

Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids

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Summary

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• Stable isotope abundance analyses recently revealed that some European green orchids and pyroloids (Ericaceae) are partially myco-heterotrophic, exploiting mycorrhizal fungi for organic carbon and nitrogen. Here we investigate related species to assess their nutritional mode across various forest and climate types in Germany and California.

• C- and N-isotope signatures of five green pyroloids, three green orchids and several obligate myco-heterotrophic species (including the putatively fully myco-heterotrophic *Pyrola aphylla*) were analysed to quantify the green plants' nutrient gain from their fungal partners and to investigate the constancy of enrichment in ¹³C and ¹⁵N of fully myco-heterotrophic plants from diverse taxa and locations relative to neighbouring autotrophic plants.

• All green pyroloid and one orchid species showed significant ¹⁵N enrichment, confirming incorporation of fungi-derived N compounds while heterotrophic C gain was detected only under low irradiance in *Orthilia secunda*. *Pyrola aphylla* had an isotope signature equivalent to those of fully myco-heterotrophic plants.

• It is demonstrated that primarily N gain from mycorrhizal fungi occurred in all taxonomic groups investigated across a wide range of geographical and ecological contexts. The ¹³C and ¹⁵N enrichment of obligate myco-heterotrophic plants relative to accompanying autotrophic plants turned out as a fairly constant parameter.

Key words: carbon (C) and nitrogen (N) nutrition, Ericaceae, monotropoids, mycorrhiza, partial and full myco-heterotrophy, orchids, *Pyrola*, stable isotopes.

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Introduction

More than 400 species of vascular plants have only traces of chlorophylls, and are almost completely dependent on their mycorrhizal fungal symbionts for supplies of C and N. Such 'myco-heterotrophic' (MH; Leake, 1994) plants occur frequently in the Orchidaceae, and one of their ecological attributes is that they are independent of irradiance and able

to colonize deeply shaded forest habitats. In contrast to the majority of autotrophic orchids that form mycorrhizas with fungi of the polyphyletic rhizoctonia group (Bernard, 1909), many MH orchids form mycorrhizas with fungi that are ectomycorrhizal (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004).

Fungal tissues and the nutrients derived from heterotrophic sources are enriched in the heavy stable isotopes of N (Gebauer & Dietrich, 1993) and C (Gleixner *et al.*, 1993) relative to

those of accompanying autotrophs. Trudell *et al.* (2003) observed that the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of a diverse range of MH species were similar to those of their ectomycorrhizal fungal associates. Similarly, Gebauer & Meyer (2003) concluded on the basis of natural stable isotope abundance that some green and hence putatively autotrophic orchids gained C and N from their fungal symbionts in addition to the C obtained by photosynthesis and the N from the soil. This mixed mode of nutrition, which involves the acquisition of C and N through association with fungi as well as through autotrophic processes, has been referred to as partial myco-heterotrophy (Gebauer & Meyer, 2003).

While partial myco-heterotrophy has been well documented in the Orchidaceae (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Leake, 2004; Selosse *et al.*, 2004; Bidartondo, 2005; Gebauer, 2005; Julou *et al.*, 2005; Abadie *et al.*, 2006), its occurrence beyond monocotyledonous plants was found only recently within the Ericaceae (Tedersoo *et al.*, 2007). The dicotyledonous family Ericaceae contains lineages of very closely related tribes which, as in the case of Orchidaceae, contain some species that are fully MH and others that are autotrophic. The tribes Monotropeae and Pterosporeae (Kron *et al.*, 2002) (subsequently referred to here as 'monotropoids') consist entirely of ectomycorrhizal basidiomycete-dependent MH species that form distinctive monotropoid mycorrhizas (Duddridge & Read, 1982), while the tribe Pyroleae (subsequently referred to here as 'pyroloids') consists, with the exception of one putatively MH species (*Pyrola aphylla*), of green and hence putatively autotrophic species, forming ectendo or arbutoid mycorrhizas (Smith & Read, 1997). All other tribes of the Ericaceae are, as far as is known, fully autotrophic. Tedersoo *et al.* (2007) identified several endophytic and ectomycorrhizal fungal associates on pyroloid roots, and found that the C and N nutrition of four green pyroloid species (*Chimaphila umbellata*, *Orthilia secunda*, *Pyrola chlorantha*, *Pyrola rotundifolia*) from two Estonian boreal coniferous forests relies partly on fungi.

This study on nutrient gain from fungi extends the spectrum of species investigated to a wide range of geographical and ecological circumstances, and is the first work to include isotope data of *P. aphylla* – the sole putatively obligate MH pyroloid. Material of five green pyroloid species (*C. umbellata*, *O. secunda*, *P. chlorantha*, *Pyrola minor*, *Pyrola picta*) and three green orchids (*Epipactis gigantea*, *Goodyera oblongifolia*, *Platanthera leucostachys*), and of neighbouring autotrophic and MH plants, was collected from 12 sites in Germany, northern California and southern California varying from temperate to Mediterranean-type climate, and from dense and very dark deciduous forest sites at low elevations to open mixed evergreen forest sites at elevations between 1500 and 1900 m asl. Mycorrhizal fungi of the green pyroloids investigated were identified using molecular analysis, and leaf isotope signatures of pyroloid and orchid species were compared to autotrophic and MH reference plants. For each pyroloid or orchid that

showed evidence of nutrient gain from fungi, the percentage of fungi-derived N and C was calculated. To standardize one parameter of these calculations, the stability of enrichment in ^{15}N and ^{13}C of obligate MH species from various taxonomic groups and locations (*Corallorhiza maculata*, *Neottia nidus-avis*, albino *Cephalanthera damasonium*, albino *Cephalanthera longifolia*, *Monotropa hypopitys*, *Pterospora andromedea*, *Sarcodes sanguinea*) relative to accompanying obligate autotrophic plants was investigated, yielding a mean enrichment factor. This uniform enrichment factor represents a suitable reference point for such calculations if fully myco-heterotrophic plants are missing at a study site.

