

*Rapid report*Reciprocal carbon and nitrogen transfer between an ericaceous dwarf shrub and fungi isolated from *Piceirhiza bicolorata* ectomycorrhizasGwen-Aëlle Grelet<sup>1,2</sup>, David Johnson<sup>1</sup>, Eric Paterson<sup>2</sup>, Ian C. Anderson<sup>2,3</sup> and Ian J. Alexander<sup>1</sup><sup>1</sup>Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK; <sup>2</sup>The Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, UK; <sup>3</sup>Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia

## Summary

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- The overstorey coniferous trees and understorey ericaceous dwarf shrubs of northern temperate and boreal forests have previously been considered to form mycorrhizas with taxonomically and functionally distinct groups of fungi.
- Here, we tested the hypothesis that *Meliniomyces variabilis* and *Meliniomyces bicolor*, isolated from *Piceirhiza bicolorata* ectomycorrhizas of pine, can function as ericoid mycorrhizal symbionts with *Vaccinium vitis-idaea*. We used split-compartment microcosms to measure the reciprocal exchange of <sup>13</sup>C and <sup>15</sup>N between *V. vitis-idaea* and three fungal isolates in the *Hymenoscyphus ericae* aggregate isolated from Scots pine ectomycorrhizas (*M. variabilis* and *M. bicolor*) or *Vaccinium* roots (*M. variabilis*).
- The extramatrical fungal mycelium of labelled mycorrhizal plants was significantly enriched in <sup>13</sup>C, and the leaves were significantly enriched in <sup>15</sup>N, compared with nonmycorrhizal and nonlabelled controls.
- These findings show for the first time that fungi in the *H. ericae* aggregate, isolated from pine ectomycorrhizas, can transfer C and N and can thus form functional ericoid mycorrhizas in an understorey ericaceous shrub.

## Introduction

Ericaceous plants occur in many biomes. Most form ericoid mycorrhizas with ascomycetous fungi (Smith & Read, 2008) and this is thought to explain their success in nutrient-poor habitats (Cairney & Meharg, 2003). In the last decade, molecular techniques have improved our understanding of the fungal community in ericaceous hair roots. In particular, sequence analysis of the nuclear ribosomal regions has allowed the taxonomic status of the fungi in ericaceous roots to be resolved (e.g. Zhang & Zhuang, 2004), and it is now evident that ericaceous hair roots host a fungal community that is much more diverse than previously thought (Allen *et al.*, 2003; Bougoure *et al.*, 2007). How these diverse fungal taxa interact with their host plant is still largely unknown, and only in a few cases have the mycorrhizal

status of taxa isolated from ericaceous hair roots been confirmed by back-inoculation (Smith & Read, 2008).

Our understanding of functional aspects of the ericoid mycorrhizal (ERM) symbioses is primarily based on the behaviour of *Rhizoscyphus ericae* (Read) Zhang & Zhaung (formerly *Hymenoscyphus ericae* (Read) Korf & Kernan). Vrålstad *et al.* (2000, 2002a) showed that *R. ericae* is part of the 'H. ericae aggregate', a species complex that also includes fungi which form the ectomycorrhizal (ECM) morphotype *Piceirhiza bicolorata*, common on many northern temperate and boreal trees. This finding was significant because ECM and ERM fungi had previously been thought to be genetically and functionally distinct.

Vrålstad *et al.* (2002a) recognized four main clades in the *H. ericae* aggregate, including *R. ericae* and *Cadophora finlandica* (Wang & Wilcox) (Harrington & McNew, 2003).

Hambleton & Sigler (2005) subsequently described three new species within the aggregate: *Meliniomyces variabilis*, *Meliniomyces vraolstadae* and *Meliniomyces bicolor* (which is closely related to *C. finlandica*). They suggested that at least five others could be described if isolates became available for examination. Re-synthesis experiments, summarized by Hambleton & Sigler (2005), suggest that fungi in the *R. ericae* clade only form ericoid mycorrhizas. Some isolates of *M. variabilis* colonize the roots of both ERM and ECM hosts. They form typical ericoid hyphal complexes in ERM hosts, and intracellular and extracellular hyphae but no true mantle in ECM hosts (e.g. Piercey *et al.*, 2002; Vohnik *et al.*, 2007). Other *M. variabilis* isolates fail to colonize ECM or ERM hosts (e.g. Piercey *et al.*, 2002; Vrålstad *et al.*, 2002b). Isolates assigned to the *C. finlandica*/*M. bicolor* clade form ectomycorrhizas or ectendomycorrhizas with *Betula*, *Picea* and *Pinus* (Wilcox & Wang, 1987; Vrålstad *et al.*, 2002b). Villarreal-Ruiz *et al.* (2004) synthesized a tripartite association among *Pinus sylvestris*, *Vaccinium myrtillus* and an isolate in the *M. bicolor*/*C. finlandica* clade obtained from a *P. bicolorata* ectomycorrhiza. The fungus grew out from the pine ectomycorrhizas and formed typical ericoid fungal coils in the *Vaccinium* hair roots.

