of stable isotopes in plants is a powerful tool to distinguish carbon sources and metabolic pathways (Farquhar *et al.*, 1989; Dawson *et al.*, 2002). Previous work has shown that obligate myco-heterotrophic plants that associate with ectomycorrhizal fungi are significantly enriched in the heavy isotopes of C and N compared to autotrophic understory plants, and have C and N isotope signatures similar to ectomycorrhizal fungi, their sole carbon and nitrogen source (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005). It has also been reported that some green orchids and pyroloids that associate with ectomycorrhizal fungi have carbon isotope values that are intermediate between autotrophs and myco-heterotrophs (Gebauer & Meyer, 2003; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). This finding indicates that these green plants can utilize at least two different trophic pathways and therefore tap into isotopically distinct C and N sources. One trophic pathway available to these plants is C gains through ectomycorrhizal fungi and N gains through a distinct (but undetermined) pathway compared to autotrophs, while the other pathway available is similar to that of autotrophic mycorrhizal plants. Plants that are capable of gaining nutrition through both of these complementary routes are referred to as mixotrophs or partial myco-heterotrophs (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). The relative enrichment in ^{13}C of mixotrophic orchids and pyroloids compared with neighboring autotrophic plants appears to be site specific and possibly influenced by light availability (Bidartondo *et al.*, 2004; McCormick *et al.*, 2004; Julou *et al.*, 2005; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Mixotrophic plants that associate with ectomycorrhizal fungi are also enriched in ^{15}N compared with surrounding autotrophic plants (Gebauer & Meyer, 2003). The mixotrophic abilities of pyroloids have been at the center of current debate because based on carbon stable isotope abundances the same species from different geographic regions appear to have varying degrees of mixotrophy (Tedersoo *et al.* 2007; Zimmer *et al.* 2007). The potential reasons for this variability among green pyroloids are further addressed here.

The goal of this study was to determine the trophic strategies of the green pyroloid *P. picta* and the achlorophyllous *P. aphylla*. In a previous study (Zimmer *et al.*, 2007), both *P. aphylla* and *P. picta* were analysed for their stable isotope values of C and N from a single site in northern California. The results of this work found *P. aphylla* to have isotope signatures for both elements that were similar to other ericaceous myco-heterotrophs;

while *P. picta* had a C isotope signature similar to surrounding autotrophs, but was enriched in ^{15}N similar to myco-heterotrophs that associate with ectomycorrhizal fungi. However, this study was based on a small sampling of the two *Pyrola* species, so the relevance of these findings to the overall distribution of the species is currently unknown. In the present study we sought to confirm these findings by determining the stable isotope signatures of C and N for *P. picta* and *P. aphylla* from more intensively sampled populations as well as sampling over a wider geographic region, and including an additional green pyroloid species (*Chimaphila umbellata*) whose isotope values have only been previously examined from a Bavarian forest. We then compared the isotope signatures of *P. aphylla*, *P. picta*, and *C. umbellata* with each other, and with autotrophic and obligate myco-heterotrophic plants to test for myco-heterotrophy and mixotrophy in the Pyroleae.

Materials and Methods

Study sites

To examine the trophic strategies of pyroloids from a wide geographic area of their natural ranges samples were collected from six National Forests in northern California and southern Oregon (USA) including El Dorado, Tahoe, Plumas, Lassen, Shasta and Willamette. The selection of sampling sites (P1–P10) was based on the presence of the target Pyroleae species: *P. aphylla* Sm. and *P. picta* Sm. All sites are dominated by second-growth mixed conifer forest at elevations between 700 m and 1400 m. Locations and species collected are summarized in Table 1.

Sampling scheme and species investigated

All samples were collected within an 8-d period from 30 June to 7 July 2006. Collection of target species' leaves or flower stalks, autotrophic reference plants' leaves and myco-heterotrophic plants' flower stalks was limited to an area of 2 m from a target species individual and sampling of autotrophic references was done only from understory saplings. This strategy was used to limit the variability of environmental factors such as atmospheric CO_2 concentrations and isotope signatures that could affect plant C isotope values or soil type that could affect N isotope values (Gebauer & Schulze, 1991). However, variation in the N isotope values of our samples that could result from possible differences in rooting depths of the plants were not accounted for (Robinson, 2001). Each collection site contained *P. aphylla* or *P. picta*, or both, plus a minimum of five individuals of at least one species that could be used as reference plants representing the autotrophic understory (Table 1). To test for differences in the isotope values between plant organs, whenever possible flowering stalks from *P. picta* were collected and analysed separately from leaves (Table 1). Four sites (P1, P2, P6 and P7) contained the obligate myco-heterotrophic species *Pterospora andromedea* Nutt. and *Corallorhiza maculata* (Raf.) Raf. and four sites (P1, P5, P6 and P7) contained the green pyroloid *Chimaphila umbellata* (L.) W. Bartram, (Table 1). A total of 37 *P. aphylla*, 42 *P. picta*, 18 *C. umbellata* individuals along with 17 obligate myco-heterotrophic plants of two different species, and 65 autotrophic reference plants of six species were collected.