Materials and Methods

Study sites

Samples were collected from six forest sites in Germany (G1–G6) and six in California (C1–C6). The German sites are located in north-east Bavaria (49°40' to 49°55' N and 10°49' to 11°32' E) at 300–520 m elevation with mean annual precipitation of 650–1000 mm, mean annual temperature of 6–9°C (German weather service, <http://www.dwd.de>) and a large gradient in relative light availability to the forest understorey. Site G1 is a dense and very dark broadleaf forest dominated by *Fagus sylvatica* with a sparse and patchy cover of understorey vegetation. Site G2 is a clearing in the same forest. Sites G3–G5 are located in an open *Pinus sylvestris* stand with a species-rich understorey. The overstorey is interspersed with some *Quercus rubra*, *Quercus robur* and *Fagus sylvatica* trees. Site G6, an ancient sandpit, is an open stand mainly composed of *P. sylvestris*, *Picea abies*, *F. sylvatica*, *Q. robur* and *Corylus avellana* with an understorey dominated by mosses. Five of the Californian sites (C1–C5) are located in the San Bernardino and San Jacinto Mountains in southern California (34°04' to 34°14' N and 116°47' to 117°14' W) at elevations between 1500 and 1900 m asl, and they are open mixed pine forests dominated by *Pinus ponderosa* and *Quercus kelloggii* with a sparse understorey of herbs and shrubs that vary in composition between sites (Allen *et al.*, 2007). Mean annual precipitation accounts for 600–1100 mm and mean annual temperature is 9–15°C (<http://www.cimis.water.ca.gov>, <http://www.jamesreserve.edu>). The six German sites (Harrison *et al.*, 2000), and the sites in southern California (Allen *et al.*, 2007) are characterized by considerably high atmospheric N-deposition rates. Site C6 is a Sierran mixed conifer forest situated at the University of California's Blodgett Experimental Forest in northern California (38°54' N, 120°39' W) at an elevation of approx. 1340 m, a mean annual precipitation of 1600 mm and mean annual temperature of 11.2°C (<http://ecology.cnr.berkeley.edu/properties/blodgett.html>). The forest is dominated by *Abies concolor* and *Pseudotsuga menziesii* with a sparse understorey and a midstorey composed mainly of *Q. kelloggii* and *A. concolor* saplings.

Sampling scheme and species investigated

Sampling was performed in 2001 (southern California, June/July) and in 2005 (Germany, June/July; northern California, August). Five 1-m² plots were selected for all 12 sites, with the exception of C6 where three plots were selected. Each plot included a green pyroloid and/or orchid species and at least two autotrophic reference plant species (excepting pyroloids and orchids) of different functional groups and growth forms. Most of the plots (except for sites G6 and C5) also contained fully myco-heterotrophic plants. Leaf material of all plants was taken following the criteria described by Gebauer & Meyer (2003). In total, 48 plant species ($n = 386$), including five green pyroloids, three green orchids, one MH pyroloid species, two MH orchids and three MH monotropoids, were sampled (Table S1 in Supplementary material). The reference species were grouped into five functional types according to their mycorrhiza (Wang & Qiu, 2006) or their ability to utilize atmospheric N₂: ectomycorrhizal plants (ECM, $n = 99$), ericoid mycorrhizal plants (ERI, $n = 20$), arbutoid mycorrhizal plants (ARB, $n = 23$), plants forming arbuscular mycorrhizas or nonmycorrhizal plants (AM/NON, $n = 109$), and plants potentially living in symbiosis with N₂-fixing microbes (FIX, $n = 15$). Sampled pyroloids, orchids and monotropoids were categorized as either green (GP/GO, $n = 64$) or MH (MHP/MHO/MHM, respectively, $n = 56$). At each site, soil samples from the organic layer were taken ($n = 100$). Root samples were collected from all green pyroloid individuals at sites G1–G6 and C6 for identification of mycorrhizal fungi.

Light measurements

At the beginning of July, relative light availability under the forest canopy (%) at sites G1–G6 was calculated by comparing PAR measurements (Quantum Sensor, Li-Cor, Lincoln, NE, USA) at the forest ground and outside the forest.

Stable isotope abundance analysis

Leaf and soil samples were oven-dried and ground to a fine powder. Relative N and C isotope abundances of all samples from sites G1–G6 and C1–C5 were measured using a dual element analysis mode with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer as described by Bidartondo *et al.* (2004). Samples from northern California (C6) were analysed in a dual element analysis mode with a continuous flow mass spectrometer (20/20 mass spectrometer, PDZ Europa Ltd, Northwich, UK). Measured abundances are denoted as δ values, calculated according to the following equation:

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

where R_{sample} and R_{standard} are the ratios of heavy isotope to light isotope of the samples and the respective standard.