As emphasized by Read (2000), when commenting on Vrålstad *et al.* (2000), the ecological role of these fungi can only be resolved by cross-inoculation experiments, which examine the fitness-related responses of the potential partners. To address this, we inoculated *Vaccinium vitis-idaea* L. seedlings with isolates of *M. variabilis* and *M. bicolor* obtained from *P. bicolorata* ectomycorrhizas, and examined reciprocal exchange of C and N between fungi and the host plant.

## Materials and Methods

### Isolation and identification of fungi

Three isolates were obtained from roots in a *Vaccinium vitis-idaea* L./*Pinus sylvestris* L. stand in north east Scotland (57°02'N, 2°52'W). The trees were between 100 and 250 yr of age. Two isolates (E and Hc) were obtained from different *P. bicolorata* ectomycorrhizas. Isolate E was assigned to *Meliniomyces bicolor* Hambleton & Sigler and isolate Hc was assigned to *Meliniomyces variabilis* Hambleton & Sigler, based upon their internal transcribed spacer (ITS) sequences (see below). An additional strain of *M. variabilis* (isolate F), obtained from a *Vaccinium* hair root, was included for comparison. DNA extraction and ITS-PCR were performed as described in Grelet *et al.* (2009), and species designation was based on matching ITS sequences to those of known origin in GenBank. The position of each isolate within the '*H. ericae* aggregate' was confirmed by phylogenetic analysis, including representatives of each of the clades/species described by Hambleton & Sigler (2005). For this purpose a neighbour-joining analysis (Saitou & Nei, 1987) with 1000

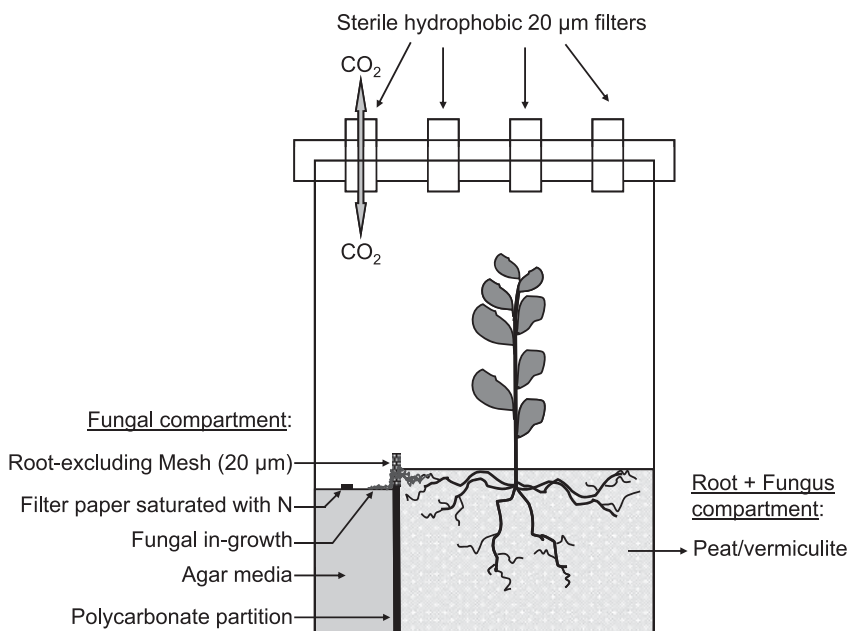
bootstrap replicates was conducted in MEGA4 (Tamura *et al.*, 2007) using the maximum composite likelihood method (Tamura *et al.*, 2004). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 521 positions in the final data set.

