# Interaction between an isolate from the *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*

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## Summary

- A fungal isolate was obtained from *Piceirhiza bicolorata*-like ectomycorrhizas on *Pinus sylvestris* in a 160-yr-old natural woodland.
- The fungus was identified by sequencing the PCR-amplified rDNA ITS regions. The sequence was compared with similar known taxa and grouped with *Cadophora finlandia* in the *Hymenoscyphus ericae* aggregate.
- The fungus formed *P. bicolorata*-like ectomycorrhizas in aseptic synthesis with *P. sylvestris* seedlings. When seedlings of *Vaccinium myrtillus* were exposed to mycelium arising from these ectomycorrhizas, or to mycelium in pure culture, the hyphae entered the cells of the hair roots and formed coils characteristic of ericoid mycorrhizas. The presence of the fungus stimulated *Vaccinium* root growth and altered root architecture.
- This is the first full report of the ability of a fungus from the *H. ericae* aggregate simultaneously to form both ectomycorrhizas and what appear to be ericoid mycorrhizas.

**Key words:** *Cadophora finlandia*, ectomycorrhizas, ericoid mycorrhizas, *Hymenoscyphus ericae* aggregate, *Pinus sylvestris*, *Vaccinium myrtillus*.

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## Introduction

In the forests of the north temperate and boreal zone, the ectomycorrhizal trees in the overstorey often occur with an understorey of ericaceous dwarf shrubs including *Calluna vulgaris* (L.) Hull and *Vaccinium* spp., which form ericoid mycorrhizas. A distinctive black ectomycorrhiza, *Piceirhiza bicolorata* (Brand *et al.*, 1992), is a common component of the ectomycorrhizal fungal communities of these forests, and has been recorded on a number of ectomycorrhizal host species (Vrålstad *et al.*, 2000). It is, for example, frequent in the native *Pinus sylvestris* L. forests of north-east Scotland (L. Villarreal-Ruiz, unpublished data).

Vrålstad *et al.* (2000) were able to show that the fungi forming *P. bicolorata* ectomycorrhizas were genetically similar to the ascomycete *Hymenoscyphus ericae* (Read) Korf and Kernan. This was a significant discovery because *H. ericae* is known to form structurally different ericoid mycorrhizas on ericaceous host plants. On this basis, Vrålstad *et al.* (2000) hypothesized that ericoid and ectomycorrhizal plants share some common mycobionts in the *H. ericae* aggregate. One of the fungi in this aggregate is the so-called dark septate endophyte (Jumpponen, 2001) of tree roots, formerly known as *Phialophora finlandia* Wang and Wilcox and transferred by Harrington & McNew (2003) to *Cadophora* as a new combination following Gams (2000). A strain of *Cadophora finlandia* (Wang & Wilcox) Harrington & McNew (UAMH7454) has been shown by Wilcox & Wang (1987a, 1987b) to form ectomycorrhizas and ectendomycorrhizas on woody hosts. Monreal *et al.* (1999) subsequently reported that the same strain 'in limited trials' formed typical ericoid mycorrhizas with the ericaceous shrub *Gaultheria shallon* Pursh. If this were to be confirmed, it would add weight to Vrålstad *et al.*'s (2000) hypothesis. However, in commenting on these findings Read (2000) sounded a note of caution, pointing out that a test of Vrålstad's hypothesis would require 'cross-inoculation experiments, to demonstrate first that under ecologically realistic conditions mutually compatible associations are formed by identified genotypes, and second that similar nutritional or other fitness-related responses are seen in both plant partners'.

Vrålstad *et al.* (2002a) subsequently performed inoculation trials between selected isolates of the *H. ericae* aggregate and

potential ectomycorrhizal and ericoid hosts. Ectomycorrhizal formation was largely restricted to *C. finlandia*-like isolates of ectomycorrhizal origin, and none of the ectomycorrhiza-forming isolates produced any structures that resembled ericoid mycorrhizas when inoculated onto *Vaccinium vitis-idaea* L. Overall, their data supported the idea that both the ericoid and the ectomycorrhizal habit have evolved within the *H. ericae* aggregate, but there was no support for the hypothesis that single isolates could form both types of mycorrhiza. However, genetically identical fungi have been found in both ectomycorrhizal and ericoid mycorrhizal roots in two instances: *Quercus robur* L. (ectomycorrhizal) and *Calluna vulgaris* (ericoid); or *Salix herbaceae* L. (ectomycorrhizal) and *Vaccinium vitis-idaea* (ericoid) (Vrålstad *et al.*, 2002b). This finding is in line with that of Bergero *et al.* (2000), who found that ectomycorrhizal roots of *Quercus ilex* L. harboured fungi capable of forming typical ericoid mycorrhizas with *Erica arborea* L.