Standard gases were calibrated with respect to international standards using the reference substances N1 and N2 for the nitrogen isotopes and ANU sucrose and NBS19 for the carbon isotopes. The samples from site C6 were analysed using standards N2 and NIST 1577 bovine liver, or NIST 1547 peach leaf and corn flour, respectively.

Statistics

Data sets from the three major regions (Germany, southern California and northern California) were tested for differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ using the Kruskal–Wallis nonparametric test and Bonferroni-corrected Mann–Whitney *U*-tests for *post hoc* comparisons. Afterwards, δ values within resulting groups were tested for normal distribution and homogeneity of variances, and compared by ANOVA and Tukey's honestly significant difference.

Calculation of C and N gains from fungi

For all green pyroloids and orchids that were significantly different from reference plants in $\delta^{15}\text{N}$ and/or $\delta^{13}\text{C}$, a linear two-source isotopic mixing model, as described by Gebauer & Meyer (2003), was used to calculate the relative contribution of N or C derived from fungal material to the N or C content of the green pyroloids and orchids (% x_{df} where $x = \text{N or C}$). The model is based on individual δ values of the green pyroloids and orchids (δx_{GP} or δx_{GO}), mean δ values of co-occurring reference plants (δx_{REF}), and the mean enrichment factor (ϵ) of fully MH plants ($\epsilon_{\text{MH-REF}} = \delta x_{\text{MH}} - \delta x_{\text{REF}}$; Table 1): % $x_{\text{df}} = (\delta x_{\text{GO/GP}} - \delta x_{\text{REF}}) / \epsilon_{\text{MH-REF}} \times 100$. Mean % x_{df} values were tested for significant difference from zero (no N or C gain from fungi) using Student's *t*-test. Data are given as means ± 1 SD.

Molecular identification of mycorrhizal fungi

Individual fine root tips from single *Pyrola*, *Orthilia* and *Chimaphila* plants that appeared to be colonized by fungi during microscopical examination were separated from the rhizome and placed in lysis buffer. Samples were frozen and thawed three times before grinding the softened tissue with a micropestle. Genomic DNA of samples from German sites (G1–G6) was extracted following methods described elsewhere (Gardes & Bruns, 1993) but using GeneClean (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. For DNA extraction from *P. picta* roots (C6), Qiagen DNeasy kit (Qiagen, Valencia, CA, USA) was used following the manufacturer's instructions, with the extracted DNA eluted into 50 μl AE buffer. Using PCR, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F and ITS4 and PCR conditions described by Gardes & Bruns (1993). Positive PCR products were purified using QIAquick 96 kits (Qiagen) (samples from G1–G6), or 0.5 μl Exosap-IT (GE Healthcare, Piscataway,

Table 1 Relative enrichment for ^{15}N and ^{13}C in leaves of fully myco-heterotrophic orchids (MHO), pyroloids (MHP) and monotropoids (MHM)

Site	Myco-heterotrophic species (functional group)	^{15}N $\epsilon_{\text{MH-REF}}$ (‰)	^{13}C $\epsilon_{\text{MH-REF}}$ (‰)
<i>Fagus sylvatica</i> forest (G1)	<i>Neottia nidus-avis</i> (MHO)	7.6	7.9
<i>Fagus sylvatica</i> forest clearing (G2)	<i>Neottia nidus-avis</i> (MHO)	10.5	7.9
<i>Pinus sylvestris/Quercus robur</i> forest (G3–G5)	<i>Monotropa hypopitys</i> (MHM)	12.1	7.9
Mixed pine forest (C1)	<i>Corallorhiza maculata</i> (MHO)	11.9	6.7
	<i>Sarcodes sanguinea</i> (MHM)	15.7	4.8
Mixed pine forest (C2)	<i>Corallorhiza maculata</i> (MHO)	13.6	7.8
	<i>Sarcodes sanguinea</i> (MHM)	14.3	6.6
Mixed pine forest (C3)	<i>Pterospira andromedeae</i> (MHM)	9.5	5.3
Mixed pine forest (C4)	<i>Pterospira andromedeae</i> (MHM)	8.9	4.7
	<i>Sarcodes sanguinea</i> (MHM)	12.0	4.3
Mixed conifer forest (C6)	<i>Pterospira andromedeae</i> (MHM)	8.0	6.5
	<i>Pyrola aphylla</i> (MHP)	13.8	7.8
<i>Fagus sylvatica</i> forest (S.E. Germany)	<i>Neottia nidus-avis</i> (MHO)*	12.2	8.4
	<i>Neottia nidus-avis</i> (MHO)†	11.6	8.1
<i>Quercus robur/Corylus avellana</i> forest (N. France)	<i>Cephalanthera damasonium</i> albino (MHO)‡	13.9	9.2
<i>Juniperus communis/Pinus sylvestris</i> shrubland (Estonia)	<i>Cephalanthera longifolia</i> albino (MHO)§	11.8	6.6
Mean \pm 1 SD		11.7 \pm 2.3	6.9 \pm 1.5

The table includes samples from this and other studies: *, Gebauer & Meyer (2003); †, Bidartondo *et al.* (2004); ‡, Julou *et al.* (2005); §, Abadie *et al.* (2006). Enrichment factors ($\epsilon_{\text{MH-REF}}$) represent the difference between mean δ values in leaves of reference plants and mean δ values of myco-heterotrophic plants collected at the respective sites. Mean $\epsilon_{\text{MH-REF}}$ are used for further calculations (Table 3). For numbers of replicates and site codes see Table S1. Values from the literature are based on $n = 5$.