Stable isotope analysis

Plant samples were oven-dried at 37°C and ground to a fine powder. Dried and ground samples were analysed for N and C stable isotope abundances using elemental analyser/continuous flow isotope ratio mass spectrometry at either the BayCEER–Laboratory of Isotope Biogeochemistry University of Bayreuth, Germany, as described by Bidartondo *et al.* (2004), or at the Center for Stable Isotope Biogeochemistry at University of California Berkeley, USA. Both laboratories used a dual-element analysis mode with a continuous-flow mass spectrometer coupled to an elemental analyser.: analysis at Berkeley was performed using a Europa ANCA-SL elemental analyzer coupled to a PDZ 20/20 Mass Spectrometer (Europa Scientific, Crewe, UK) while analysis at BayCEER was performed with a Carlo Erba 1108 (Carlo Erba Milan, Italy) coupled via a ConFlo III interface to a delta S (Finnigan MAT, Bremen, Germany). Abundances measured are denoted as δ values and are calculated according to the equation:

$$\delta^{15}\text{N or } \delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000\ [‰]$$

(R_{sample} and R_{standard} are the ratios of heavy isotope to light isotope of the samples and the respective standard). At the University of Bayreuth standard gases were calibrated with respect to international standards by using the reference substances N1 and N2 for the N isotopes and ANU sucrose and NBS 19 for the C isotopes (standards from the International Atomic Energy Agency, Vienna, Austria). At the University of California Berkeley, standards N2 and NIST 1577 bovine liver, or NIST 1547 peach leaf and corn flour, were used for N and C isotope calibrations, respectively (standards from the National Institute of Standards and Technology, Gaithersberg, MD, USA). In the Bayreuth laboratory the reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measures of the test substance acetanilide (Gebauer & Schulze, 1991). At least six test substances with varying sample weights were routinely analysed within each batch of 50 samples. Maximum variation of δ^{13}C and δ^{15}N within as well as between batches was always below 0.2‰. In the Berkeley laboratory the long-term precisions for δ^{13}C and δ^{15}N based on the laboratory's working standards (NIST 1577 bovine liver and sucrose solution) are: 0.1‰ for δ^{13}C and 0.2‰ for δ^{15}N. Differences between the two laboratories are not to be expected because both laboratories refer to internationally accepted standards.

Table 1 Location, species, number of individuals (*n*), plant parts collected for stable isotope analysis at each sampling site (P1–P10), and mean δ^{15}N (‰) and δ^{13}C (‰) values ± 1 SD

