

# Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants

Steven A. Trudell<sup>1</sup>, Paul T. Rygielwicz<sup>2</sup> and Robert L. Edmonds<sup>1</sup>

<sup>1</sup>Division of Ecosystem Sciences, College of Forest Resources, Box 352100, University of Washington, Seattle, WA 98195–2100, USA; <sup>2</sup>Western Ecology Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, 200 SW 35th Street, Corvallis, OR 97333, USA

## Summary

Author for correspondence:  
Robert L. Edmonds  
Tel: +1206 6850953  
Fax: +1206 5433254  
Email: bobe@u.washington.edu

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- Over 400 species of achlorophyllous vascular plants are thought to obtain all C from symbiotic fungi. Consequently, they are termed 'myco-heterotrophic.' However, direct evidence of myco-heterotrophy in these plants is limited.
- During an investigation of the patterns of N and C stable isotopes of various ecosystem pools in two old-growth conifer forests, we sampled six species of myco-heterotrophic achlorophyllous plants to determine the ability of stable isotope ratios to provide evidence of myco-heterotrophy and host-specificity within these symbioses.
- Dual-isotope signatures of the myco-heterotrophic plants differed from those of all other pools. They were most similar to the signatures of ectomycorrhizal fungi, and least like those of green plants.  $\delta^{15}\text{N}$  values of the myco-heterotrophic plants correlated strongly and positively with those of putative mycobionts.
- Used in conjunction with other techniques, N and C stable isotope ratios can be used to demonstrate myco-heterotrophy and host-specificity in these plants when other ecosystem pools are well characterized. They also appear promising for estimating the degree of heterotrophy in photosynthetic, partially myco-heterotrophic plants.

**Key words:** stable isotopes,  $^{15}\text{N}$ ,  $^{13}\text{C}$ , myco-heterotrophic plants, achlorophyllous plants, Monotropoideae, *Corallorhiza*, ectomycorrhiza.

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## Introduction

Worldwide, more than 400 species of parasitic angiosperms in 87 genera, are achlorophyllous and myco-heterotrophic (Leake, 1994), that is, they obtain C from a symbiotic fungus (mycobiont) rather than directly from a photosynthetic host (photobiont) as is the case for plants that are root or stem parasites. In many instances a tripartite symbiosis is involved, where the mycobiont receives photosynthate from a photobiont (or photobionts) and the myco-heterotrophic plant (MHP) in turn receives C from the mycobiont, probably in a different form from that transferred from the photobiont. Although the relationship between MHP and mycobiont in these tripartite symbioses generally has been thought of as mycorrhizal, some feel it is more accurate to refer to the MHP as epiparasites on

the photobiont (Björkman, 1960; Cullings *et al.*, 1996; Taylor & Bruns, 1997, 1999; Bidartondo *et al.*, 2000; Bidartondo & Bruns, 2001, 2002). The mycobionts in these associations include both ecto- and arbuscular mycorrhizal taxa (Leake, 1994; Imhof, 2001; Bidartondo *et al.*, 2002). However, those dealt with in this paper are all ectomycorrhizal (EcM). Although MHP are most abundant in the tropics (Leake, 1994), those occurring in north temperate and boreal forests have received most study, especially members of the subfamily Monotropoideae (Ericaceae), and orchids in the genera *Corallorhiza*, *Cephalanthera*, and *Neottia*.

There are four main lines of evidence supporting myco-heterotrophy. First is the consistent association between the roots of some achlorophyllous plants and fungi, and lack of direct connection between the roots of the achlorophyllous

plants and those of photosynthetic plants (Björkman, 1960; Trappe & Berch, 1985). Second, field isolation experiments demonstrated the importance of maintaining connections between mycobionts and photosynthetic plants. For example, when putative fungal connections to surrounding plants were severed in *Monotropia hypopitys*, individuals did not develop, or developed very poorly compared to control plants (Björkman, 1960). Third, radioisotope tracer studies in the field showed translocation of  $^{14}\text{C}$  and  $^{32}\text{P}$  from trees to *M. hypopitys* and *Sarcodes sanguinea* (Björkman, 1960; Vreeland *et al.*, 1981), but not (or in significantly lower concentration) to nearby photosynthetic plants. Fourth, radioisotope tracer experiments in the laboratory demonstrated transfer of  $^{14}\text{C}$  from mycobiont to MHP (Smith, 1966, 1967) and from photobiont to MHP via mycobiont (McKendrick *et al.*, 2000).

Although these examples provide strong circumstantial evidence for myco-heterotrophy, few studies show a direct physiological interaction between photobiont, mycobiont, and MHP; only the tracer data provide direct evidence that putative MHP receive C from photosynthetic plants via mycorrhizal fungi. Inconclusive observations also exist. Not all attempts to demonstrate fungal connections have been successful and, in some instances, radioisotope tracer studies in the field failed to detect tracer in MHP (Vreeland *et al.*, 1981, Duddridge *et al.* unpublished data, cited by Smith & Read, 1997).

The logistical difficulties of conducting radioisotope tracer studies in the field make it unlikely that they will be common in the future. Although laboratory-based studies are easier to implement and offer greater experimental control, it is difficult to assess the relevance of their results to natural systems (Read, 2002). However, measurement of the natural abundances of stable isotopes in field-collected samples could provide an important means of corroboration and assist in elucidating the physiological relationships within MHP systems. Most biologically important elements occur as two or more stable

isotopes, with one being far more abundant than the other(s). Fractionation of the isotopes by biological and physical processes leads to concentration differences in substances of biological interest, and these differences can provide insights into fluxes among organisms, between organisms and their abiotic environment, and among compartments of the abiotic environment. An important advantage in using natural abundance stable isotope ratios for ecosystem studies is their ability to present a time-integrated picture of functional processes that often are difficult to examine directly (Robinson, 2001).