NJ, USA) combined with 3.5 μl PCR product and 1 μl dH_2O (samples from C6). DNA sequencing was performed on an ABI3100 Genetic Analyzer using BigDYE ver. 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using SEQUENCE NAVIGATOR ver. 1.0.1 (Applied Biosystems) or SEQUENCHER ver. 4.2.2 (Gene Codes Corporation, Ann Arbor, MI, USA). All samples with strong PCR amplification of single templates were blasted in GenBank to ascertain taxonomic affinity. For C6 samples, all the sequences that appeared to contain more than one template were cloned using the TOPO TA Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Positive clones were picked, amplified with plasmid primers T3 and T7, sequenced, and visually aligned at 95% similarity using SEQUENCHER ver. 4.2.2. From the clone library, the longest fragment of each dominant haplotype was then blasted in GenBank to ascertain taxonomic affinity to the genus level. The GenBank accession numbers are EF372399–EF372410 (samples from G1–G6) and EF101760–EF101777 (C6 samples).

Results

Comparison of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between functional groups

Within most of the sites, leaves of plants belonging to the functional groups ECM, AM/NON, ERI and ARB showed

similar ^{15}N and ^{13}C abundances. Consequently, data of these groups were pooled for each site, forming one group of reference plants (REF) and describing one endpoint of the linear two-source isotopic mixing model. Because of their association with N-fixing bacteria, FIX plants can use atmospheric N_2 ($\delta^{15}\text{N} = 0\text{‰}$), in addition to soil mineral N, which leads to $\delta^{15}\text{N}$ values generally closer to zero and makes them unsuitable as N references. Data from fully MH plants (contributing to the other endpoint of the mixing model) were pooled as well for each site (MH).

Except for G6 and C5, which did not include MH plants, all sites showed significant differences in N and C isotopic composition between functional groups (Table S2). Compared to the respective autotrophic references, MH plants were significantly and highly enriched in ^{15}N and ^{13}C at all sites. All green pyroloids from Germany and California were consistently and significantly enriched in ^{15}N compared to the respective REF plants (Figs 1, 2). The $\delta^{15}\text{N}$ values in leaves of some green pyroloids – *O. secunda* at a dark beech forest (Fig. 1a), *P. chlorantha* (Fig. 1d), *P. picta* (Fig. 2c,d,f) – were even not statistically different from those of MH plants at the same sites. All other green pyroloids had an N isotopic composition intermediate between the REF and the MH group. The green orchid *P. leucostachys* (C2, C5) was also significantly enriched in ^{15}N compared to the respective autotrophic references, and depleted in ^{15}N compared to the MH plants, whereas the $\delta^{15}\text{N}$ values of *E. gigantea* (C4) and *G. oblongifolia* (C6) were not significantly different from those of REF plants. Concerning ^{13}C abundances, the effects on isotopic composition