Site	Location	Species (*n*)	Plant part	Mean δ^{15}N ± 1 SD	Mean δ^{13}C ± 1 SD
P1	El Dorado N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–3.4 ± 0.9	–31.0 ± 0.3
	38°54′01.70″N 120°34′26.77″W	*Chimaphila umbellata*[a] (4)	Leaves	6.7 ± 0.9	–31.2 ± 0.4
		Corallorhiza maculata[b] (1)	Stalk/flower	10.9	–20.7
		Pterospora andromedea[b] (1)	Stalk/flower	5.7	–24.2
		Pyrola aphylla[a] (6)	Stalk/flower	16.4 ± 2.3	–24.0 ± 0.5
		Pyrola aphylla (1)	Leaves	12.4	–24.7
		Pyrola picta[a] (1)	Leaves	5.6	–31.9
P2	El Dorado N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–4.0 ± 0.7	–30.6 ± 0.7
	38°54′3.47″ N 120°34′28.40″ W	*Pterospora andromedea*[b] (3)	Stalk/flower	4.8 ± 1.1	–24.9 ± 0.6
		Pyrola aphylla[a] (1)	Stalk/flower	17.9	–22.2
		Pyrola picta[a] (4)	Leaves	4.9 ± 1.2	–31.9 ± 0.5
		Ribes roezlii[c] (5)	Leaves	–4.3 ± 1.2	–31.4 ± 0.6
P3	Tahoe N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–3.9 ± 0.7	–31.0 ± 0.8
	39°31′2.84″ N 120°59′26.46″ W	*Lithocarpus densiflora*[c] (5)	Leaves	–4.1 ± 1.5	–30.1 ± 0.9
		Pyrola aphylla[a] (3)	Stalk/flower	13.8 ± 0.2	–24.3 ± 0.5
P4	Tahoe N.F., CA	*Lithocarpus densiflora*[c] (5)	Leaves	–3.7 ± 1.0	–30.9 ± 0.4
	39°31′37.64″ N 120°59′25.47″ W	*Pyrola aphylla*[a] (6)	Stalk/flower	13.9 ± 3.4	–23.6 ± 0.2
		Pyrola aphylla (1)	Leaves	9.0	–27.1
		Pyrola picta[a] (3)	Leaves	8.6 ± 1.0	–29.1 ± 2.0
P5	Plumas N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–3.8 ± 1.1	–30.4 ± 1.0
	40°03′36.02″ N 120°51′32.99″ W	*Chimaphila umbellata*[a] (4)	Leaves	6.2 ± 1.9	–30.4 ± 1.6
		Pyrola aphylla[a] (3)	Stalk/flower	13.7 ± 1.5	–23.0 ± 0.4
P6	Plumas N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–5.5 ± 0.9	–30.0 ± 1.0
	40°03′29.94″ N 120°51′28.86″W	*Chimaphila umbellata*[a] (5)	Leaves	6.3 ± 1.8	–30.5 ± 0.9
		Pterospora andromedea[b] (3)	Stalk/flower	5.4 ± 0.9	–28.0 ± 0.1
		Pyrola aphylla[a] (2)	Stalk/flower	10.4	–24.7
			Stalk/flower	10.1	–23.9
		Pyrola picta[a] (12)	Leaves	5.0 ± 0.8	–30.3 ± 0.9
		Pyrola picta (2)	Stalk/flower	5.3	–28.5
			Stalk/flower	4.5	–30.6
P7	Plumas N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–3.7 ± 1.0	–31.6 ± 1.0
	40°04′00.17″ N 120°51′4.17″ W	*Corallorhiza maculata*[b] (4)	Stalk/flower	11.9 ± 1.1	–25.8 ± 0.3
		Chimaphila umbellata[a] (5)	Leaves	6.5 ± 1.5	–32.8 ± 0.6
		Pterospora andromedea[b] (5)	Stalk/flower	5.5 ± 1.0	–26.6 ± 0.4
		Pyrola aphylla[a] (2)	Stalk/flower	15.0	–25.2
			Stalk/flower	14.1	–25.7
		Pyrola picta[a] (6)	Leaves	8.2 ± 1.4	–32.0 ± 1.5
		Pyrola picta (1)	Stalk/flower	7.6	–31.7
P8	Lassen N.F., CA, USA	*Abies concolor*[c] (5)	Leaves	–2.9 ± 0.9	–30.9 ± 0.7
	40°13′39.97″ N 121°11′03.99″ W	*Pyrola aphylla*[a] (5)	Stalk/flower	13.7 ± 1.1	–23.9 ± 0.7
		Pyrola picta[a] (10)	Leaves	7.7 ± 3.1	–29.8 ± 1.4
P9	Shasta N.F., CA	*Pyrola aphylla*[a] (5)	Stalk/flower	15.5 ± 0.9	–24.3 ± 1.5
	41°00′44.90″ N 121°39′13.35″ W	*Pseudotsuga menziesii*[c](5)	Leaves	–5.3 ± 0.8	–30.7 ± 0.7
		Quercus kelloggii[c] (5)	Leaves	–2.5 ± 1.0	–30.8 ± 0.3
P10	Willamette N.F., OR, USA	*Pyrola aphylla*[a] (4)	Stalk/flower	14.6 ± 1.8	–24.6 ± 0.2
	44°18′36.00″ N 122°00′36.02″ W	*Pyrola picta*[a] (5)	Leaves	7.8 ± 1.1	–31.2 ± 1.0
		Tsuga heterophylla[c] (5)	Leaves	–2.2 ± 0.8	–31.9 ± 1.9

[a], Pyroloid; [b], myco-heterotroph; [c], autotroph; N.F., National Forest.