During the course of a larger comparative study of N and C stable isotope patterns in two old-growth conifer forests, samples of MHP were collected and analysed. This allowed us to assess whether natural abundance stable isotope ratios could be of value in confirming the myco-heterotrophic nature of MHP, and elucidating other aspects of their biology. To our knowledge, only three N stable isotope values for MHP have been reported previously, and those were from studies comparing N-isotope signatures in N-fixing and nonN-fixing plants (Delwiche *et al.*, 1979; Virginia & Delwiche, 1982). Thus, this is the first report of C stable isotope ratios in MHP, and the first stable isotope study focused on their symbiotic association. In addition to the lack of previous data, understanding of the physiology of the mycorrhizas formed by MHP, including their N and C transfer mechanisms, is limited (Leake, 1994; Smith & Read, 1997; Taylor *et al.*, 2002). Combined, these factors make it difficult to predict relationships between the stable isotope ratios of MHP and ecosystem pools such as green plants, fungi, and soils. Thus, we developed three simple models against which to compare our results, based on reported stable isotope patterns among ecosystem pools (Table 1).

The EcM model derives from several studies demonstrating that sporocarps, and in some cases mycorrhizal mantle tissue, of EcM fungi are enriched in both  $^{13}\text{C}$  and  $^{15}\text{N}$  relative to photobionts. Based on this model, we would expect MHP to

**Table 1** Three working models for evaluating the relationships among natural abundance stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of myco-heterotrophic plants (MHP), ectomycorrhizal plants (EcMP), and ectomycorrhizal fungi (EcMF)

Model	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$			
	Reported relationship ( $\Delta$ (‰))	Ref.	Predicted relationship	Reported relationship ( $\Delta$ (‰))	Ref.	Predicted relationship
Ectomycorrhiza	EcMF > EcMP (3–6)	1, 2, 3, 4, 5	MHP > EcMF	EcMP < EcMF (5–15)	1, 2, 4, 5, 6, 7, 8	MHP ≈ EcMP < EcMF
Mistletoe	Host > mistletoe (1–3)	9, 10, 11, 12, 13	MHP ≤ EcMF	Host > mistletoe (< 1)	12, 14	MHP ≤ EcMF
Food chain	$\text{TL}_{A+1} \geq \text{TL}_A$ (< 2)	15, 16, 17, 18	MHP ≥ EcMF	$\text{TL}_{A+1} \geq \text{TL}_A$ (3–5)	18, 19, 20	MHP > EcMF

The  $\Delta$ -values represent typical values from the literature. See Materials and Methods section for explanation of  $\delta$  and  $\Delta$  notation. TL, trophic level. References: (1) Hobbie *et al.* (1999); (2) Högberg *et al.* (1999); (3) Henn & Chapela, 2001; (4) Kohzu *et al.* (1999); (5) Trudell, unpublished data; (6) Gebauer & Dietrich (1993); (7) Taylor *et al.* (1997); (8) Lilleskov *et al.* 2002; (9) Ehleringer *et al.* (1985); (10) Press *et al.* (1987); (11) Marshall & Ehleringer (1990); (12) Schulze *et al.* (1991); (13) Lüttge *et al.* (1998); (14) Bannister & Strong (2001); (15) DeNiro & Epstein (1978); (16) Gearing *et al.* (1984); (17) Ponsard & Ardit, 2000; (18) Scheu & Falca, 2000; (19) DeNiro & Epstein (1981); (20) Minagawa & Wada (1984).

be depleted in  $^{15}\text{N}$  compared to their mycobionts, as are EcM plants, because the flow of N in both cases is from fungus to plant. However, because C flows in opposite directions – EcM plant to mycobiont and mycobiont to MHP – applying the model to the mycobiont-MHP relationship for  $^{13}\text{C}$  is problematic. Thus, we will evaluate the EcM model primarily on the basis of the  $^{15}\text{N}$  abundance data.

The mistletoe model is based on observations that both  $^{13}\text{C}$  and  $^{15}\text{N}$  are often depleted in xylem-tapping mistletoes and root parasites compared to levels in their hosts. The observed differences in  $^{13}\text{C}$  abundance usually are attributed to differences in water-use efficiency or  $\text{CO}_2$  utilization during photosynthesis, and the degree of difference has been used to estimate the degree of heterotrophy exhibited by these hemiparasites (Ehleringer *et al.*, 1985; Marshall & Ehleringer, 1990; Schulze *et al.*, 1991; Richter *et al.*, 1995; Bannister & Strong, 2001). Based on these observations, a holoparasitic (fully heterotrophic) plant should exhibit a  $^{13}\text{C}$  abundance very close to that of its host. Indeed, the similarity in C stable isotope signatures has been used to identify a holoparasitic mistletoe species (Kraus *et al.*, 1995). The achlorophyllous MHP in our study are presumably holoparasitic and, consequently, we have assumed under the mistletoe model that a MHP would exhibit  $^{13}\text{C}$  and  $^{15}\text{N}$  abundances similar to those of its mycobiont.

The food chain model is based on a considerable body of experimental and empirical data that has led to widespread acceptance of the phrase, 'you are what you eat, plus a few %' (DeNiro & Epstein, 1976 and additional references cited in Table 1; but cf; Gannes *et al.*, 1997; Eggers & Jones, 2000). Although complexities such as omnivory can complicate application of this model to food webs, the simplicity of the system with which we are concerned (one or two linear trophic transfers) suggests this should not be an issue here. Thus, under the food chain model, we would expect to see a MHP similar to, or slightly higher in,  $^{13}\text{C}$  abundance and higher in  $^{15}\text{N}$  abundance than its mycobiont.