In summary, there is good evidence that fungi in the *H. ericae* aggregate which form ericoid or ectomycorrhizas are closely related, and that fungi capable of forming ericoid mycorrhizas are found in ectomycorrhizal root systems. However, the only evidence for a single isolate forming both types of mycorrhiza is that of Monreal *et al.* (1999), where the synthesis was widely separated in time and the evidence for ericoid mycorrhizal formation was said to be limited and still to be confirmed.

In an attempt to clarify further the nature of ericoid and ectomycorrhizal formation by fungi in the *H. ericae* aggregate, we report here the outcome of dual and tripartite *in vitro* culture of a fungal isolate from a *P. bicolorata*-like ectomycorrhiza with seedlings of *P. sylvestris* and *Vaccinium myrtillus* L.

## Materials and Methods

### Site description and sampling procedure

*Piceirhiza bicolorata*-like ectomycorrhizas were extracted in November 2000 from soil cores randomly sampled from a permanent plot in a 160-yr-old stand (plot 11: 57°01′45.5″N, 002°53′27.9″W, 333 m a.s.l.) in Glen Tanar National Nature Reserve, Aboyne, Aberdeenshire, north-east Scotland. Soil cores were wrapped with aluminium foil and placed in sealed plastic bags, stored in a cold room at 4°C, and processed within 72 h. Samples were soaked with tap water and cleaned by wet sieving. Ectomycorrhizal tips were cleaned of soil particles and organic debris under a dissecting microscope and placed in petri dishes with deionized water.

### Mycobiont isolation and culture

Vital *P. bicolorata*-like root tips (*sensu* Brand *et al.*, 1992: tissue light in colour when sectioned) were shaken for 1 min in 0.2% aqueous solution of Tween 20 (v/v) and rinsed in sterile distilled water, then transferred to 30% aqueous $H_2O_2$ (v/v) for 30 s and immediately rinsed three times in sterile deionized water. Surface-sterilized ectomycorrhizal tips were placed on sterilized microscope slides and dissected into small pieces, then placed in petri dishes with modified Melin–Norkrans agar media (MMN; Marx, 1969; Marx & Bryan, 1975) plus chlorotetracycline (30 mg $l^{-1}$) and incubated in the dark at 20°C. Emergent fungal colonies were transferred individually to fresh MMN agar petri dishes without antibiotics. Adjacent *P. bicolorata*-like ectomycorrhizas from the same core were fixed in FEA solution (formaldehyde : ethanol 70% : acetic acid (5 : 90 : 5 v/v/v)) as voucher material (Agerer *et al.*, 2000).

### DNA extraction

DNA was extracted from a 30-d-old axenic culture of isolate LVR4069 and from a synthesized ectomycorrhiza. Briefly, 0.5 g mycelium or one synthesized ectomycorrhizal tip was placed in a red lysing matrix tube (Qbiogene, Cambridge, UK) along with 0.5 ml hexadecyltrimethylammonium bromide (CTAB) extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol) and 0.5 ml phenol/chloroform/isoamyl alcohol (25 : 24 : 1, pH 8.0). The sample was then lysed for 2 × 15 s at a speed of 5.5 m $s^{-1}$ in a FastPrep bead beating system (Qbiogene, Cambridge, UK) and the aqueous phase was separated by centrifugation at 13 500 r.p.m. for 7 min at 4°C. The sample was extracted further with 600 µl chloroform before precipitating the nucleic acids with sodium acetate (3 M) and isopropanol by centrifugation at 13 500 r.p.m. for 30 min. Pelleted nucleic acids were then washed with cold 70% (v/v) ethanol and air-dried overnight before resuspension in 100 µl TE buffer (pH 7.4).