Statistics

Once δ values were obtained for all samples (Table 1), the δ^{15}N and δ^{13}C values of all reference plants for each collection site were tested for inter-site variation with a one-way ANOVA and Tukey's HSD. Owing to significant differences at an ∝0.05 among δ^{15}N values of the reference plants between sites (P6–P8 $P = 0.036$; P6–P10 $P = 0.002$) the δ values could not be pooled to make comparisons across sites. In order to make these comparisons δ values for both elements and all samples were converted into site-independent enrichment factors (ε). The calculation of enrichment factors is a useful method that eliminates the majority of the influence of spatial variation on isotope abundances and therefore allows for comparison among samples from different sites (Emmett *et al.*, 1998; Preiss & Gebauer, 2008) or substrates (Gebauer & Taylor, 1999). First, for each site the δ^{15}N and δ^{13}C values of all species of reference plants were averaged. Then, on a per site basis these averages were subtracted from all samples (pyroloids, reference and myco-heterotrophic plants) to create site-independent enrichment factors ($\varepsilon = \delta x_S - \delta x_R$) for each sample, where $\delta x_S = \delta^{15}$N or δ^{13}C of individual sample per site and δx_R = mean δ^{15}N or δ^{13}C of all reference plants per site. Thus, the resulting means of both the ε^{13}C and ε^{15}N factors of the reference plants is equal to 0‰ and individual samples' ε factors represent their difference from this mean. To test appropriately for differences between trophic groups (pyroloids, references and myco-heterotrophic plants) the variance around the mean δ values of the autotrophic references used to calculate ε for pyroloids and myco-heterotrophs must be retained. This is done through calculating ε not for only pyroloids and myco-heterotrophs but also the reference samples. Where the individual ε^{15}N and ε^{13}C factors of each autotrophic reference plant sampled represents the variance of these samples' δ values from the mean δ^{15}N or δ^{13}C of all references per site. Furthermore, both the intersite and intrasite standard deviation of the ε^{15}N and ε^{13}C factors for all reference species is small (≤ 1‰ for both ^{15}N and ^{13}C, Table 1). Statistical comparisons between all ε factors per group (pyroloids, myco-heterotrophic plants and autotrophic references) were made using nonparametric Kruskal–Wallis and sequential Bonferroni-corrected Mann–Whitney *U*-tests for *post hoc* comparisons. To make more robust comparisons between the pyroloids and obligate myco-heterotrophs in addition to the two myco-heterotrophic species (*P. andromedea* and *C. maculata*) collected at our sites we included the ε factors of seven fully myco-heterotrophic species: *C. maculata* ($n = 12$), *Sarcodes sanguinea* Torr. ($n = 14$), *P. andromedea* ($n = 13$), *Neottia nidus-avis* (L.) Rich. ($n = 31$), *Monotropa hypopitys* L. ($n = 9$), *Cephalanthera damasonium* L. albino ($n = 10$) and *Cephalanthera longifolia* (L.) Fritsch albino ($n = 9$) from previously published data (Preiss & Gebauer, 2008). For clarity, the ε factors of all species collected are reported in the results section and presented in Fig. 2 as species means ± 1 SD. In addition, the ε factors of the flowering stalks of *P. picta* were compared with those of their leaves and *P. aphylla* stalks from plots P6 and P7 using independent *t*-tests.

Results

Comparison of isotope signatures between trophic groups

The δ values of reference plants and myco-heterotrophic plants collected at our sites were within the range of previous records from temperate forests (Trudell *et al.*, 2003; Zimmer *et al.*, 2007 and Table 1). The enrichment factors (ε) of individual reference plants clustered *c.* 0‰, reflecting the small interspecific and intraspecific variations in their isotope signatures that were not significantly different between sites, while enrichment factors for the other groups (pyroloids and myco-heterotrophs) separated out into distinct groups based on the difference of their δ values from the mean of their respective references (Fig. 2). Across all sites the two green Pyroleae species were as strongly enriched in ^{15}N as the obligate myco-heterotrophs (*C. umbellata* average ε^{15}N = 10.6 ± 1.6‰; *P. picta* average ε^{15}N = 10.6 ± 1.9‰, Fig. 2). However, these two species were not enriched in ^{13}C compared to autotrophic reference plants (*C. umbellata* average ε^{13}C = −0.5 ± 1.0‰; *P. picta* average ε^{13}C = 0.3 ± 1.4‰; Fig. 2). By contrast, across all sites the achlorophyllous *P. aphylla* had a ^{13}C signature typical for myco-heterotrophic species associated with ectomycorrhizal fungi (average ε^{13}C = 6.9 ± 0.9‰, Fig. 2; see the Supporting Information, Table S1) and was enriched in ^{15}N (average ε^{15}N = 18.0 ± 2.2‰ Fig. 2, Table S1) compared with other pyroloids and surrounding autotrophs, similar to the findings of Zimmer *et al.* (2007). Interestingly, two *P. aphylla* plants in sites P1 and P4 had very small basal leaves (Fig. 1). These leaves were analysed separately for their isotope abundances. They were found to be similar to stalks of other *P. aphylla* collections for N (P1 ε^{15}N = 15.8‰, P4 ε^{15}N = 12.8‰), and similar (P1 ε^{13}C = 6.3‰) or less enriched in ^{13}C (P4 ε^{13}C = 3.8‰) indicating that at least in the latter individual extremely low levels of photosynthesis may still be taking place, similar to the leafless stems of *Corallorhiza trifida* (Zimmer *et al.*, 2008).