We used inter-simple-sequence-repeat PCR amplification (ISSR-PCR) in order to test whether or not the two *M. variabilis* isolates were identical genotypes. Two ISSR primers were used: GTG5 (5'-GTGGTGGTGGTGGTG-3') and CGA5 (5'-DHB-CGACGACGACGACGA-3'). Amplification reactions were carried out in 25- $\mu$ l volumes containing 5  $\mu$ M primer, 250  $\mu$ M each dNTP, 2 mM MgCl<sub>2</sub>, 1  $\times$  green GoTaq reaction buffer, 1.25 U GoTaq DNA polymerase (Promega) and 0.5  $\mu$ l of DNA extract. The PCR conditions were as follows: for GTG5, 30 cycles of 2 min at 94°C, 45 s at 63°C and 1 min at 72°C, followed by 10 min at 72°C; for CGA5, 1 min at 94°C, followed by 30 cycles of 1 min 30 s at 94°C, 45 s at 55°C and 1 min at 72°C, followed by 10 min at 72°C. At least two duplicate extracts per isolate were subjected to ISSR-PCR with each primer. The amplified products were run on a 15  $\times$  15 cm 1.5% agarose gel at 110 volts for 2.5 h. The presence/absence and size of bands were determined for each isolate using GelCompar® II version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). A total of 40 bands were scored: 21 for CGA5 and 19 for GTG5.

### Experimental microcosms

Seeds of *V. vitis-idaea* (Chiltern seeds, Ulverston, UK) were surface sterilized for 10 min in 10% hydrogen peroxide and germinated in the light (photoperiod 16 h light/8 h dark, 20°C) on sterile water agar containing 200 ppm gibberellic acid 3 (GA-3). When 10 wk old, seedlings were transferred to a low-mineral nutrient and carbon agar (LSM agar), of composition 0.600 mM NH<sub>4</sub>NO<sub>3</sub>, 0.599 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.662 mM KH<sub>2</sub>PO<sub>4</sub>, 0.170 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.299 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 0.080 mM K<sub>2</sub>SO<sub>4</sub>, 0.041 mM FeNaEDTA, 0.006 mM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 3.69  $\mu$ M MnSO<sub>4</sub>·H<sub>2</sub>O, 0.04  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 9.55  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 1  $\mu$ M CuSO<sub>4</sub>·5H<sub>2</sub>O and 100 mg l<sup>-1</sup> glucose. The seedlings were then inoculated by overlaying their root systems with sterile charcoal-activated filter paper, colonized by one of the three *Meliniomyces* isolates or with no fungus (nonmycorrhizal treatment). After 4 months, each seedling was transferred to Magenta® dishes (PhytoTechnology Laboratories, Lenexa, KS, USA) modified to allow gas exchange using a sterile hydrophobic 20  $\mu$ m filter (AMD® filter, Tissue Quick Plant Laboratories, New Milton, Hampshire, UK) under axenic conditions (Fig. 1). A vertical polycarbonate partition (1.5 cm height, 2 mm thickness), supporting a 20  $\mu$ m polyester mesh, was inserted in the dish to create a compartment accessible only by mycorrhizal hyphae. The main root compartment was filled with sterile peat : vermiculite : LSM without glucose (1:4:1.8, v/v/v),

**Fig. 1** Schematic representation of the microcosm used in this study. Four sterile filters (AMD®) were placed in the lid of the Magenta® dish to ensure maximal CO<sub>2</sub> exchange between inner and outer atmospheres. The fungal compartment was separated from the root + fungal compartment by a partition, the lower part comprising solid polycarbonate and the upper part comprising 20 µm nylon mesh. This permitted extension of external fungal mycelium into the fungal compartment but prevented the diffusion of nutrients between compartments.



whilst the fungal compartment was filled with LSM agar at double nutrient concentration and 100 mg l<sup>-1</sup> glucose. The upper surface of the fungal compartment was 2 mm lower than that of the root compartment to prevent diffusion of nutrients from the fungal compartment to the main root compartment. The plants were grown for 2 months in a growth cabinet with 16 h light (at 15°C)/8h dark (at 10°C) and 70% relative humidity. Light was provided by a mixture of fluorescent tubes and incandescent bulbs, and photon flux density was set at 150 µmol m<sup>-2</sup> s<sup>-1</sup> to avoid excessive heat in the Magenta® dishes, bearing in mind that the *Vaccinium* photosynthetic rate saturates at *c.* 300 µmol m<sup>-2</sup> s<sup>-1</sup> (Kolari *et al.*, 2006). When hyphal growth into the fungal compartment extended 1 cm beyond the root-excluding mesh, the plants were labelled (described in the following section). At the end of the labelling period, hyphae covered *c.* 10% of the surface of the agar in the fungal compartment, which in itself represented one-third of the total substrate available to the fungus. Most of the fungal biomass was located in the root compartment.