Because little is known of the physiology of the mycorrhizas formed by monotropes and achlorophyllous orchids, it is difficult to select one of the models as most probable. Studies of the ultrastructure of mycorrhizas formed by species of *Monotropa*, *Pterospora*, and *Sarcodes* (Lutz & Sjolund, 1973; Duddridge & Read, 1982; Robertson & Robertson, 1982) have shown that they clearly differ from EcM. The presence of characteristic pegs formed by the mycobiont suggests a possible analogy with the haustoria formed by parasitic fungi and plants, including mistletoes. If these structures do function in a similar fashion, it would argue for the mistletoe model being the most relevant of the three models. However, the pegs are formed by the host, whereas haustoria are formed by the parasite. Thus, the resemblance could be superficial, and important functional differences could exist between pegs and haustoria (Smith & Read, 1997).

Regardless of which model is best supported by our data, we hypothesize that the relationship between the stable isotope

signatures of MHP and EcM fungi will be much stronger than that between MHP and green plants, reflecting the direct physiological connection between MHP and their EcM mycobionts.

Recent DNA-based identifications of the mycobionts of monotropes and achlorophyllous orchids show that a high degree of host-specificity exists (Cullings *et al.*, 1996; Bidartondo & Bruns, 2001, 2002; Taylor *et al.*, 2002; Young *et al.*, 2002). In addition, we have shown significant differences in  $^{15}\text{N}$  abundance among different genera and species of EcM fungi in our study areas. For instance, species of *Russula* exhibit significantly lower  $\delta^{15}\text{N}$  values than species in *Tricholoma* and *Hydnellum* (Trudell *et al.*, 2001 and unpublished data). Thus, in addition to the prediction that the isotope signatures of MHP will show closer relationships to those of EcM fungi than to those of green plants, we hypothesize that the signatures of individual MHP will reflect the signatures of their respective mycobionts.

## Materials and Methods

### Study areas and sites

The study was conducted in two areas within Olympic National Park, western Washington, USA. The first, the upper Hoh River Valley (Hoh), is located on the western (windward) side of the Olympic Peninsula, approximately 30 km east of the Pacific Ocean coast. The forest zone in this area is Sitka spruce *Picea sitchensis* (Henderson *et al.*, 1989). The second, lower Deer Park Road (DP), is located on the northeastern side of the Peninsula, approximately 15 km south of the Strait of Juan de Fuca. The forest zone in this area is western hemlock *Tsuga heterophylla* (Henderson *et al.*, 1989). The two study areas are separated by the Olympic Mountains, which rise to nearly 2500 m, creating a rain shadow that produces a steep climatic gradient between the two study areas within a reasonably short distance (~55 km). In addition to precipitation and forest zone, the two areas differ in bedrock geology, soils, subordinate vegetation, and other environmental characteristics (Table 2).

Five sites were selected in each of the two study areas. At each site, three 400-m<sup>2</sup> plots were established; thus a 0.6-ha area was sampled at each of the two areas.

### Sample collection and handling

We visited the study sites 24 times from April 2000 until November 2001 to collect samples from nine ecosystem pools, including fungal sporocarps, plant foliage, coarse woody debris, and soils (Table 3). Shoots, and in some cases, root masses of achlorophyllous plants (*Allotropia virgata* T. & G., *Hemitomes congestum* Gray, *M. hypopitys* L., *M. uniflora* L., *Pterospora andromedea* Nutt., and *Corallorhiza maculata* Raf.) were collected opportunistically during these sampling trips. Because these plants

**Table 2** Environmental characteristics of two study areas – upper Hoh River Valley (Hoh) and lower Deer Park Road (DP) – in Olympic National Park, Washington, USA

	Hoh River Valley	Deer Park Road
Latitude/longitude	47°50' N/124°02' W	47°59' N/123°19' W
Altitude (m a.s.l.)	170–250	750–1025
Mean (range) annual temperature (°C)	10 (4.5–16)	9 (2–16)
Mean annual precipitation (mm)	3300–3600	1000–1300
Environmental zone (1–12)*	1 (wettest)	10 (dry)
Dominant overstorey species	<i>Tsuga heterophylla</i> , <i>Picea sitchensis</i> , <i>Pseudotsuga menziesii</i>	<i>Pseudotsuga menziesii</i> , <i>Tsuga heterophylla</i> , <i>Thuja plicata</i> , <i>Alnus rubra</i>
Main understory species	<i>Vaccinium</i> spp., <i>Polystichum munitum</i> , <i>Oxalis oregana</i> , mosses, and liverworts	<i>Gaultheria shallon</i> , mosses
Maximum tree age (year)	> 600	> 300
Bedrock†	Western Olympic lithic assemblage. Late Eocene and Oligocene marine sandstone and minor conglomerate with generally less than 40% siltstone and argillite.	Blue Mountain Unit. Middle Eocene marine sandstone and argillite. Volcanic rich with rare conglomerate, limestone pods, and coaly plant fragments.
Soils	Entisols, Inceptisols, and incipient Spodosols; thin to thick somewhat distinct organic layer; moderate colour and horizon development within mineral soil; loamy, high organic matter, cold, acidic.	Entisols and Inceptisols; thin to medium-thick distinct organic layer; pale colour and little horizon development within mineral soil; sandy loamy, low organic matter, cold, acidic.

\*Reference: Henderson *et al.* (1989). †Reference: Tabor & Cady (1978).

appeared unpredictably and in low numbers (we limited collecting of these relatively rare plants for conservation reasons), we were not able to control sample size to the extent we did with the other sample types. Nomenclature for plants follows Hitchcock & Cronquist (1973), except for *Hypopitys monotropa* Crantz. *M. hypopitys* is used for consistency with previous studies of MHP. For North American fungi, no single nomenclatural source exists, and so names follow prevailing regional usage. Decay classes of down logs were assigned using the five-class system developed by Maser *et al.* (1979). Classes 1–5 reflect increasing decomposition.