### ITS PCR, sequencing and phylogenetic analysis

Internal transcribed spacer (ITS) regions were amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). Polymerase chain reactions were carried out on a PTC-200 Thermal Cycler (MJ Research, Reno, NV, USA) using 50 µl reaction volumes each containing *c.* 50 ng template DNA, 20 pmol of each primer, 2 mM $MgCl_2$, 250 µM of each dATP, dCTP, dGTP and dTTP, 10× buffer [20 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween 20 (v/v), 0.5% Nonidet P40 (v/v), 50% glycerol (v/v)] and 2.5 U *Taq* DNA polymerase (Bioline, London, UK). Cycling parameters were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. Reactions were performed in duplicate, and negative controls (containing no DNA) were included in each reaction. Products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. PCR products were purified using the Qiaquick purification kit (Qiagen, Crawley, UK) and sequenced using the BigDye Terminator Cycle Sequencing Kit with an automated DNA sequencer (ABI model 377, Applied Biosystems, Warrington, UK) using ITS1 and ITS4 primers. DNA sequences generated from isolate LVR4069

**Table 1** GenBank accession number, FASTA closest matches of isolate LVR4069, mycobiont origin and mycotrophic status of ascomycetes included in the phylogenetic analysis

| GenBank accession number | FASTA SS (%)/no (bp)† | Origin | Mycotrophic status‡ (resynthesis confirmation references) |
|---|---|---|---|
| Ingroup | | | |
| AY579413 | | Axenic culture (LVR4069) from *Piceirhiza bicolorata*-like on *Pinus sylvestris*, Scotland | This study: ECM and ERM |
| AJ430136 | 98.9/476 | DNA extract from *P. bicolorata* on *Betula pubescens,* Norway | NT |
| AJ430144 | 98.5/476 | DNA extract from *P. bicolorata* on *B. pubescens*, Norway | NT |
| AJ292203 | 98.3/476 | Axenic culture from *P. bicolorata* on *Quercus robur*, Norway | Vrålstad *et al*. (2002a): ECM |
| AF481386 | 97.8/512 | DNA extract from ectomycorrhiza in mixed coniferous forest, Sweden | NT |
| AJ308340 | 97.5/477 | Axenic culture from *P. bicolorata* on *Picea abies* roots, Norway | Vrålstad *et al*. (2002a): ECM |
| AJ308341 | 97.5/476 | Axenic culture from *P. bicolorata* on *Populus tremula*, Norway | Vrålstad *et al*. (2002a): ECM |
| AJ430174 | 97.1/477 | Axenic culture from ectomycorrhiza on *P. sylvestris*, Norway | NT |
| AF476977 | 97.0/533 | DNA extract from ectomycorrhiza from B2 horizon in mixed coniferous forest, Sweden | NT |
| AJ430160 | 96.8/477 | DNA extract from *P. bicolorata* on *P. sylvestris*, Norway | NT |
| AF476973 | 96.7/515 | DNA extract from ectomycorrhiza in mixed coniferous forest, Sweden | NT |
| AF011327 | 96.1/542 | *Cadophora finlandia*, axenic culture from *P. sylvestris* ectomycorrhizas, Finland | Wilcox & Wang (1987a,b): ECM and EEM; Monreal *et al*. (1999): ERM? |
| AJ292202 | ND | Axenic culture from *P. abies* ectomycorrhiza, Sweden | Vrålstad *et al*. (2002a): ECM |
| AJ430119 | ND | Axenic culture from *Vaccinium vitis-idaea* roots, Norway | NT |
| AJ292201 | ND | Axenic culture from ectomycorrhiza on *P. sylvestris*, Norway | Vrålstad *et al*. (2002a): NM |
| AJ308339 | ND | Axenic culture from *P. bicolorata* on *Q. robur*, Norway | Vrålstad *et al*., 2002a): NM |
| AJ292199 | ND | Axenic culture from ectomycorrhiza on *B. pubescens*, Norway | NT |
| AJ292200 | ND | Axenic culture from ectomycorrhiza on *B. pubescens*, Norway | Vrålstad *et al*. (2002a): ECM |
| AF069505 | ND | *Hymenoscyphus ericae* axenic culture from *Calluna vulgaris* roots (Read 101), England | Monreal *et al*. (1999): ERM |
| AJ319078 | ND | *H. ericae* axenic culture from *C. vulgaris* roots (isolate UAMH6735 from type specimen), England | Vrålstad *et al*. (2002a): ERM |
| AJ308337 | ND | Axenic culture from *C. vulgaris* roots, Norway | Vrålstad *et al*. (2002a): ERM |
| AJ430168 | ND | Axenic culture from *P. sylvestris* ectomycorrhizas, Norway | NT |
| AJ430159 | ND | DNA extract from *P. bicolorata* on *P. sylvestris*, Norway | NT |
| AJ319077 | ND | *Scytalidium vaccinii* axenic culture from *Vaccinium angustifolium* roots (isolate UAMH5828 from type specimen), USA | Dalpé *et al*. (1989); Vrålstad *et al*. (2002a): ERM |
| Outgroup | | | |
| AJ430222 | ND | *Mollisia cinerea* on *P. abies* decaying cone, axenic culture from ascospores, Norway | |
| AY394921 | ND | *Phialocephala fortinii*, axenic culture from hemlock root tip, Canada | |