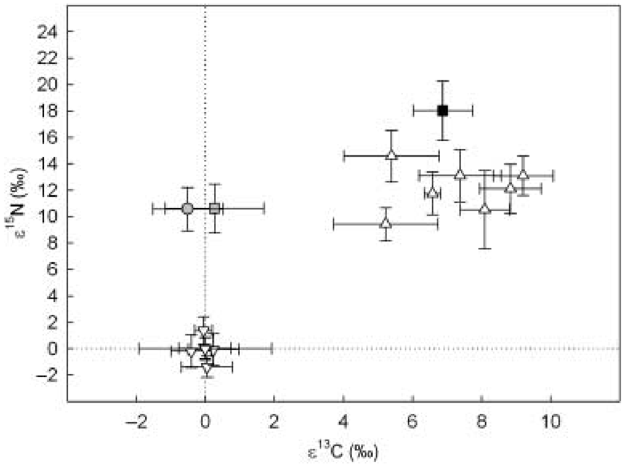

Fig. 2 Mean ^{13}C and ^{15}N enrichment factors (ε) of all species analysed: open downward triangles, autotrophic reference plants (two species values overlap); tinted circle, *Chimaphila umbellata*; tinted square, *Pyrola picta*; closed square, *Pyrola aphylla*; open upward triangles, myco-heterotrophic plants including *Pterospora andromedea* and *Corallorhiza maculata* from this and previously published studies (Preiss & Gebauer, 2008) and five additional species (*Sarcodes sanguinea*, *Neottia nidus-avis*, *Monotropa hypopitys*, *Cephalanthera damasonium* albino, and *Cephalanthera longifolia* albino) from Preiss & Gebauer (2008). Error bars represent 1 SD.

Independent *t*-tests revealed that comparisons of the enrichment factors of the flowering stalks of *P. picta* (average $\varepsilon^{15}N = 10.7 \pm 0.6‰$, $\varepsilon^{13}C = 0.2 \pm 1.1‰$) and *P. aphylla* (average $\varepsilon^{15}N = 17.0 \pm 1.5‰$, $\varepsilon^{13}C = 5.9 \pm 0.5‰$) at an $\propto 0.05$ were significantly different from each other for both elements ($\varepsilon^{13}C$ $P < 0.001$ and $\varepsilon^{15}N$ $P = 0.008$) and the isotope signatures of the stalks from *P. picta* were not statistically different from the leaves (average $\varepsilon^{15}N = 11.1 \pm 1.3‰$, $P = 0.639$; $\varepsilon^{13}C = -0.4 \pm 1.2‰$, $P = 0.452$). However, these tests were done with very low sample sizes as flowering stalks of *P. picta* were only collected from three plants in two sites (Table 1).

Discussion

Pyrola aphylla exhibited enrichment in ^{15}N that exceeded that of associated photosynthetic plants, other species in the Pyroleae, and even most other myco-heterotrophs analyzed. While the cause of this enrichment is unclear, it follows both the pattern of ^{15}N enrichment found in green mixotrophic Pyroleae species (Tedersoo *et al.* 2007; Zimmer *et al.* 2007; and data presented here) and all previously analysed myco-heterotrophic plants that associate with ectomycorrhizal fungi (Gebauer & Meyer, 2003; Trudell *et al.* 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Zimmer *et al.*, 2007). Possible mechanisms that could be driving the high ^{15}N enrichment found in myco-heterotrophs relative to autotrophs include a difference in the physiological processing of N by mycorrhizal fungi when in association with myco-heterotrophs and differences in N fractionation between fungal species (Gebauer & Taylor, 1999; Taylor *et al.*, 2003, 2004; Trudell *et al.* 2003; Nygren *et al.*, 2007). Similar to other ericaceous myco-heterotrophs, the N enrichment seen in *P. aphylla* is coupled with a less dramatic, though significant, enrichment in ^{13}C. Enrichment in ^{13}C is a well established pattern in ectomycorrhizal myco-heterotrophs where carbon is passed from autotrophs to ectomycorrhizal fungi and finally to the myco-heterotroph (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Leake, 2004).