As soon as possible after collection, samples were placed on ice in coolers for transport to the field laboratory in Forks, Clallam Co., Washington (~1 h driving time from the study areas). Fungus and plant samples of known identity were placed immediately into food dehydrators and dried at ~40°C until brittle. Remaining samples were refrigerated at ~4°C until they could be identified. All samples were dried within 7 d after collection. Plant samples were divided into leaf and twig fractions, and dead and diseased portions were discarded. Woody petioles were included in the twig fraction. Soil samples were sieved and the < 2-mm fraction was used for analysis.

### Stable isotope analyses

Dried samples of all types were ground to a fine (60-mesh; < 250-µm diameter) powder using an electric coffee grinder and pestle and mortar. Grinding instruments and sieves were cleaned carefully between samples to prevent cross-contamination. Stable N and C isotope abundances were determined by

continuous-flow, elemental analysis/isotope-ratio mass spectrometry. Fungal sporocarps and plant foliage were analysed at the US Environmental Protection Agency (EPA) Integrated Stable Isotope Research Facility, Corvallis, Oregon, USA (some sporocarps were analysed at the University of Arkansas Stable Isotope Laboratory during a period when the EPA instrument was off-line). Coarse woody debris and soil samples were analysed at the University of California, Berkeley, Center for Stable Isotope Biogeochemistry because of their low N concentrations (< 0.5%).

Blanks, standards, replicate samples, and spiked samples were included in each analytical run, typically comprising ~1/3 of the total samples, in accordance with the quality assurance plan for the project (EPA, 1998). National Institute of Standards and Technology (NIST) standard reference materials were used for quality assurance assessment during plant, fungus, coarse woody debris, and O-horizon analyses. Selection of the particular standard reference material used in each case was based on similarity of N concentration in the standard and the samples being analysed. Because standard reference materials certified for isotope abundances are not available, the NIST materials were self-certified by EPA for isotope concentrations. Standards prepared by the University of Washington College of Forest Resources Soils Analysis Laboratory were used for mineral soil samples. Post hoc quality control calculations indicated that data from each laboratory met the precision ( $\pm 0.5\%$ ) and accuracy ( $\pm 0.5\%$ ) criteria specified by the data quality objectives set forth in the quality assurance plan. Results of replicate analyses typically were within 0.2‰, both for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ . Thus, we were able to combine data from the three laboratories.

**Table 3** Summary of samples collected in two areas – upper Hoh River Valley (Hoh) and lower Deer Park Road (DP) – Olympic National Park, Washington, USA

Pool (n)	Sample Description	Taxon or Type (n) or (no. spp., n)
MHP (13)	Fresh shoots from single plant	<i>Allopora virgata</i> (3), <i>Corallorhiza maculata</i> (1), <i>Hemitomes congestum</i> (3), <i>Monotropa hypopitys</i> (2), <i>M. uniflora</i> (3), <i>Pteropora andromedea</i> (1)
AMP (55)	Fresh leaves or shoots from single plant; tree samples from lower crown of canopy individuals	Trees: <i>Thuja plicata</i> (5); shrubs/subshrubs: <i>Berberis nervosa</i> (10), <i>Linnaea borealis</i> (5); forbs: <i>Maianthemum dilatatum</i> (5); <i>Oxalis oregana</i> (5), <i>Tiarella trifoliata</i> (5), <i>Trillium ovatum</i> (10); ferns: <i>Polystichum munitum</i> (10)
EcMP (20)	Fresh leaves from lower crown of single canopy individuals	(All trees) <i>Picea sitchensis</i> (5), <i>Pseudotsuga menziesii</i> (5), <i>Tsuga heterophylla</i> (10)
ErMP (20)	Fresh leaves from single plant	(All shrubs) <i>Gaultheria shallon</i> (10), <i>Vaccinium parvifolium</i> (10)
EcMF (550)	One or more whole sporocarps from single collections	<i>Albatrellus</i> (1, 6), <i>Amanita</i> (4, 21), <i>Boletopsis</i> (1, 4), <i>Boletus</i> (6, 20), <i>Cantharellus</i> (2, 20), <i>Chroogomphus</i> (1, 13), <i>Clavulina</i> (1, 10), <i>Cortinarius</i> (20, 102), <i>Gomphidius</i> (1, 5), <i>Gomphus</i> (1, 3), <i>Hebeloma</i> (2, 6), <i>Hydnellum</i> (3, 20), <i>Hydnum</i> (1, 3), <i>Hygrophorus</i> (6, 22), <i>Inocybe</i> (7, 36), <i>Laccaria</i> (1, 9), <i>Lactarius</i> (7, 50), <i>Lyophyllum</i> (1, 5), <i>Naucoria</i> (1, 3), <i>Phaeocollybia</i> (7, 18), <i>Ramaria</i> (1, 8), <i>Russula</i> (12, 81), <i>Sarcodon</i> (2, 10), <i>Suillus</i> (2, 19), <i>Tricholoma</i> (13, 54), <i>Tylopilus</i> (1, 2)
SapF (169)	One or more whole sporocarps from single collections	<i>Armillaria</i> (1, 2), <i>Chrysomphalina</i> (1, 2), <i>Clitocybe</i> (1, 3), <i>Cystoderma</i> (2, 6), <i>Fomitopsis</i> (1, 2), <i>Ganoderma</i> (2, 6), <i>Gymnopilus</i> (1, 3), <i>Gymnopus</i> (1, 11), <i>Hygrophoropsis</i> (1, 5), <i>Hypholoma</i> (3, 17), <i>Inonotus</i> (1, 4), <i>Laetiporus</i> (1, 3), <i>Lepiota</i> (1, 2), <i>Lyophyllum</i> (1, 6), <i>Mycena</i> (7, 22), <i>Oligoporus</i> (2, 2), <i>Paxillus</i> (1, 2), <i>Phaeolus</i> (1, 6), <i>Pholiota</i> (5, 27), <i>Pleurocybella</i> (1, 5), <i>Pluteus</i> (1, 4), <i>Polyporus</i> (1, 2), <i>Pseudoplectania</i> (1, 3), <i>Rhodocollybia</i> (2, 8), <i>Tricholomopsis</i> (1, 4), <i>Xeromphalina</i> (2, 12)
CWD (20)	Grab from down log; composite of several subsamples	Decay classes 2–3* – slightly to moderately decomposed (10); decay classes 4–5* – moderately to highly decomposed (10)
Ohor (30)	Grab encompassing entire thickness of O-horizon (Oi + Oe + Oa); composite of three random subsamples from within –3-m radius of sample point	–
Mhor (30)	Grab from upper 10 cm of mineral soil; composite of three random subsamples from within –3-m radius of sample point	–