†SS, Sequence similarity; no, nucleotide overlap from FASTA search closest matches between LVR4069 isolate ITS sequence and sequences from GenBank nucleotide database; ND, not determined.
‡NM, nonmycorrhizal; ECM, ectomycorrhizal; EEM, ectendomycorrhizal; ERM, ericoid mycorrhizal; ?, to be confirmed; NT, not tested.

and synthesized ectomycorrhiza were edited, and a consensus sequence obtained for both, using the SEQUENCHER software package (ver. 3.0; Gene Codes Corporation, Ann Arbor, MI, USA). Closest matches were identified in the GenBank/EMBL/DDBJ nucleotide databases using the FASTA 3.0 program (Pearson & Lipman, 1998) and were included in the phylogenetic analysis along with other ITS sequences of taxa within the *H. ericae* aggregate (Vrålstad *et al*., 2002b) (Table 1). All sequences were aligned using CLUSTALW (ver. 1.8.2; Thompson *et al*., 1994) and manual adjustments were made to the alignment where necessary. The transition/transversion ratio and the gamma distribution parameter were estimated using TREE-PUZZLE (ver. 5.0) before conducting a neighbour-joining analysis using the F84 model in PAUP (ver. 4.0b10; Swofford, 2002) with 1000 bootstrap replicates. *Mollisia cinerea* (Batsch:Fr.) P. Karst. (AJ430222) and *Phialocephala fortinii* Wang and Wilcox (AY394921) were used as the outgroup taxa in the analysis.

## Mycorrhizal synthesis

Ectomycorrhizas were synthesized aseptically using a low-carbon and -nutrient agar culture system. Plastic petri plates (14 cm diameter) were prepared containing autoclaved modified Ingestad's solution for *P. sylvestris* (Ingestad, 1979; Ingestad & Kähr, 1985) solidified with 0.8% agar, supplemented with 0.01% glucose (MISAG), and overlaid with sterilized cellophane. From the edge of a 30-d-old mycobiont colony, six mycelium plugs (5 mm) were spaced over two-thirds of a MISAG–cellophane petri plate surface. The dish was sealed and incubated for 2 wk at constant temperature (20°C) in the dark to promote hyphal growth. Surface-sterilized Scots pine

seeds were placed in petri plates with autoclaved MISAG, and germinated in the dark for 2 wk. Five axenic germinated seedlings were transferred to the petri plates previously inoculated with the fungi, sealed and placed in a growth chamber (photoperiod 18 h; light 500 μmol $m^{-2}$ $s^{-1}$ PAR; temperature 20°C/15°C day/night; RH 75%) for 8 months.

Axenically cultured *V. myrtillus* seedlings were placed in petri plates with autoclaved MISAG in the growth chamber until used. The cross-infection experiments were performed in culture systems similar to those described above for ectomycorrhizal synthesis. In the dual synthesis, eight *V. myrtillus* seedlings were transferred aseptically to each of the petri plates previously inoculated with nine mycelial plugs on fresh autoclaved MISAG–cellophane. In the tripartite synthesis, six *V. myrtillus* seedlings were transferred with one eight month-old Scots pine seedling bearing *P. bicolorata*-like ectomycorrhizas, synthesized as described above, to three replicated fresh MISAG–cellophane plates. Two plates with six to eight non-inoculated *V. myrtillus* seedlings were left as a control. Plates were placed for 3 months in a growth chamber (photoperiod 18 h; light 200 μmol $m^{-2}$ $s^{-1}$ PAR; temperature 18°C/8°C day/night; RH 75%).