### Stable isotope labelling and harvest

Seven or eight plants of each mycorrhizal treatment, plus seven nonmycorrhizal controls, were labelled. Five nonlabelled microcosms were used to determine background <sup>13</sup>C and <sup>15</sup>N. Two of these were nonmycorrhizal, plus one of each mycorrhizal treatment.

Before labelling, all microcosms were checked to ensure that no contaminants (either bacterial or fungal) were visible in the root compartment or in the fungal compartment.

Sterile filter-paper triangles (Whatman #541, Maidstone, UK, 10 mm<sup>2</sup>) were saturated with nutrient solution con-

taining 11 µg of N as nitrate and ammonium at 3.4 and 6 mM, respectively, with a <sup>15</sup>N : <sup>14</sup>N ratio of 95.4 atom %, or at natural abundance for control unlabelled dishes. Two triangles per dish were placed onto the surface of the agar, 5 mm away from the leading edge of extending fungal hyphae in the fungal compartment. All the dishes, except the nonlabelled controls, were then transferred to a clear acrylic labelling chamber (Paterson *et al.*, 2007) situated within a controlled-temperature growth room. Light intensity, temperature, humidity and photoperiod were the same as in the growth cabinet, where the unlabelled control dishes remained. The atmosphere of the chamber was renewed at the start of every photoperiod by flushing the chamber with a mix of CO<sub>2</sub>-free air and 98 atom % excess <sup>13</sup>CO<sub>2</sub> (400 µmol mol<sup>-1</sup>) at 10 l min<sup>-1</sup> for 1 h. The chamber was then sealed, with a maximum draw-down of CO<sub>2</sub> concentration to 270 µmol mol<sup>-1</sup> over the course of each photoperiod. After 6 d, leaves were harvested. The upper 0.5 mm of agar in the fungal compartment (with surface hyphae in mycorrhizal treatments, without hyphae in nonmycorrhizal controls) was removed. In the mycorrhizal treatments, this ensured that most of the fungal hyphae which had grown beyond the root-excluding mesh were collected. Leaves and agar samples were freeze-dried and weighed, and their <sup>13</sup>C and <sup>15</sup>N contents were measured using a continuous-flow isotope ratio mass spectrometer interfaced to an elemental analyser (Thermo Finnigan, Bremen, Germany). Isotopic enrichment results were expressed as δ<sup>13</sup>C and δ<sup>15</sup>N in parts per thousand (‰) relative to their international standards, namely Vienna Pee Dee Belemnite (V-PDB) for C and atmospheric N<sub>2</sub> for N, using the following calculation:

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

**Table 1** Effect of labelling and mycorrhizal treatment on plant biomass, leaf nitrogen (N) status, uptake and fixation of  $^{13}\text{C}$ -labelled  $\text{CO}_2$  in plant leaves and transfer of  $^{15}\text{N}$  from  $\text{NH}_4\text{NO}_3$  patches to host plants from compartments accessible only to external mycelium

	Treatments					
	Labelled	<i>M. variabilis</i>			Nonmycorrhizal	Nonlabelled <sup>1</sup>
		<i>Meliniomyces bicolor</i> (from ECM)	(from ECM)	(from ERM)		
Number of plants per treatment	7	8	7	7	5	
Leaf dry matter (mg DW per plant)	38 ± 7 <sup>a</sup>	36 ± 7 <sup>a</sup>	27 ± 4 <sup>a</sup>	26 ± 9 <sup>a</sup>	19 ± 8 <sup>a</sup>	
Leaf percentage N	1.9 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>a</sup>	1.8 ± 0.1 <sup>a</sup>	1.8 ± 0.1 <sup>a</sup>	
$^{13}\text{C}$ fixation in green leaves (mg of $^{13}\text{C}$ g <sup>-1</sup> leaf dry mass)	42 ± 1 <sup>a</sup>	39 ± 0 <sup>b</sup>	41 ± 1 <sup>ab</sup>	39 ± 1 <sup>b</sup>	5 ± 0 <sup>c</sup>	
$^{15}\text{N}$ enrichment of mycelium/agar in fungal compartment (atom %)	1.3 ± 0.2 <sup>a</sup>	1.6 ± 0.4 <sup>a</sup>	1.8 ± 0.4 <sup>a</sup>	2 ± 0.2 <sup>a</sup>	0.4 ± 0 <sup>b</sup>	

Values are means (± SE) and those sharing a letter within a row are not significantly different ( $P > 0.05$ ).