\*Reference: Maser *et al.* (1979). MHP, myco-heterotrophic plants; AMP, arbuscular mycorrhizal plants; EcMP, ectomycorrhizal plants; ErMP, ericoid mycorrhizal plants; EcMF, ectomycorrhizal fungi; SapF, saprotrophic fungi; CWD, coarse woody debris; Ohor, soil O-horizon; Mhor, upper mineral soil.

**Table 4** Summary of one-way ANOVA comparing mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of nine ecosystem pools (listed in Table 3) in two areas – upper Hoh River Valley (Hoh) and lower Deer Park Road (DP) – Olympic National Park, Washington, USA

Effect	SS	df	MS	F	P
$\delta^{15}\text{N}$					
Pool	10567.9	8	1321.0	129.6	< 0.0001
Error	9153.2	898	10.2	–	–
$\delta^{13}\text{C}$					
Pool	5082.1	8	635.3	534.3	< 0.0001
Error	1067.6	898	1.2	–	–

The stable isotope composition of the samples is expressed in differential notation as parts per thousand (per mille; ‰), relative to a standard reference material. For example, for the two stable isotopes of N this is:

$$\delta^{15}\text{N}(\text{‰}) = \left[ \frac{\left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{sample}} - \left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{standard}}}{\left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{standard}}} \right] * 1000$$

Atmospheric N and PeeDee Belemnite C were used as the standards. Differences between the  $\delta$ -values of different pools are noted as  $\Delta$ , also in ‰ units.

### Statistical analyses

The statistical package Statistica 6.0® (StatSoft, Oklahoma City, Oklahoma, USA) was used to calculate descriptive statistics (mean, SD, CI) and analyse the relationships among the data. Mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of the nine ecosystem pools were compared separately using one-way ANOVA, followed by multiple comparison tests (Tukey HSD for unequal  $n$ ) at overall  $\alpha = 0.05$ . Data for the two study areas were combined. Although between-area differences in the isotope signatures of many individual ecosystem pools were evident (data not shown), these were much smaller than those among different pools regardless of area, and the overall pattern of isotope signatures at Hoh and DP separately was essentially the same as the combined pattern.

The degree of association between the  $\delta^{15}\text{N}$  of MHP and putative mycobionts was examined using Pearson's product-moment correlation coefficient. Correlation analysis was used instead of linear regression because small  $n$  for MHP taxa precluded assessment of homoscedasticity, and because the existence of an independent-dependent variable relationship was equivocal. Thus, we selected the more conservative approach. Not all possible mycobiont data were used in the correlation analysis. For *A. virgatum*, *H. congestum*, and *M. hypopitys*,

**Table 5** Multiple comparison test results (Tukey HSD for unequal  $n$ , overall  $\alpha = 0.05$ ) comparing mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of nine ecosystem pools (listed in Table 3) in two areas – upper Hoh River Valley (Hoh) and lower Deer Park Road (DP) – Olympic National Park, Washington, USA

	MHP	EcMF	SapF	CWD	Ohor	Mhor	AMP	ErMP	EcMP
MHP	–	***	***	***	***	***	***	***	***
EcMF	1.00	–	***	***	***	0.47	***	***	***
SapF	***	***	–	0.84	0.69	***	1.00	1.00	0.09
CWD	0.65	0.69	***	–	1.00	0.02	0.52	0.37	***
Ohor	***	***	***	***	–	***	0.29	0.42	***
Mhor	0.10	**	***	0.94	***	–	***	***	***
AMP	***	***	***	***	***	***	–	1.00	0.29
ErMP	***	***	***	***	***	***	0.01	–	0.42
EcMP	***	***	***	***	***	***	**	***	–

P-values and codes for  $\delta^{15}\text{N}$  are listed in the upper right, above the diagonal; those for  $\delta^{13}\text{C}$  are listed in the lower left, below the diagonal. MHP, myco-heterotrophic plants; EcMF, ectomycorrhizal fungi; SapF, saprotrophic fungi; CWD, coarse woody debris; Ohor, soil O-horizon; Mhor, upper mineral soil; AMP, arbuscular mycorrhizal plants; ErMP, ericoid mycorrhizal plants; EcMP, ectomycorrhizal plants. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ .