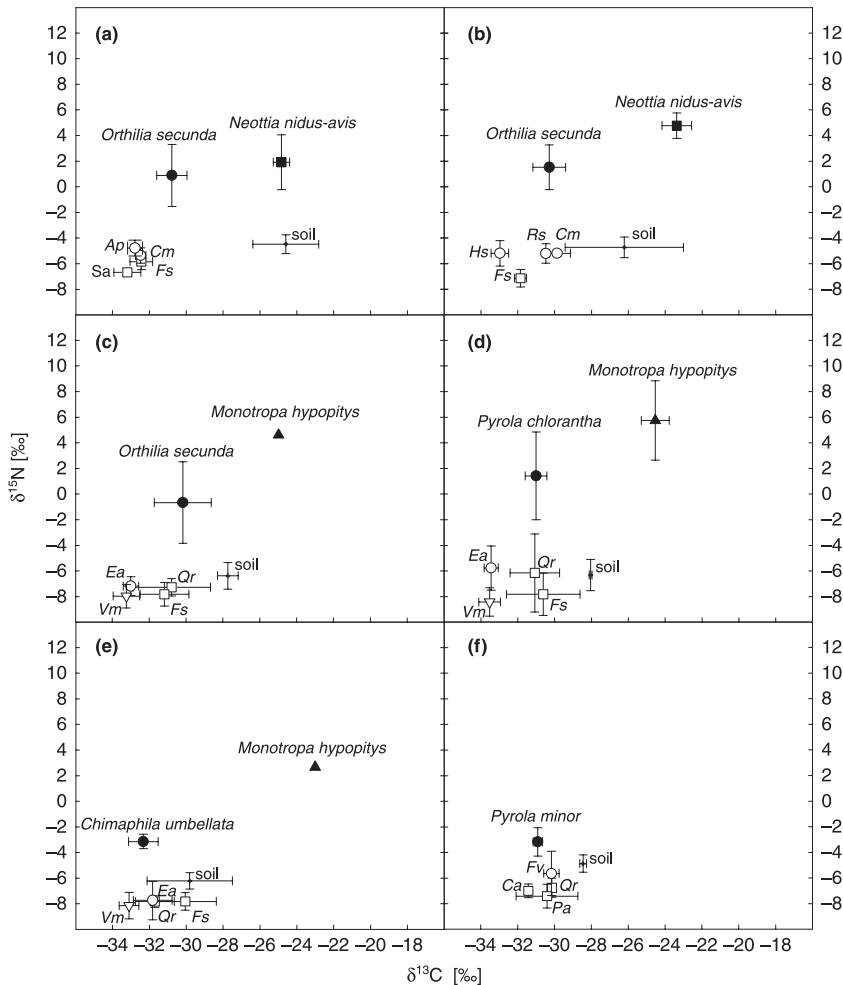


Fig. 1 Mean (± 1 SD) values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in leaves of four green pyroloid species, two fully myco-heterotrophic species, 12 fully autotrophic reference species and in soil samples collected from six forest sites in north-eastern Bavaria, Germany: (a) *Fagus sylvatica* forest G1; (b) *F. sylvatica* forest clearance G2; (c–e) mixed *Pinus sylvestris* forest G3–G5; (f) mixed conifer forest G6. Symbols represent classification according to functional groups: ectomycorrhizal reference plants (ECM, \square), arbuscular mycorrhizal or nonmycorrhizal reference plants (AM/NON, \circ), ericoid mycorrhizal reference plants (ERI, ∇), green pyroloids (GP, \bullet), myco-heterotrophic orchids (MHO, \blacksquare), myco-heterotrophic monotropoids (MHM, \blacktriangle). For numbers of replicates see Table S1. Error bars are missing if $n < 3$. Plant species: Ap, *Acer pseudoplatanus*; Ca, *Corylus avellana*; Cm, *Convallaria majalis*; Ea, *Epilobium angustifolium*; Fs, *Fagus sylvatica*; Fv, *Fragaria vesca*; Hs, *Hieracium sylvaticum*; Pa, *Picea abies*; Qr, *Quercus robur*; Rs, *Rubus saxatilis*; Sa, *Sorbus aucuparia*; Vm, *Vaccinium myrtillus*.

within green pyroloids and green orchids were weak. Except for *O. secunda* from a very dark beech forest (G1), which had an isotopic position intermediate between REF and MH plants (Fig. 1a), no significant differences in $\delta^{13}\text{C}$ were detected between green pyroloids, green orchids and their references.

Comparison of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between sites

According to the Kruskal–Wallis test ($P < 0.001$ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and *post hoc* comparisons, leaf $\delta^{15}\text{N}$ values of reference plants from the three major regions (Germany, southern California, northern California) differed significantly from each other and were positively correlated with the mean $\delta^{15}\text{N}$ values of the respective soil samples. Because of the Mediterranean climate, $\delta^{13}\text{C}$ signatures of reference plants from southern California were significantly less negative than those of northern California and Germany. Differences were also found between samples from southern and northern California (Mann–Whitney *U*-test, $P < 0.001$ after Bonferroni correction) but not between samples from Germany and

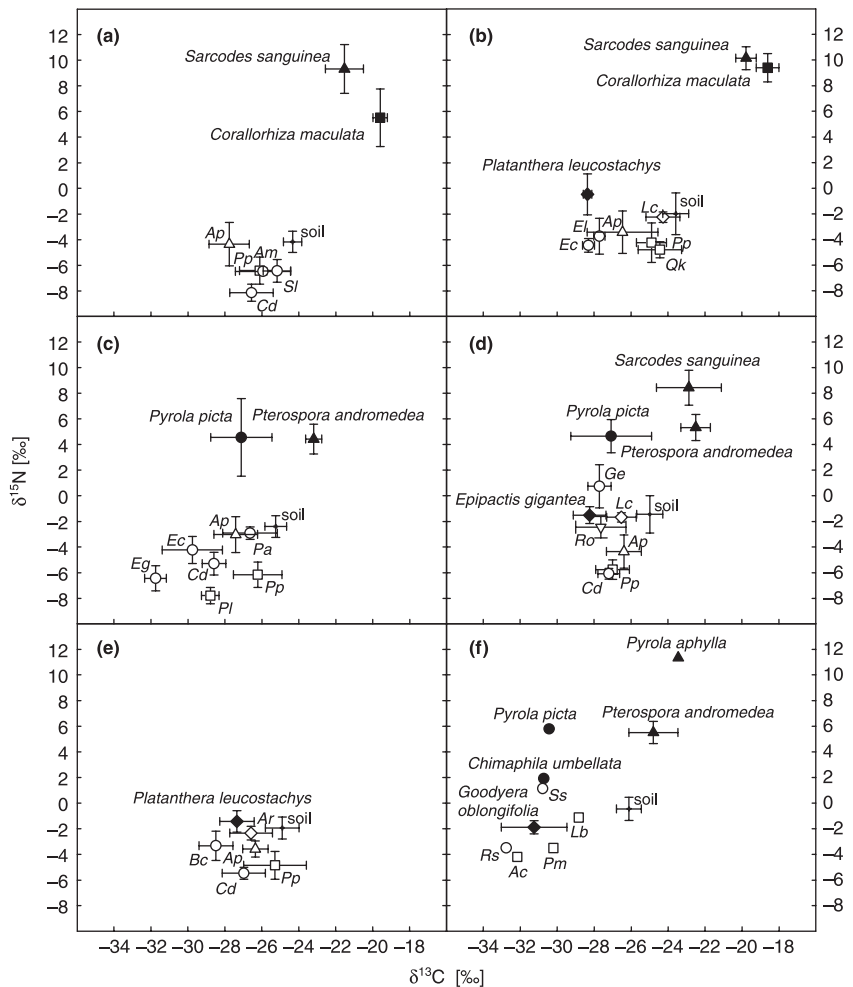
northern California (Mann–Whitney *U*-test, $P = 1$ after Bonferroni correction). Subsequent ANOVAs within regions were conducted with respect to the results of the Mann–Whitney *U*-tests.