### Growth response of *Vaccinium myrtillus*

After 3 months the *V. myrtillus* seedlings were harvested and the shoot and root carefully separated with a scalpel. Shoots were oven-dried at 60°C for 48 h and their dry weight recorded. The excised hair root system of each seedling was digitized using a Win-Rhizo LA 1600 Scanner (Regent Instruments, Quebec, Canada). The analysis of digitized hair root images was performed using WIN-RHIZO ver. 3.9 software at a scanning resolution of 400 dpi (Polomski & Kuhn, 2002). In order to describe hair root architecture, the following morphometric variables were measured: (a) total hair root length; (b) total hair root surface area; (c) total hair root volume; (d) total number of hair root tips; (e) hair root branch density (root tip number/total root length) (Gamalero *et al.*, 2002). Data were obtained for each seedling, and the mean value for all surviving seedlings in a dish ($n = 5–8$) was used for statistical comparisons.

### Staining and microscopy

A sample of synthesized ectomycorrhizal root tips were excised and split into three subsamples: (1) preserved at 4°C as voucher material in 3% glutaraldehyde in 0.1 M phosphate buffer pH 6.8; (2) placed in CTAB extraction buffer and frozen at −81°C for molecular analyses; (3) morpho-anatomically characterized using freehand longitudinal and cross sections and mantle scrapes, mounted in 85% lactic acid and observed and photographed using a Carl Zeiss Axiophot D-7082 photomicroscope.

Each *Vaccinium* hair root system from the synthesis plates was cleared for 5 min with 10% (w/v) KOH in a water bath (Grant Instruments, Barrington, Cambridge, UK) at 90°C, acidified with 0.1 M hydrochloric acid for 1 h, stained in 0.05% (w/v) trypan blue for 15 min at 90°C, and destained overnight with lactic acid : glycerol : deionized water (14 : 1 : 1 v/v/v). Whole roots were mounted in the destaining solution, observed, and photographed as above.

### Percentage of infection

The magnified intersections method (McGonigle *et al.*, 1990) was adapted to quantify the percentage of infection of *Vaccinium* hair roots, using hyphal coils in epidermal cells as the sampling target. From each petri plate, three *Vaccinium* seedlings were chosen at random and the whole root system was examined under a microscope equipped with a cross-hair eyepiece graticule using differential interference contrast at ×1000 magnification. Where possible, at least 100 intersections per seedling root system were scored; on the smaller root systems a minimum of 44 intersections were scored. Counts were recorded as percentage of root length colonized (RLC) by the mycobiont using the formula:

$$\%RLC = 100 \times \text{number of intersections with coils/total number of intersections counted.}$$

### Mycobiont reisolation

Synthesized ectomycorrhizal root tips and 1 cm fragments of *Vaccinium* hair roots (control and uninfected) from each cross-infection experimental plate were aseptically excised and transferred in triplicate to PDA agar, grown in the dark at 20°C for 4 wk, and screened every week for comparisons with the original inoculated fungal strain. The fungal ITS region from synthesized ectomycorrhiza was PCR-amplified and sequenced for comparison with the inoculated fungus.

## Results

### Molecular identification and phylogeny

FASTA analysis of the ITS sequence of the *P. bicolorata*-like isolate LVR4069 (GenBank accession number AY579413) revealed that it was most closely related to ITS sequences of ascomycetes within the *H. ericae* aggregate (Table 1). Phylogenetic analysis using ITS sequences of the closest database matches, along with sequences representing the four main clades of the *H. ericae* aggregate (as described by Vrålstad *et al.*, 2002b), clustered the *P. bicolorata*-like mycobiont isolate LVR4069 in clade IV along with fungi referable to *C. finlandia* (Fig. 1). The clade was strongly supported in a bootstrap analysis (96% bootstrap support) and the sequences within this group were 94.7–99.7% similar to each other. The *P. bicolorata*-like (LVR4069) sequence was most closely related (98.9% sequence similarity) to AJ430136 (*P. bicolorata* root tip on *Betula pubescens* Ehrh., Norway), and the two sequences clustered together within clade IV. The remaining sequences