<sup>1</sup>Nonlabelled plants included two nonmycorrhizal plants and one plant inoculated with each of the isolates tested in this experiment. ECM, ectomycorrhizal; ERM, ericoid mycorrhizal.

where R is the molar ratio ( $^{13}\text{C} : ^{12}\text{C}$  or  $^{15}\text{N} : ^{14}\text{N}$ ) of the leaf or of the fungal sample. Statistical analysis of leaf biomass, and of C and N data were performed using ANOVA and Fisher's PLSD tests in GENSTAT (version 10; VSN International Ltd, Hemel Hempstead, UK).

To test for fungal anapleurotic uptake of  $^{13}\text{C}$  (dark fixation), duplicate 6-wk-old colonies of each fungal isolate were exposed to  $^{13}\text{C}$ -labelled  $\text{CO}_2$  at 50 atom % and 800  $\mu\text{mol mol}^{-1}$  in the dark for 16 h. An identical set of colonies was maintained in the dark at ambient  $\text{CO}_2$ . There was no evidence of enrichment of  $^{13}\text{C}$  in the colonies exposed to  $^{13}\text{C}$ -labelled  $\text{CO}_2$  (Table S1).

Root systems of labelled plants were stored in 3% glutaraldehyde in 0.2 M phosphate buffer at 4°C. They were then stained with Trypan blue, as described by Villarreal-Ruiz *et al.* (2004), mounted in lactic acid and observed under light microscopy using a ×100 oil-immersion objective. Plants were scored as mycorrhizal whenever fungal coils were observed in the epidermal cells of the hair roots.

## Results

### Strain identification and genetic relatedness

Isolates F and Hc had identical ITS sequences (i.e. 100% similarity in the ITS1-5.8S-ITS2 regions), while isolate E shared 90.5% sequence similarity with the ITS sequences of both isolates F and Hc. Sequences were deposited in GenBank under accession numbers FN179335 (E) and FN179336 (Hc = F). Subsequent phylogenetic analyses identified isolates F and Hc as *M. variabilis* (Hambleton & Sigler, 2005) (clade 1 as defined by Vrålstad *et al.*, 2002a) and isolate E as *M. bicolor* (Hambleton & Sigler, 2005) (clade 4 as

defined by Vrålstad *et al.*, 2002a) (Fig. 2). Isolate E shared 98.5% sequence identity with *M. bicolor* strain type ARON 2805.S. Isolates F and Hc shared 98.9% sequence identity with *M. variabilis* UAMH 8862.