As shown by the site ANOVAs (Tables S3 & S4), ^{15}N and ^{13}C abundances in the soil and in leaves of REF plants varied in a highly significant manner between sites, even within major regions ($P < 0.001$). These variations demonstrate that data from different sites cannot be pooled and must be analysed separately. However, despite varying δ values between sites in reference plants and soil samples, the enrichment factor of obligate MH plants relative to accompanying autotrophic plants ($\epsilon_{\text{MH-REF}}$) remained considerably stable, irrespective of taxonomic groups or climatic conditions.

Mycorrhizal fungi of pyroloid roots

Light microscope observations revealed that roots of all pyroloid species investigated were colonized by hyphae forming a Hartig net and intracellular coils restricted to the

Fig. 2 Mean (± 1 SD) values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in leaves of two green pyroloid species, three green orchids, four fully myco-heterotrophic species, 22 fully autotrophic reference species and in soil samples collected from five forest sites in southern California: (a–e) mixed pine–oak forest C1–C5; and from one site in northern California: (f) mixed conifer forest C6. Symbols represent classification according to functional groups: ectomycorrhizal reference plants (ECM, \square), arbuscular mycorrhizal or nonmycorrhizal reference plants (AM/NON, \circ), arbutoid mycorrhizal references (ARB, \triangle), ericoid mycorrhizal references (ERI, ∇), reference plants potentially living in symbiosis with N_2 -fixing microbes (FIX, \diamond), green orchids (GO, \blacklozenge), green pyroloids (GP, \bullet), myco-heterotrophic orchids (MHO, \blacksquare), myco-heterotrophic pyroloids/monotropoids (MHP/MHM, \blacktriangle). For numbers of replicates see Table S1. Error bars are missing if $n < 3$. Plant species: *Ac*, *Abies concolor*; *Am*, *Acer macrophyllum*; *Ap*, *Arctostaphylos patula*; *Ar*, *Alnus rhombifolius*; *Bc*, *Bromus carinatus*; *Cd*, *Calocedrus decurrens*; *Ee*, *Elymus elymoides*; *Eg*, *Elymus glaucus*; *Ec* (part c), *Epilobium canum*; *Ec* (part b), *Eriophyllum confertifolium*; *Ge*, *Glyceria elata*; *Lb*, *Linnea borealis*; *Lc*, *Lotus crassifolius*; *Pa*, *Pteridium aquilinum*; *Pl*, *Pinus lambertiana*; *Pm*, *Pseudotsuga menziesii*; *Pp*, *Pinus ponderosa*; *Qk*, *Quercus kelloggii*; *Rs*, *Ribes* sp.; *Ro*, *Rhododendron occidentale*; *Ss*, *Smilacina* sp.; *Sl*, *Stipa lettermanii*.



para-epidermal cells. Whereas no fungal sheath was found for species collected at German sites, colonization in *P. picta* (C6) resembled ectendomycorrhizae, with a sparse, netted fungal mantle. As described by Robertson & Robertson (1985), we observed individual cells with varying degrees of fungal colonization. Accordingly, 'pyroloid' mycorrhizas have been described as arbutoid (Robertson & Robertson, 1985; Molina *et al.*, 1992) with an intracellular infection similar to that seen in ericoid mycorrhizas (Read, 1983). Molecular identification of fungal partners revealed that both basidiomycetes and ascomycetes associate with pyroloid species (Table 2). The majority of the fungi detected are obligate mycorrhizal basidiomycetes that form ectomycorrhizas with trees. In contrast to root samples of *P. picta*, only a few positive amplifications were obtained from samples of pyroloid species collected in Germany. This may be caused by degradation during the longer storage of root samples from sites G1–G6 before molecular analyses.

N and C gain of GP and GO from fungal association

The relative contribution of N or C derived from fungi to the N or C content of *P. leucostachys* and the green pyroloid species was calculated *via* the linear two-source mixing model, using the mean enrichment factor of fully MH plants ($\epsilon_{\text{MH-REF}} = 11.7 \pm 2.3\text{‰}$ for ^{15}N ; $\epsilon_{\text{MH-REF}} = 6.9 \pm 1.5\text{‰}$ for ^{13}C), which was determined using the present data set and all suitable isotope data for MH plants described in the literature (Table 1).

The results show that *P. leucostachys* and all green pyroloid species gained a remarkable amount of N from their fungal associations – varying between $25 \pm 7\%$ and $32 \pm 14\%$ for *P. leucostachys*, and ranging from $30 \pm 9\%$ (*P. minor*) to $83 \pm 26\%$ (*P. picta*) for the pyroloid species (Table 3). By contrast, a significant C gain ($28 \pm 12\%$) could be detected only for *O. secunda* growing under extremely low light conditions at site G1.