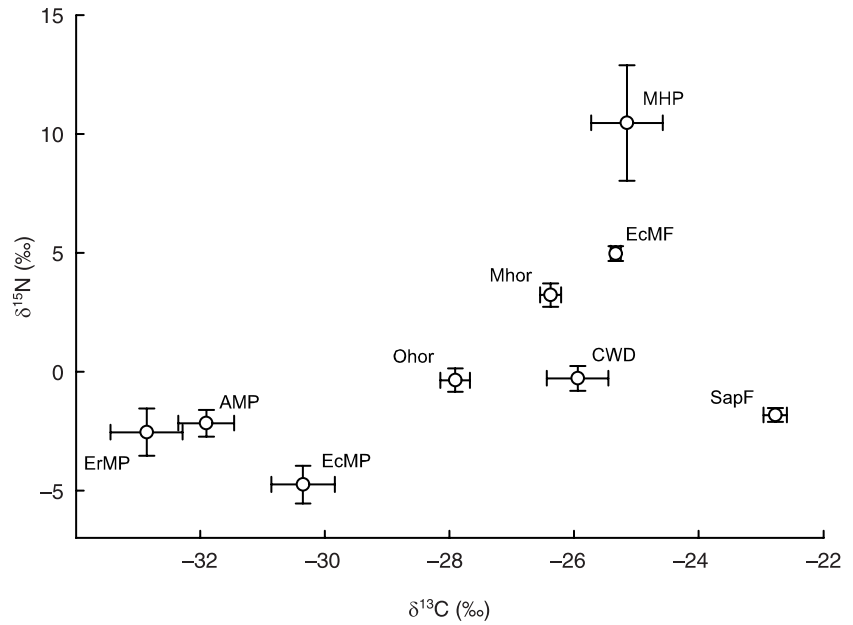
average values for all collections of *Tricholoma magnivelare*, the two species of *Hydnellum*, and three species of *Tricholoma*, respectively, were used. However, for *C. maculata*, the average value of all DP collections in the Russulaceae was used. Although we have data for *R. xerampelina*, one of the mycobionts reported in the literature (Table 6), a DNA sequence from roots of one collection indicated the mycobiont was a species of *Russula* not collected at DP during our study (Martin Bidartondo, unpublished data). For the same reason, the average value for all Hoh Russulaceae was used for *M. uniflora*, rather than the value for *R. brevipes*. Use of the Russulaceae values is warranted because previous studies (e.g. Taylor *et al.*, 1997; Hobbie *et al.*, 1999; Kohzu *et al.*, 1999) as well as other results from this project (Trudell *et al.*, 2001; Trudell, unpublished data) have shown that, although there are significant differences in  $\delta^{15}\text{N}$  among different genera of EcM fungi including *Russula*, *Hydnellum*, and *Tricholoma*, values for *Russula* and *Lactarius* (the two genera in Russulaceae collected in this study) are very similar and thus could be combined.

## Results

### Isotope signatures of ecosystem pools

The nine ecosystem pools sampled – MHP, arbuscular mycorrhizal plants, EcM plants, ericoid mycorrhizal plants, EcM fungi, saprotrophic fungi, coarse woody debris, soil O-horizon, and upper mineral soil – exhibit distinctive combinations of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values (Fig. 1 and Tables 4 and 5). MHP are significantly ( $P < 0.001$ ) greater than all other pools with respect to  $\delta^{15}\text{N}$ . They are significantly ( $P < 0.001$ ) greater than all green

**Fig. 1** Nitrogen and carbon stable isotope values for nine ecosystem pools from two areas – upper Hoh River Valley (Hoh) and lower Deer Park Road (DP) – Olympic National Park, Washington, USA. Data points represent means, error bars 95% CI. Sample types and *n* for each pool are given in Table 3. AMP, arbuscular mycorrhizal plants; CWD, coarse woody debris; EcMF, ectomycorrhizal fungi; EcMP, ectomycorrhizal plants; ErMP, ericoid mycorrhizal plants; Mhor, upper mineral soil; MHP, myco-heterotrophic plants; Ohor, soil O-horizon; SapF, saprotrophic fungi.



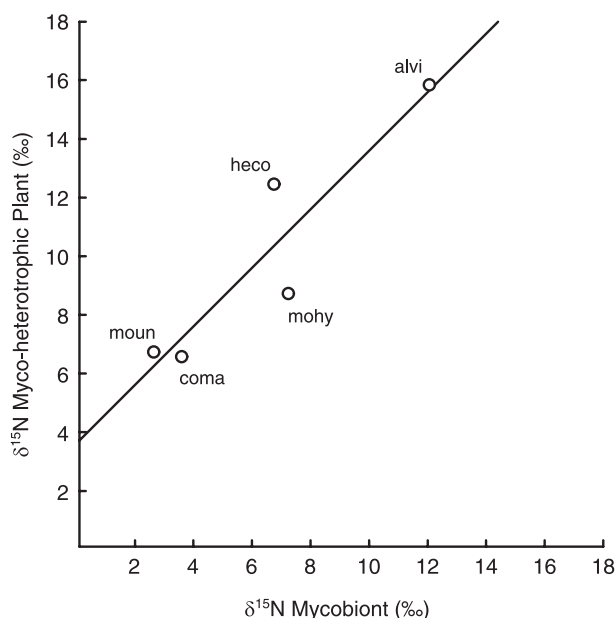
**Table 6** Summary of reported host-specificity between myco-heterotrophic plants and mycobionts, possible mycobionts from the upper Hoh River Valley (Hoh) and lower Deer Park Road (DP), Olympic National Park, Washington, USA, and stable C- and N-isotope data. nrITS sequence identity is included if reported in the original source