It is interesting that even the green pyroloids from this study have a significant enrichment in ^{15}N compared with surrounding autotrophs as recently there has been debate regarding the mixotrophic abilities of green Pyroleae species (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Similar to the findings of Zimmer *et al.* (2007), this study found no evidence for C gain via mixotrophic means in either *P. picta* or *C. umbellata* adult plants. However, compared with *C. umbellata* individuals from Bavaria the samples from the western USA were more enriched in ^{15}N than autotrophic reference plants and the ^{15}N enrichment of both green pyroloid species from this study were more similar to myco-heterotrophic taxa other than *P. aphylla* (Fig. 2). We propose two potential possibilities for this pattern. First, although all pyroloid seedlings are myco-heterotrophic, once they develop leaves C gains are primarily through photosynthesis, but they continue to gain nitrogen through an unknown uptake mechanism similar to myco-heterotrophs. A second possibility is that C gains via a myco-heterotrophic strategy are still present, but the analysis of plants' bulk tissue C isotope abundances is not a sensitive enough method to detect these gains, which may only take place during certain seasonal, or plant developmental periods (Taylor *et al.*, 2004).

In previous studies a linear isotopic mixing model has been used to estimate per cent C and N gains via fungi in green pyroloids. This model is based on the enrichment factors of pyroloids that are statistically distinct (for either element) from those of surrounding autotrophs, which are then compared with the relative C and N enrichment of obligate myco-heterotrophs (Gebauer & Meyer, 2003; Preiss & Gebauer, 2008). However, because of the variability in isotope signatures of obligate myco-heterotrophs it is difficult to determine what species accurately represent the isotope signatures of the C and N pools actually accessed by the plants, and the choice of myco-heterotrophic end-members can affect the estimated levels of myco-heterotrophy in the new species being investigated. In the case of *P. aphylla*, if the per cent C and N gains via fungi were calculated using the mixing model first described by Gebauer & Meyer (2003) and a myco-heterotrophic end-member based on the mean relative enrichment of seven fully myco-heterotrophic plants associated with ectomycorrhizal fungi (Preiss & Gebauer, 2008) the estimated per cent C derived from fungal material would be 96 ± 12%. This indicates that *P. aphylla* essentially gains all of its C from a source that is similar to other fully myco-heterotrophic plants associated with ectomycorrhizal fungi. This conclusion fits well with the morphology of *P. aphylla*, which lacks photosynthetic organs. By contrast, if the same mixing model is used to calculate per cent N gain via myco-heterotrophy, *P. aphylla* would gain over 100% (149 ± 18%) of its N from the source(s) utilized by the myco-heterotrophic end-members. Thus the myco-heterotrophic species used as end-members in this scenario obviously do not fully represent the extent of variability in ^{15}N signatures of myco-heterotrophs. Until there is more definitive information on what factors drive the isotopic variability of myco-heterotrophs – especially in the case of ^{15}N enrichment – and the isotope signatures of the nutrient pools accessed by myco-heterotrophs, calculations of per cent C and N gains via fungi in putative mixotrophs and myco-heterotrophs must be viewed as rough estimates.

Final remarks

The evidence for myco-heterotrophy in *P. aphylla* is now compelling. Our results based on the isotope signatures of *P. aphylla* and *P. picta* support one of the hypotheses put forth by Camp (1940) and others that *P. aphylla* does indeed behave as a parasite 'deriving their food from the fungous [*sic*] mycelia associated with their roots'. Whereas, Haber (1987) assumed that *P. aphylla* was one of many morphological forms of *P. picta* connected by a rhizome to nearby leafy rosettes. Confirming these connections in the field between *P. aphylla* and surrounding *P. picta* plants is difficult as individual rhizomes can stretch for many meters in the soil. However, because of significant differences in the isotope signatures of *P. picta* and *P. aphylla* (Fig. 2) this study provides no substantiating evidence for rhizomatous connections between the two. Although there are reported differences among $\delta^{13}C$ values of plant organs (Willmer & Roksandic, 1980; Gebauer & Schulze, 1991; Badeck *et al.*, 2005; Bowling *et al.*, 2008) these differences are small compared with those found here between the leaves of *P. picta* and the flowering stalks of *P. aphylla*. Furthermore, when a small number of samples from the same plant organ (flowering stalks) from both plants were analysed for C and N isotope abundances they were significantly different from each other for both elements.