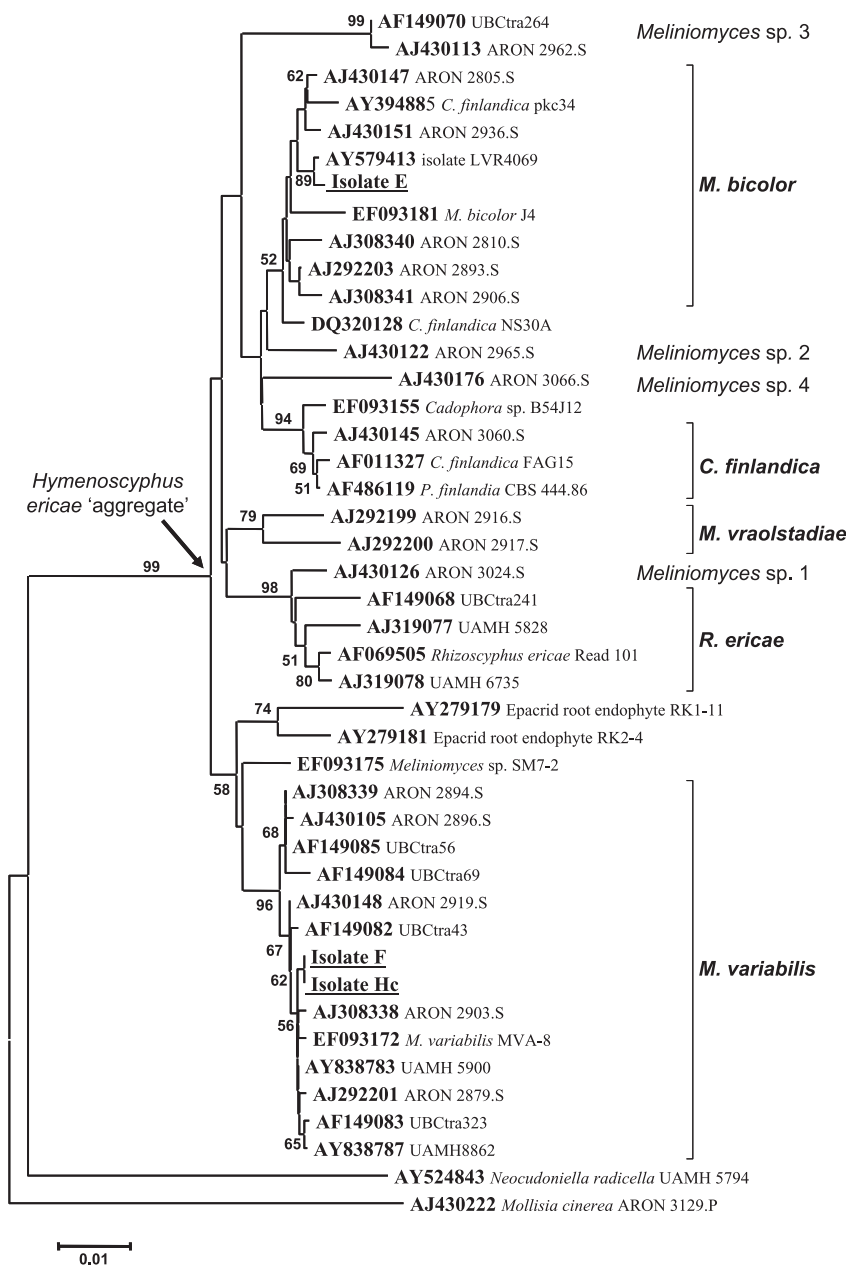
The ISSR-PCR profiles generated using the two primers were sufficient to distinguish between the two *M. variabilis* isolates. (Fig. S1). In total, 40 bands were detected across the three isolates. The ISSR profiles for isolate E were 42.5% similar to those for isolates F or Hc. The ISSR profiles for the two *M. variabilis* isolates (F and Hc), which had identical ITS sequences, were only 75% similar to each other, confirming that these two isolates represent different *M. variabilis* genotypes.

### Growth and mycorrhizal infection

There was no difference in leaf biomass between treatments (Table 1), whether mycorrhizal or not. All three isolates formed intracellular coils in the epidermal cells of *Vaccinium* hair roots. All inoculated seedlings were colonized, but colonization was uneven within each root system, with entire root branches remaining uncolonized. We did not attempt to measure the overall level of infection in each plant. The fungi also formed occasional aggregations of hyphae on the surface of the hair roots. There was no fungal colonization (neither intracellular nor extracellular) of the hair roots of uninoculated plants.

### Recovery of $^{15}\text{N}$ and $^{13}\text{C}$ in plant leaves and in the fungal compartment

Regardless of the fungal strain, *V. vitis-idaea* leaves were significantly more enriched with  $^{15}\text{N}$  ( $P = 0.005$ ) and the



**Fig. 2** Neighbour-joining phylogenetic analysis of internal transcribed spacer (ITS) sequences showing the position of the three isolates E, F and Hc within the *Hymenoscyphus ericae* aggregate. Values next to branches represent bootstrap values (1000 replicates). The scale bar represents the number of base substitutions per site.

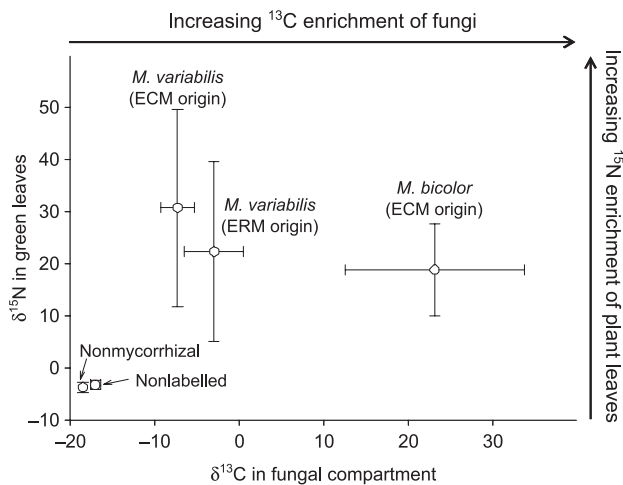
fungal compartment was significantly more enriched with  $^{13}\text{C}$  ( $P < 0.001$ ), than either inoculated nonlabelled controls or nonmycorrhizal labelled controls (Fig. 3).  $^{13}\text{C}$  enrichment of labelled *V. vitis-idaea* leaves averaged 10 atom %. By contrast, the uptake/diffusion of  $^{15}\text{N}$ -labelled ammonium/nitrate out from the filter paper was low because  $^{15}\text{N}$  enrichment of the samples taken from the fungal compartment did not exceed 2 atom % (Table 1).