Myco-heterotrophic plant ( <i>n</i> )	Area	δ <sup>13</sup> C	δ <sup>15</sup> N	Reported mycobionts (% nrITS sequence identity)	Ref.*	Possible study area mycobionts ( <i>n</i> )
<i>Allotropia virgata</i> (3)	DP	-24.5 ± 0.6	15.8 ± 1.4	<i>Tricholoma magnivelare</i> (99)	1	<i>T. magnivelare</i> (11)
<i>Corallorhiza maculata</i> (1)	DP	-24.7	6.6	<i>Russula xerampelina</i>	2	<i>R. xerampelina</i> (5)
				Many other spp. in Russulaceae	2	All DP Russulaceae (72)
<i>Hemitomes congestum</i> (3)	DP	-25.5 ± 0.2	12.5 ± 2.2	<i>Hydnellum diabolus</i> (99)†	1	<i>H. peckii</i> (10)†
				<i>H. aurantiacum</i> (98)	1	<i>H. aurantiacum</i> (5)
<i>Monotropa hypopitys</i> (2)	DP	-26.3 ± 0.3	8.7 ± 0.1	<i>Tricholoma cingulatum</i> (99)	1	nf
				<i>T. flavovirens</i> (99)	1	<i>T. flavovirens</i> (2)
				<i>T. portentosum</i> (99)	1	nf
				<i>T. sejunctum</i> (99)	1	nf
				<i>T. terreum</i> (99)‡	1	<i>T. myomyces</i> (3)‡
				<i>T. columbetta</i> (98)	1	nf
				<i>T. saponaceum</i> (97)	1	<i>T. saponaceum</i> (4)
<i>M. uniflora</i> (3)	Hoh	-24.7 ± 1.5	6.7 ± 1.5	<i>Russula brevipes</i> (100)	1	<i>R. brevipes</i> (3)
				<i>Lactarius theiogalus</i> (100)	1	nf
				<i>R. paludosa</i>	1	nf
				<i>Russula</i> spp.	1	All Hoh <i>Russula</i> (36)
				Several taxa in Russulaceae	3	All Hoh Russulaceae (55)

See Materials and Methods section for explanation of δ and Δ notation. All δ and Δ-values are in ‰. δ-values are reported as mean ± SD. nf, not found during this study. \*References: (1) Bidartondo & Bruns (2001); (2) Taylor & Bruns (1999); (3) Young *et al.* (2002). †*Hydnellum peckii* and *H. diabolus* are very similar in appearance. Although generally regarded as separate species (e.g. Baird, 1986; Harrison & Grund, 1987), they are considered to be synonymous (as *H. peckii*) by some mycologists (e.g. Maas Geesteranus, 1969). Assuming the two species are distinct, available distribution data (Hall & Stuntz, 1972; Tylutki, 1987; Franklin, 1999), suggest that collections from western North America, where *Hemitomes congestum* is endemic, probably represent *H. peckii*. ‡The name *Tricholoma terreum* frequently has been misapplied to similar-looking species such as *T. myomyces* (Shanks, 1997). Records of '*T. terreum*' contained in public databases such as GenBank should be interpreted with caution.

plant pools and soil O-horizon, and less than saprotrophic fungi, with respect to δ<sup>13</sup>C. They are not significantly different (*P* > 0.05) from EcM fungi, coarse woody debris, and mineral soil with respect to δ<sup>13</sup>C (Table 5). Of all pools, MHP differ

most from the three groups of green plants with respect to δ<sup>15</sup>N and δ<sup>13</sup>C (Fig. 1). The MHP are most similar to the EcM fungi, with respect to both δ<sup>15</sup>N and δ<sup>13</sup>C, although the MHP have a significantly higher δ<sup>15</sup>N (Fig. 1).



**Fig. 2** Correlation between the  $\delta^{15}\text{N}$  values of myco-heterotrophic plants and ectomycorrhizal fungi that represent possible mycobionts. Data are given in Table 6. *alvi*, *Allotropa virgata*; *coma*, *Corallorhiza maculata*; *heco*, *Hemitomes congestum*; *mohy*, *Monotropa hypopitys*; *moun*, *M. uniflora*. The Pearson correlation coefficient is 0.92 ( $P = 0.026$ ). The equation for the best-fit line is,  $\delta^{15}\text{N}_{\text{MHP}} = 3.620 + 0.998 \delta^{15}\text{N}_{\text{Mycobiont}}$ .

### Isotope signatures of individual myco-heterotrophs and fungal symbionts

Several of the MHP species included in previous investigations (Table 6) were collected during our study, as were sporocarps of EcM fungal taxa reported to be symbiotic with them. The MHP have  $\delta^{15}\text{N}$  values from 0.7 to 6.2‰ higher than, and  $\delta^{13}\text{C}$  values very similar to, those of their putative mycobionts (Table 6). The  $\delta^{15}\text{N}$  values of MHP are strongly and positively correlated with those of the mycobionts (Fig. 2); those for  $\delta^{13}\text{C}$  are less strongly and negatively correlated. Pearson correlation coefficients for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  are 0.92 ( $P = 0.026$ ) and  $-0.79$  ( $P = 0.113$ ), respectively.

## Discussion

### Myco-heterotrophy and the nature of MHP mycorrhizas

The stable isotope ratios of MHP are most similar to those of EcM fungi and they are greatly different from those of green (including EcM) plants and the soil O-horizon. The simplest explanation for these patterns is that MHP are receiving both C and N from their mycobionts. The large differences in isotope values supports the current understanding that they are not directly parasitic on green plants and are not saprophytes obtaining nutrition directly from soil organic matter. The specific

relationships seen –  $\delta^{13}\text{C}$  of MHP similar to that of the EcM fungi and  $\delta^{15}\text{N}$  of the MHP on average 3–4‰ higher than that of the EcM fungi – best fit the food-chain model. The worst fit is with the EcM model. In EcM, transfer of C and N seems to involve selective processes in which isotopically depleted substances are transferred by both symbionts (Hobbie & Colpaert, 2003). The higher  $\delta^{15}\text{N}$  in MHP relative to EcM fungi, and the similarity in the  $\delta^{13}\text{C}$  values of the two groups strongly suggest that transfer in MHP symbioses differs from that in EcM.