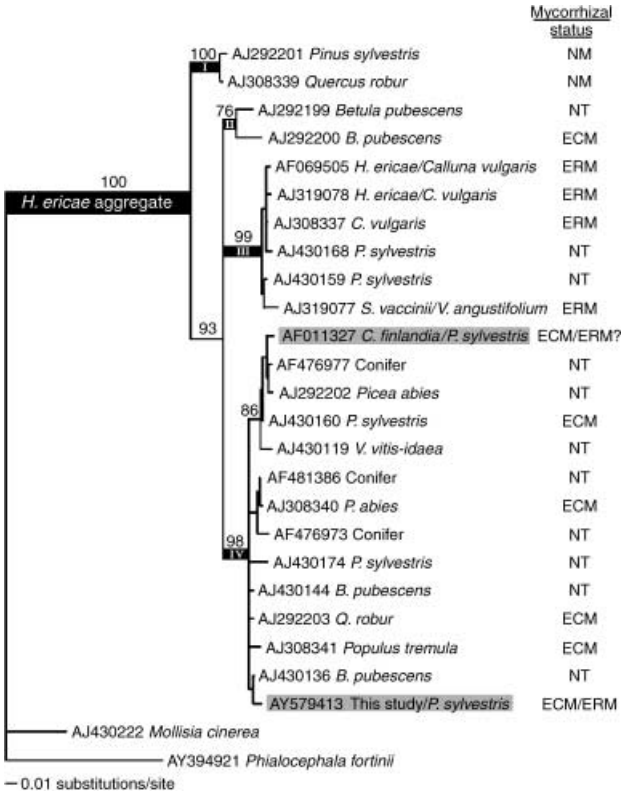


**Fig. 1** Phylogenetic relationship of fungal isolate LVR4069 to other members of the *Hymenoscyphus ericae* aggregate and their mycorrhizal status. Clades (*sensu* Vrålstad *et al*., 2002b) are indicated by Roman numerals inside the bars. Bootstrap values are given for branches of the tree supporting the main clades. NM, nonmycorrhizal; ECM, ectomycorrhizal; ERM, ericoid mycorrhizal; ?, to be confirmed; NT, not tested.

clustered into three further clades representing clades I, II and III described by Vrålstad *et al.* (2002b). The ITS sequence for the *P. bicolorata*-like mycobiont had 91.6–92.8%, 93.3–94.1% and 89.9–90.0% similarity with sequences in clades I, II and III, respectively.

## Synthesis of mycorrhizas

After 8 months a complete series of ectomycorrhizal developmental stages were present on Scots pine seedlings, ranging from unbranched, through dichotomously branched, to sparsely branched (Figs 2a, 3b–d) with or without a hyaline tip. The colour and appearance changed with age from orange-brown, grey, dark-grey with emanating hyaline hyphae to fully melanized charcoal black and dark-reddish-brown ectomycorrhizas. The emanating hyphae from the sheath (up to 1025 μm long, 1.5–2.0 μm diameter) had rounded tips and were narrow and septate, hyaline, amber to dark brown, thick walled, smooth or asperulose to sparsely verrucose (individual warts up to 0.5 μm) connected to a dense dark mantle (12.5–45 μm) over tannin cells. A well developed, palmetti-type Hartig net was observed. The morpho-anatomical characteristics of synthesized mycorrhizas were similar to those of mycorrhizas collected in the field (Fig. 3a).

In both dual and tripartite inoculations (Fig. 2b,c), the fungus produced structures that appeared to be typical ericoid mycorrhizas in the epidermal cells of hair roots of healthy *Vaccinium* plants (Fig. 3e,f). The control plants remained uninfected during the experiment. In the dual inoculation dishes (*V. myrtillus* + fungus) the mycobiont formed vigorous mycelial fans covering parts of the hair roots (Fig. 2d). In the tripartite inoculation (*V. myrtillus* + *P. sylvestris* + fungus) these vigorous mycelial fans were not observed. In this case the