The  $^{13}\text{C}$  and  $^{15}\text{N}$  enrichments were indistinguishable in the two *M. variabilis* treatments, even though one strain had originally been isolated from an ECM root while the other was isolated from an ERM root. Greater  $^{13}\text{C}$  enrichment in the fungal compartment ( $P = 0.015$ , Table 1) and a higher

rate of  $^{13}\text{C}$  fixation in the *V. vitis-idaea* leaves ( $P = 0.006$ , Table 1) were observed in *M. bicolor*-inoculated microcosms compared with either *M. variabilis* treatment (Table 1).

## Discussion

Since the landmark papers of Vrålstad *et al.* (2000, 2002a), evidence has been accumulating that ericaceous hair roots contain a group of closely related ascomycetes in the Helotiales, some of which can also form ectomycorrhizas. Here we show, for the first time, that one of these fungi (*M. bicolor*), isolated from a Scots pine ectomycorrhiza, can form functional ericoid mycorrhizas in *V. vitis-idaea*, a ubiquitous understory



**Fig. 3** The abundance of  $^{15}\text{N}$  in plant green leaves and the abundance of  $^{13}\text{C}$  in the fungal compartment. Data are expressed in delta units (‰) and are means  $\pm$  SE of five to eight replicates. Plants subjected to different mycorrhizal treatments (nonmycorrhizal or mycorrhizal with *Meliniomyces bicolor* or *Meliniomyces variabilis*) were exposed to both  $^{15}\text{NH}_4^{15}\text{NO}_3$  and  $^{13}\text{CO}_2$  or to unlabelled (controls). ECM, ectomycorrhizal; ERM, ericoid mycorrhizal.

ericaceous shrub in northern temperate and boreal forest ecosystems. The basis for this conclusion is the formation of typical ERM structures in *Vaccinium* hair roots, the absence of negative effects on growth of the *Vaccinium* seedlings, and the reciprocal transfer of C and N between the fungus and the host plant. *M. bicolor* has previously been shown to form ectomycorrhizas on a number of tree hosts (Vrålstad *et al.*, 2002b). Villarreal-Ruiz *et al.* (2004) showed that the hyphae of a *M. bicolor* isolate (LVR4069; accession code AY579413; 99.5% similarity with isolate E) grew out from pine ectomycorrhizas and formed coils in *Vaccinium* hair roots. Taken together, these studies suggest that the morphology of the mycorrhizal association (ERM or ECM) is under the control of the host plant, leading to the formation of characteristic structures in or on the roots. This provides further evidence that the distinction between 'ECM' and 'ERM' fungi is not clear.

The nature of the interaction between our *M. variabilis* isolates and the pine roots from which they were isolated is unknown. Ohtaka & Narisawa (2008) showed that a *M. variabilis* strain isolated from barley roots could colonize both *Azalea* (ERM) and *Eucalyptus* (ECM) roots. However, the fungus enhanced shoot growth only in the case of *Azalea*. *M. variabilis* strains isolated from ECM hosts by Schild *et al.* (1988) colonized *Picea* (ECM) with no detrimental effects on seedling growth. Clearly, the nature of the relationship between *M. variabilis* and its non-ericaceous hosts requires further study. Nevertheless, in our study, *M. variabilis* interacted in a similar manner with *Vaccinium* regardless of whether it originated from an ECM or an ERM root.