Food-chain transfer of C and N is essentially a mass-flow process, as a result of which little or no fractionation occurs. Studies of the ultrastructure of MHP mycorrhizas (Duddridge & Read, 1982; Robertson & Robertson, 1982) suggest that mass flow from mycobiont to MHP might occur. Formation of the fungal pegs involves invagination of the plant cell wall and plasma membrane. An opening is formed at the tip of the peg and the fungus appears to extend through it into a membranous sac. Whether this sac eventually bursts resulting in a mass transfer of the fungal hyphal contents into the MHP, is still unclear (Leake, 1994; Smith & Read, 1997). Should bursting occur, causing mass transfer of a substantial portion of total MHP C input, then the similarity in  $\delta^{13}\text{C}$  we observed would be readily explainable. However, difficulty arises in explaining the enrichment in  $^{15}\text{N}$  in MHP. In food chains, the enrichment between trophic levels is usually attributed to metabolic fractionation, especially during deamination and transamination reactions, and excretion of  $^{15}\text{N}$ -depleted products such as urea (Minagawa & Wada, 1984). Presumably, this N-loss mechanism does not operate in MHP. However, enrichment would occur if the material transferred to the MHP was higher in  $^{15}\text{N}$  than the fungal mycelium as a whole. Chitin, a major component of basidiomycete and ascomycete cell walls, is highly depleted in  $^{15}\text{N}$  (~10‰) relative to other cellular constituents such as proteins and amino acids (Taylor *et al.*, 1997). Thus, if a larger proportion of cellular contents than cell wall material is transferred from mycobiont to MHP, then enrichment of the MHP in  $^{15}\text{N}$  would occur. The ‘bursting sac’ mechanism appears to be one, although not necessarily the only, means by which this enrichment could occur. By contrast to the situation for N, chitin in saprotrophic fungi seems to be neither depleted nor enriched in  $^{13}\text{C}$  relative to the whole mycelium (Gleixner *et al.*, 1993) and, similarly, insect chitin is neither enriched nor depleted relative to whole-animal C (DeNiro & Epstein, 1978). Thus, mass balance considerations require that the  $\delta^{13}\text{C}$  of cell wall material and cellular contents are similar, and concurrent enrichment of MHP in  $^{13}\text{C}$  would not be expected. Other evidence suggests that bursting sacs, if they occur at all, would account for only part of the material transfer from mycobiont to MHP. The presence of abundant mitochondria, endoplasmic reticulum, microtubules, and cell wall ingrowths similar to those of transfer cells (Gunning & Pate, 1969) suggest a high degree of metabolic activity in the vicinity of the sac. Presumably this

would include active transfer of materials from mycobiont to MHP. Currently available data are insufficient to resolve the issue of whether or not bursting sacs could contribute significantly to transfer. However, irrespective of whether that is the specific operative mechanism, the isotope signatures appear to reflect relatively greater transfer of hyphal contents (or a portion of them) to the MHP than of cell wall material.

The observed similarity in  $\delta^{13}\text{C}$  values between MHP and EcM fungi is consistent with the mistletoe model; the observed 3–4‰ difference in  $\delta^{15}\text{N}$  values is not. However, if transfer involves hyphal contents that are enriched in  $^{15}\text{N}$  relative to whole-fungus values as discussed above, then the observed values would be consistent with expectations under this model, where there is little or no fractionation between host and parasite. In light of the apparently parasitic nature of MHP, it is tempting to conclude that the mistletoe model actually provides the best fit and that the physiological relationships between MHP and EcM fungi are the same as those between mistletoe and host plant. However, before such a conclusion could be reached, more rigorous investigations of the function of the fungal peg apparatus in MHP would be required, especially ones that allow functional comparisons with the haustoria of mistletoes.

#### MHP–mycobiont specificity

The observed relationships between the  $\delta^{15}\text{N}$  of individual MHP species and their putative mycobionts provide additional support for many pairings inferred in previous studies (Cullings *et al.*, 1996; Taylor & Bruns, 1999; Bidartondo & Bruns, 2001, 2002; Young *et al.*, 2002). Although our data are not from actual pairings of individual MHP with their specific mycobiont mycelia, the strong correlation between the  $\delta^{15}\text{N}$  of the MHP and their putative mycobiont(s) over a wide range of values suggests that the  $\delta^{15}\text{N}$  values reflect host-specificity. Future sampling of MHP and EcM fungi, combined with stable isotope analyses and DNA-based identification of specific mycobionts, will be needed to address this issue more fully.

#### Use of N and C stable isotope ratios in MHP biology

Beyond the use of N and C natural abundance stable isotope ratios for identifying myco-heterotrophy and host-specificity in fully MHP, we suggest that they also could be utilized for assessing degree of heterotrophy in green plants that are partially myco-heterotrophic. Myco-heterotrophy is not an either-or phenomenon. It represents a range with photosynthetic fully autotrophic plants at one extreme, nonphotosynthetic fully heterotrophic plants at the other, and photosynthetic partial heterotrophs in between (Leake, 1994; Taylor *et al.*, 2002). Because of the evolutionary importance of the variety of plant-fungus trophic relationships, the degree of myco-heterotrophy in different taxa is of great interest (Leake, 1994; Taylor *et al.*,

2002). However, we are aware of no reported data relevant to this issue. If, as they do in our study, C and N source pools for MHP, such as plant photosynthate and saprotrophic and mycorrhizal fungi, exhibit significantly different isotope signatures, it should be possible to estimate the degree of heterotrophy of particular MHP, as has been done with mistletoes and root parasites (Press *et al.*, 1987; Marshall & Ehleringer, 1990; Schulze *et al.*, 1991; Marshall *et al.*, 1994; Kraus *et al.*, 1995; Richter *et al.*, 1995). Because many partial MHP are associated with saprotrophic fungi such as *Armillaria* and *Rhizoctonia* (Leake, 1994; Taylor *et al.*, 2002),  $\delta^{13}\text{C}$  may provide more distinct source pools and thus be more informative than  $\delta^{15}\text{N}$ .

#### Conclusions

N and C stable isotope data support the generally accepted contention that the six achlorophyllous plant species examined are myco-heterotrophic. Further, the  $\delta^{15}\text{N}$  values support the existence of the MHP-mycobiont partnerships reported in the literature. Natural abundance stable isotope ratios represent a potential tool for investigating MHP biology, including estimating the degree of heterotrophy in chlorophyllous partial MHP.

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