The confirmation that *P. aphylla* is a myco-heterotroph provides some insights into the order of the evolutionary steps toward obligate myco-heterotrophy, especially because its close relatives exhibit trends towards myco-heterotrophy. These trends include pyroloids' dependency in early stages of development on fungal nutrition (Leake, 1994), an association with ectomycorrhizal fungi shared with overstory trees that could allow for epiparasitism, an enrichment in ^{15}N similar to that of all ectomycorrhizal myco-heterotrophs studied to date and, though not found in this study, some green pyroloids have been found to be enriched in ^{13}C compared to surrounding autotrophs (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Similar approaches have been used to examine the transition to myco-heterotrophy in the orchids where the loss of photosynthesis is often coupled with an increase in specificity toward particular lineages of mycorrhizal fungi (Bidartondo *et al.*, 2004). Although the identities of the fungi associated with *P. aphylla* are yet to be determined, other closely related green pyroloids, including *P. picta*, have been found to associate with a suite of ericoid, endophytic and ectomycorrhizal fungi – the ectomycorrhizal fungi most likely providing the link between these plants and surrounding autotrophs (Robertson & Robertson, 1985; Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Massicotte *et al.*, 2008; Vincenot *et al.*, 2008). The elucidation of the fungal associates of *P. aphylla* is of great interest for the study of myco-heterotrophy as it may provide further insight into the evolution of these intriguing plants.

Acknowledgements

The authors thank Paul Brooks and Stefania Mambelli at the University of California Center for Stable Isotope Biogeochemistry and Christine Tiroch and Iris Schmiedinger at the University of Bayreuth for assistance with the isotope analysis of samples as well as Rachel Adams and Diana Jolles for field assistance. The authors would also like to acknowledge the valuable input from three anonymous reviewers on earlier versions of this manuscript. Partial funding for this project was awarded to N.A.H. from the Institute of European Studies and the California Native Plant Society.

References

Abadie J-C, Püttsepp Ü, Gebauer G, Faccio A, Bonfante P, Selosse M-A. 2006. *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and nonphotosynthetic individuals. *Canadian Journal of Botany* **84**: 1462–1477.

Badeck FW, Tcherkez G, Nogues S, Piel C, Ghashghaie J. 2005. Post photosynthetic fractionation of stable carbon isotopes between plant organs – a widespread phenomenon. *Rapid Communications in Mass Spectrometry* **19**: 1381–1391.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society Biological Sciences Series B, Biological Sciences* **271**: 1799–1806.

Bowling DR, Pataki DE, Randerson JT. 2008. Carbon isotopes in terrestrial ecosystem pools and CO_2 fluxes. *New Phytologist* **178**: 24–40.

Camp WH. 1940. Aphyllous forms in *Pyrola*. *Bulletin of the Torrey Botanical Club* **67**: 453–465.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. 2002. Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics* **33**: 507–559.

Emmett BA, Kjönaas OJ, Gundersen P, Koopmans C, Tietema A, Sleep D. 1998. Natural abundance of ^{15}N in forests across a nitrogen deposition gradient. *Forest Ecology and Management* **101**: 9–18.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 503–537.

Freudenstein JV. 1999. Relationships and character transformation in Pyroloideae (Ericaceae) based on its sequences, morphology, and development. *Systematic Botany* **24**: 398–408.

Gebauer G, Meyer M. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist* **160**: 209–223.

Gebauer G, Schulze ED. 1991. Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia* **87**: 198–207.

Gebauer G, Taylor AFS. 1999. ^{15}N natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. *New Phytologist* **142**: 93–101.

Haber E. 1987. Variability distribution and systematics of *Pyrola picta* sensu-lato Ericaceae in western North America. *Systematic Botany* **12**: 324–335.

Henderson MW. 1919. *A comparative study of the structure and saprophytism of the Pyrolaceae and Monotropaceae with reference to their derivation from the Ericaceae*. PhD thesis. Philadelphia, PA, USA: University of Pennsylvania.