Table 2 Mycorrhizal fungi detected in roots of pyroloid species from six German sites and one northern Californian site

Pyroloid species	Site	<i>n</i>	Mycorrhizal fungi
<i>Orthilia secunda</i>	G1	5	<i>Piloderma</i> sp. (1), Ascomycete sp.* (1)
<i>Orthilia secunda</i>	G2	5	<i>Sebacina</i> sp. (1), Pezizales sp.* (2)
<i>Orthilia secunda</i>	G3	5	<i>Piloderma</i> spp. (2), <i>Wilcoxina</i> sp. (1)
<i>Pyrola chlorantha</i>	G4	5	<i>Russula</i> sp. (1)
<i>Chimaphila umbellata</i>	G5	5	<i>Tomentella</i> sp. (1), <i>Phialophora</i> sp.* (1)
<i>Pyrola minor</i>	G6	5	<i>Laccaria</i> sp. (1)
<i>Pyrola picta</i>	C6	5	<i>Cortinarius</i> sp. (1), <i>Hebeloma</i> sp. (1), <i>Piloderma</i> spp. (2), <i>Rhizopogon</i> spp. (2), <i>Russula</i> spp. (3), <i>Thelephoraceae</i> spp. (2), <i>Tomentella</i> spp. (2), Helotiales sp.* (1), <i>Phialocephala</i> spp. (1), <i>Wilcoxina</i> sp. (1), <i>Cadophora</i> sp. (1), <i>Leptodontidium</i> sp. (1)

Obligate ectomycorrhizal lineages are shown in bold. *, Lineages that contain some ectomycorrhizal taxa. Site codes are given in Table S1. *n*, Number of individuals of which root samples were taken. Numbers in parentheses after each fungus indicate the number of plants it was associated with.

Table 3 Percentage of nitrogen ($N_{df} \pm 1$ SD) and carbon ($C_{df} \pm 1$ SD) derived from mycorrhizal fungi in the leaves of green orchids and pyroloids, calculated from δ values and mean enrichment factors (Table 1) using a linear two-source isotopic mixing model

Pyroloid/orchid species	Site	Light availability (%)	N_{df} (%) ± 1 SD	C_{df} (%) ± 1 SD
<i>Orthilia secunda</i>	G1	1	56 \pm 21**	28 \pm 12**
<i>Orthilia secunda</i>	G2	100	62 \pm 15**	–
<i>Orthilia secunda</i>	G3	15	59 \pm 27**	–
<i>Pyrola chlorantha</i>	G4	15	74 \pm 29**	–
<i>Chimaphila umbellata</i>	G5	15	40 \pm 5***	–
<i>Pyrola minor</i>	G6	25	30 \pm 9**	–
<i>Platanthera leucostachys</i>	C2		32 \pm 14**	–
<i>Pyrola picta</i>	C3		83 \pm 26**	–
<i>Pyrola picta</i>	C4		70 \pm 11***	–
<i>Platanthera leucostachys</i>	C5		25 \pm 7***	–

Significance levels for deviations from zero based on a Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$. In accordance with the model assumptions no calculations were made for species that were not statistically different in their δ values from the respective references, or if replication was insufficient. For numbers of replicates and site codes see Table S1. Relative light availability under the forest canopy (%) was measured at sites G1–G6.

Discussion

Mycorrhizal fungi of pyroloid roots

Most of the fungi identified as mycorrhizal associates of pyroloid roots are known also to be ectomycorrhizal associates of forest trees. The pattern of symbiotic associations in this group of plants is thus seen to be analogous to that in fully MH (Taylor & Bruns, 1997; McKendrick *et al.*, 2002; Selosse *et al.*, 2002) and partially MH orchids (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004), and in monotropoids (Bidartondo & Bruns, 2001, 2002; Trudell *et al.*, 2003), which are specialized on particular fungi of a broad spectrum of ectomycorrhizal fungal taxa. It is also consistent with that recorded for members of the genera *Arbutus* and *Arctostaphylos*, the arbutoid mycorrhizas of which have been known for some time to be produced by a diverse range of ectomycorrhizal fungi (Molina & Trappe, 1982; Mühlmann & Göbl, 2006). Specialization

of pyroloid species on ectomycorrhizal fungi is consistent with the situation reported by Tedersoo *et al.* (in press), who observed the presence of ectomycorrhizal fungi in the pyroloid species *O. secunda*, *P. chlorantha* and *C. umbellata*. However, while the present study found a diverse range of ectomycorrhizal associates in these and related pyroloid species, Tedersoo *et al.* (in press) suggest a preference for fungi of the genus *Tricholoma*. It will be important to determine the extent of specificity occurring in the tribe Pyroleae.