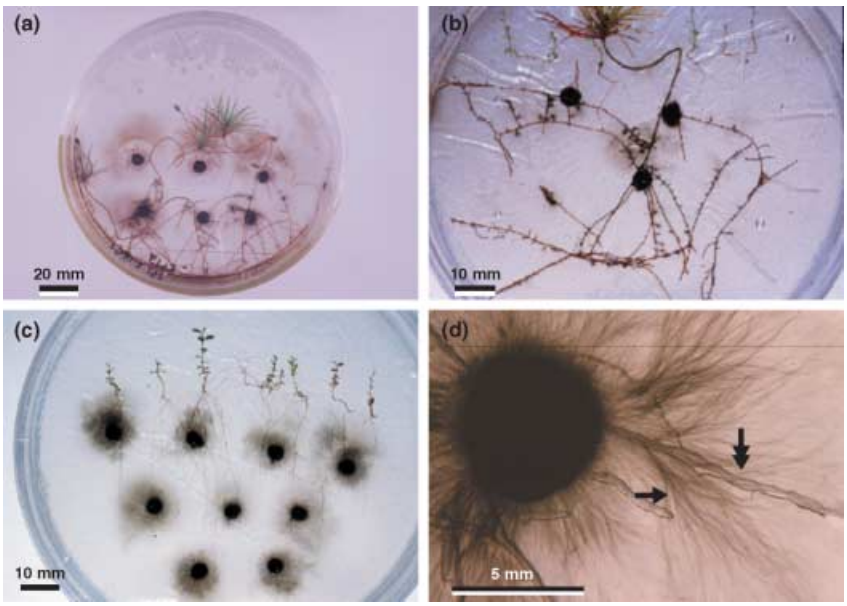


**Fig. 2** Mycorrhizal synthesis between Scots pine (*Pinus sylvestris*), *Vaccinium myrtillus* and isolate LVR4069. (a) *Piceirhiza bicolorata*-like ectomycorrhizas synthesized on 8-month-old Scots pine seedlings. (b) Cross-infection between synthesized *P. sylvestris* + *P. bicolorata*-like ectomycorrhiza and *V. myrtillus*. (c) Infection of *V. myrtillus* seedlings from fungal colonies on MISAG–cellophane. (d) Fan-like hyphal aggregations (arrow) of isolate LVR4069 interacting with *V. myrtillus* hair roots (double arrow).