Holm T. 1898. *Pyrola aphylla*: a morphological study. *Botanical Gazette* **25**: 246–254.

Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse M-A. 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. *New Phytologist* **166**: 639–653.

de Jussieu AL. 1789. *Genera Plantarum, secundum ordines naturales disposita juxta methodum in Horto Regio Parisiensi exaratam*. Apud viduam Herissant, typographum, viâ novâ B.M. sub signo Crucis Aureæ. Et Theophilum Barrois, ad ripam Augustinianorum. Paris, France: Herissant.

Kron KA, Johnson SL. 1997. Phylogenetic analysis of the monotropoids and pyroloids (Ericaceae) using nrITS and 18s sequence data. *American Journal of Botany* **84**: 205–206.

Kron KA, Judd WS, Stevens PF, Crayn DM, Anderberg AA, Gadek PA, Quinn CJ, Luteyn JL. 2002. Phylogenetic classification of Ericaceae: molecular and morphological evidence. *Botanical Review* **68**: 335–423.

Leake JR. 1994. Tansley review: the biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist* **127**: 171–216.

Leake JR. 2004. Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. *Current Opinion in Plant Biology* **7**: 422–428.

Massicotte HB, Melville LH, Tackaberry LE, Peterson RL. 2008. A comparative study of mycorrhizas in several genera of Pyroleae (Ericaceae) from western Canada. *Botany-Botanique* **86**: 610–622.

McCormick MK, Whigham DF, O'Neill J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* **163**: 425–438.

Nygren CMR, Edqvist J, Elfstrand M, Heller G, Taylor AFS. 2007. Detection of extracellular protease activity in different species and genera of ectomycorrhizal fungi. *Mycorrhiza* **17**: 241–248.

Preiss K, Gebauer G. 2008. A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes in Environmental and Health Studies* **44**: 393–401.

Robertson DA, Robertson JR. 1985. Ultrastructural aspects of *Pyrola* mycorrhizae. *Canadian Journal of Botany* **63**: 1089–1098.

Robinson D. 2001. Delta ^{15}N as an integrator of the nitrogen cycle. *Trends in Ecology and Evolution* **16**: 153–162.

Smith JE. 1814. *Pyrola*. In: Rees A, ed. *The cyclopedia or universal dictionary of arts, sciences, and literature*, vol. 29, London, UK: Longman, Hurst, Rees, Orme and Brown.

Taylor AFS, Fransson PM, Högberg P, Högberg MN, Plamboeck AH. 2003. Species level patterns in ^{13}C and ^{15}N abundance of ectomycorrhizal and saprotrophic fungal sporocarps. *New Phytologist* **159**: 757–774.

Taylor AFS, Gebauer G, Read DJ. 2004. Uptake of nitrogen and carbon from double-labelled (^{15}N and ^{13}C) glycine by mycorrhizal pine seedlings. *New Phytologist* **164**: 383–388.

Taylor DL, Bruns TD, Leake JR, Read DJ. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. In: Sanders IR, van der Heijden M, eds. *The ecology of mycorrhizas*. Ecological Studies vol. 157. Berlin, Germany: Springer-Verlag, 375–414.

Tedersoo L, Pellet P, Kõljalg U, Selosse M-A. 2007. Parallel evolutionary paths to myco-heterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. *Oecologia* **151**: 206–217.

Trudell SA, Rygiewicz PT, Edmonds RL. 2003. Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytologist* **160**: 391–401.

Vincenot L, Tedersoo L, Richard F, Horcine H, Kõljalg U, Selosse M-A. 2008. Fungal associates of *Pyrola rotundifolia*, a mixotrophic Ericaceae, from two Estonian boreal forests. *Mycorrhiza* **19**: 15–25.

Willmer CM, Roksandic Z. 1980. Carbon isotope discrimination by tissues and organs from oat and barley inflorescences. *Journal of Experimental Botany* **31**: 1493–1495.

Zimmer K, Hynson NA, Gebauer G, Allen EB, Allen MF, Read DJ. 2007. Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. *New Phytologist* **175**: 166–175.

Zimmer K, Meyer C, Gebauer G. 2008. The ectomycorrhizal specialist orchid *Corallorhiza trifida* is a partial myco-heterotroph. *New Phytologist* **178**: 395–400.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Results of sequential Bonferroni-corrected Mann–Whitney *U*-tests for *post hoc* comparisons