Some aspects of the ecology of pyroloid plants provide an indirect indication that there may be specificity in their symbiotic relationships. They are known to be particularly susceptible to disturbance. Thus, reports from the Pacific north-west (Halpern & Spies, 1995), Norway (Haugset *et al.*, 1996), Canada (Timoney *et al.*, 1997; A. Schmiedinger and co-workers, unpublished data) and central Alaska (Rees & Juday, 2002) indicate that species such as *P. picta*, *P. chlorantha*, *Pyrola asarifolia*, *C. umbellata* and *Chimaphila menziesii* are

lost from sites that experience disturbance from logging or burning. In addition to the loss of any competitive advantage that these species might possess from tolerance to the shade typical of primary forests, logging is known to cause changes in the ectomycorrhizal key fungal species (Jones *et al.*, 2003). Among other environmental factors to which both pyroloid and monotropoid plants are known to be sensitive is anthropogenic N deposition. Nitrogen inputs of up to 50 kg N ha⁻¹ yr⁻¹ in the San Bernardino Mountains have been shown to be associated with loss of *P. picta* and of *P. andromedea* since 1973 (Allen *et al.*, 2007). Again, the loss of fungal diversity associated with N deposition is well documented (Wallenda & Kottke, 1998; Taylor *et al.*, 2000), and the possibility that elimination of key fungal symbionts contributes to the loss of these species is worthy of investigation.

Isotopic evidence for N and C gain from mycorrhizal fungi

All the pyroloid species examined were significantly enriched in ¹⁵N relative to co-associated reference plants, indicating the incorporation of fungi-derived N. In the species with the highest level of such enrichment, *P. picta*, as much as 83% (±26%) of N was obtained by this route. No correlation could be found between N gain from mycorrhizal fungi and irradiance at the site of origin. The observations of N gain in the green pyroloids are consistent with those seen in the four pyroloid species examined by Tedersoo *et al.* (2007). Hence the N demand of all hitherto investigated pyroloid species is supplied by fungi-derived N.

A significant MH gain of C (28 ± 12%) could be detected in only one species (*O. secunda*), and then only at one of its sites (G1), where ground-level irradiance was particularly low. As no C gain was detected in *O. secunda* when growing at higher irradiances, it is unlikely that the single instance of C gain is species-dependent. A lower C than N gain may be attributed to greater complexity of pathways for C gain and loss than for N (Bidartondo *et al.*, 2004). The study suggests that the negative relationship between the quantity of C gain from fungi and light availability, previously described by Gebauer (2005) in partially MH orchids, may also be found in pyroloids, but further analyses are required to confirm this. Our failure, with the exception of the one species and site, to detect C gain from fungal sources stands in marked contrast to the findings of the recent study by Tedersoo *et al.* (2007), in which significant C gains of up to 68% were recorded in four pyroloid species. It is not clear whether the discrepancy between the two studies arises from differences in the light climates to which the plants were exposed, but it seems unlikely in view of the broad inter- and intrasite range of irradiances found in the German and Californian locations examined in the present study. Hashimoto *et al.* (2005) have provided preliminary evidence of C transfer from the overstorey tree *Larix kaempferi* to *Pyrola incarnata* through mycelia of co-associated

theleporoid fungi. Rigorous quantification of such C flows under controlled conditions of irradiance, preferably with parallel characterization of foliar isotopic signatures, is urgently needed.

Among the putatively autotrophic orchids, no significant C gain from fungi could be detected. However, in the case of ¹⁵N, *P. leucostachys*, in contrast to *E. gigantea* and *G. oblongifolia*, was significantly enriched. Hence it can be hypothesized that *P. leucostachys* has switched its mycorrhizal partnership from a rhizoctonia-type fungus to an ectomycorrhizal associate.

All the fully MH orchids and monotropoids showed strong enrichment in ¹⁵N and ¹³C relative to neighbouring autotrophs. This reflects their dependence on N and C sources that have been metabolized by ectomycorrhizal fungi. The mean enrichment factor ($\epsilon_{\text{MH-REF}}$) calculated from the broad spectrum of fully MH species for ¹⁵N (11.7 ± 2.3‰) and ¹³C (6.9 ± 1.5‰) can be useful for linear mixing model calculations, especially to obtain unbiased values for N and C gains of partially MH species if obligate MH plants are missing at a study site (as at sites G6 and C5). Standard deviations of $\epsilon_{\text{MH-REF}}$ may result to some degree from the MH plants' preference towards certain fungi, or may be a legacy of specificity during underground development from seed.

The isotopic analyses in this work indicate that *P. aphylla* has isotope signatures typical of a fully MH plant. If more extensive analysis of this species confirms this finding it will be the only fully MH species, besides the monotropoid lineage, in the Ericaceae. Furthermore, this study demonstrates that, irrespective of site elevation, climate or forest type, (ecto)mycorrhizal fungi supply significant proportions of the required N in two more pyroloid and one more orchid species. With the exception of *O. secunda* at the notably shaded site, no evidence was obtained for fungal augmentation of plant C supply. Further experimental investigation, particularly of the roles of irradiance in determining the polarity of C movement, are required before the nature and full extent of the dependence of pyroloid upon myco-heterotrophy can be appreciated.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Species investigated, functional groups, leaf persistence types, site codes and number of samples

Table S2 Variation in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ among functional groups tested by one-way ANOVA

Table S3 Variation in $\delta^{15}\text{N}$ values among German sampling sites (G1–G6) and variation in $\delta^{13}\text{C}$ values among German and northern Californian sampling sites (G1–G6 and C6) tested by one-way ANOVA

Table S4 Variation in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values among southern Californian sampling sites (C1–C5) tested by one-way ANOVA

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02065.x>

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