*M. bicolor*, on the other hand, can form typical ECM structures with ECM hosts (Vrålstad *et al.*, 2002b; Villarreal-Ruiz *et al.*, 2004). It may therefore be significant that, in ERM *Vaccinium* roots, our *M. bicolor* isolate behaved differently from both *M. variabilis* isolates. Although the amount of  $^{15}\text{N}$  translocated to leaves was similar between the species, the  $^{13}\text{C}$  enrichment of the fungal compartment was significantly greater in plants colonized by *M. bicolor*. Plants colonized by *M. bicolor* also had greater  $^{13}\text{C}$  enrichment in their leaves despite no increase in leaf biomass, suggesting a greater rate of C assimilation through photosynthesis. One explanation might be that the ability to transfer N was the same for both mycorrhizal species but that *M. bicolor* constituted a greater C sink than *M. variabilis* in our system. Quantitative measurements of C flux from plant to fungus would be required to test this hypothesis.

We used  $^{15}\text{NH}_4^{15}\text{NO}_3$  to trace nitrogen transfer. Based upon  $^{15}\text{N}$  enrichment values in leaves and in external fungal mycelium, we estimated that 1.54% of leaf N was derived from the patch of N after uptake and transfer by the fungal partner. Given the slow growth of *Vaccinium* species and the short period of exposure to labelled N, this flux has the potential to meet a considerable part of plant N demand. However, we recognize that ERM fungi can utilize a wide range of organic nutrient sources (Kerley & Read, 1995; Leake & Miles, 1996; Midgley *et al.*, 2004) that are often found in soil in their natural habitat. Nevertheless, our findings demonstrate the potential for these fungi to transfer N to an ericaceous host plant and provide a basis for further experiments involving more complex sources of N.

It is also important to note that ericaceous roots are colonized by more fungi than previously thought, including a wide range of helotialian ascomycetes and Sebaciales (Bougoure *et al.*, 2007; Selosse *et al.*, 2007). Clearly, the number of fungal taxa involved in ERM associations is potentially far greater than previously thought. Our findings should encourage further work to determine the functional roles of these other important groups of fungi and the interactions between them.

Our study builds on an emerging body of molecular evidence that some fungi in the boreal forest are capable of associating with the roots of host plants that differ markedly in their morphology and physiology. A similar situation is emerging in other ecosystems. For example, in the dry sclerophyll forests of eastern Australia, some ascomycete fungi, considered to form ericoid mycorrhizas in epacrid species, have been found to colonize the roots of co-occurring ECM trees (Chambers *et al.*, 2008). In grassland, a group of helotialian fungi, with potentially overlapping phylogeny and function to endophytes from the Ericaceae, were found to associate with the roots of *Deschampsia flexuosa* (Zijlstra *et al.*, 2005). In a Mediterranean oak woodland, the same fungal genotype was found in both ECM oak and neighbouring ericaceous roots (Bergero *et al.*, 2000), and several fungi

capable of forming ERM structures were shown to persist in the absence of their ericaceous hosts (Bergero *et al.*, 2003). Although our study has focused on testing the ability of *M. bicolor* and *M. variabilis* to participate in the transfer of N and C, there is clearly potential for these and other related fungi to undertake a range of ecologically important processes in situations where ericaceous plants form a substantial component of understorey vegetation. For example, the presence of ERM inoculum on the roots of ECM plant species in the lower soil horizons has the potential to act as refugia and to facilitate ericaceous seedling establishment, particularly after disturbance events like fire or freeze–thaw cycles. Functional investigations are required to determine the full extent of the interactions between these fungi and their hosts in nature.

In our microcosms, the fungi extended beyond the surface of the hair roots to exploit a patch of resource, suggesting that this particular group of ERM fungi have the potential to form extraradical mycelial networks. The extent and the functional significance of extraradical mycelium of ERM fungi under field conditions have never been examined. The ability of *M. bicolor* to form a tripartite symbiosis with Scots pine and *V. myrtilus* (Villarreal-Ruiz *et al.*, 2004), and our physiological data, suggest the intriguing possibility of a functional link in C and nutrient cycling among soil, trees and shrubs in northern temperate and boreal forests. An investigation of resource transfers between these fungi and their networked ECM and ERM hosts is clearly required.

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## Supporting Information

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

**Fig. S1** ISSR profiles generated with the primer CGA5 for isolate E (lanes 2, 3), F (lanes 4, 5) and Hc (Lanes 6, 7). M, Hyperladder II molecular weight marker (Bioline, UK) with sizes indicated in bp.

**Table S1**  $\delta^{13}\text{C}$  (atom‰) in hyphae and culture media taken from fungal colonies exposed to  $^{13}\text{C}$ -labelled or unlabelled  $\text{CO}_2$  (control)

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