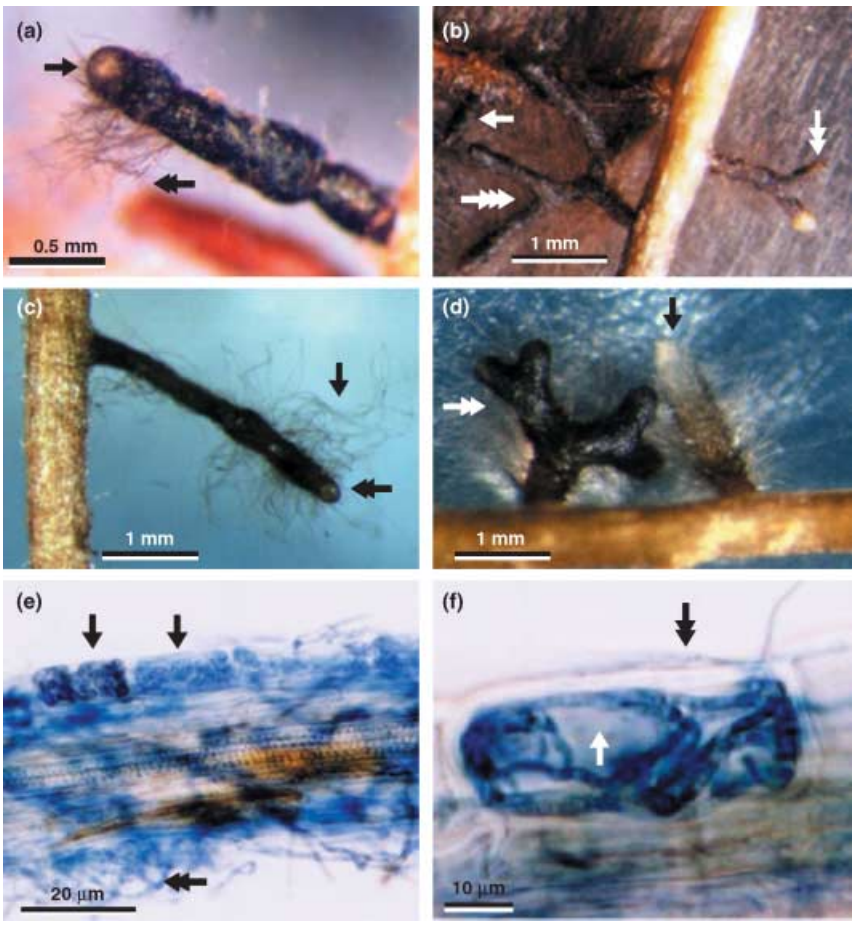


**Fig. 3** Ectomycorrhizal and putative ericoid mycorrhizal structures produced by the interaction between an isolate from the *Hymenoscyphus ericae* aggregate, *Pinus sylvestris* and *Vaccinium myrtillus* host plant roots. (a) *Piceirhiza bicolorata*-like ectomycorrhiza found on mature Scots pine with profuse radiating emanating hyphae (double arrow); note that the clear tip becomes melanized (arrow). (b) Developmental stages of synthesized *P. bicolorata*-like ectomycorrhizas: unbranched (arrow), dichotomously branched (double arrow), to sparsely branched (triple arrow). (c) Melanized charcoal black synthesized ectomycorrhiza with profuse emanating hyphae (arrow) and a hyaline tip partly covered by melanized hyphae (double arrow). (d) Unramified root tip (arrow) infected by hyaline emanating hyphae from a neighbouring sparsely branched charcoal black ectomycorrhiza (double arrow). (e) Bright-field photomicrograph (×400) of *V. myrtillus* hair root epidermal cells colonized by fungal isolate LVR 4069 intracellular hyphal coils (arrows) and external hyphal aggregates (double arrow). (f) Intracellular hyphal coil (arrow) and external runner hypha (double arrow) on *V. myrtillus* hair root epidermal cell, bright-field photomicrograph (×1000).

aerial hyphae arising from *P. sylvestris* ectomycorrhizas ran along hair root surfaces and entered the epidermal cells. In both cases the mycobiont produced melanized and verrucose runner hyphae (1.5–2.0 μm diameter) growing along the hair root system, which gave rise to fine, hyaline, smooth hyphae (1 μm). These runner hyphae gave rise in some places to dense melanized mycelial patches with some occasional loops and gangliform hyphae. The smooth, hyaline hyphae entered the epidermis and formed intracellular hyphal coils, which ranged from simple circinate hyphae with obvious organelles (Fig. 3f) to coils of convoluted hyphae (Fig. 3e), which could be either hyaline or melanized.

The infection of the *Vaccinium* hair roots was between 20 and 25% RLC, and was not significantly different whether the fungus colonized from colonies on MISAG–cellophane or from pine ectomycorrhizas. There was no relationship between the size of individual seedling root systems and RLC (data not shown).

**Table 2** Growth of *Vaccinium myrtillus* under axenic conditions (V.myr) or in the presence of a fungal (Fun) isolate from the *Hymenoscyphus ericae* aggregate inoculated as a pure culture (V.myr + Fun), or as synthesized mycorrhizas on Scots pine (Sp) seedlings (V.myr + Sp + Fun)

| Parameter | V.myr ($n$ = 2) | V.myr + Fun ($n$ = 3) | V.myr + Sp + Fun ($n$ = 3) |
|---|---|---|---|
| Shoot dry weight (mg) | 0.74a | 0.75a | 0.84a |
| Hair root length (cm) | 2.76a | 23.20b | 6.70a |
| Number of hair root tips | 9.40a | 57.68b | 24.05a |
| Hair root surface area ($cm^2$) | 0.23a | 1.77a | 0.44a |
| Hair root volume ($mm^2$) | 0.17a | 1.22a | 0.24a |
| Infection (%) | – | 25 ± 9.84 | 20 ± 5.69 |

Means with the same letter are not significantly different ($P \leq 0.05$, Student–Newman–Keuls). Replicates ($n$) are on petri plates containing five to eight *Vaccinium* seedlings.

## Fungal effect on plant growth and hair root architecture

All the *V. myrtillus* seedlings were green and healthy at the end of the 3 month growth period, and there were no differences in shoot dry weight between the treatments (Table 2). However, there were significant effects of fungal presence on *Vaccinium* root development, although these differed in intensity between dishes where the fungus alone was present, and those where the fungus was also colonizing Scots pine (Table 2). The most profound effects were on total hair root length (eightfold increase



 

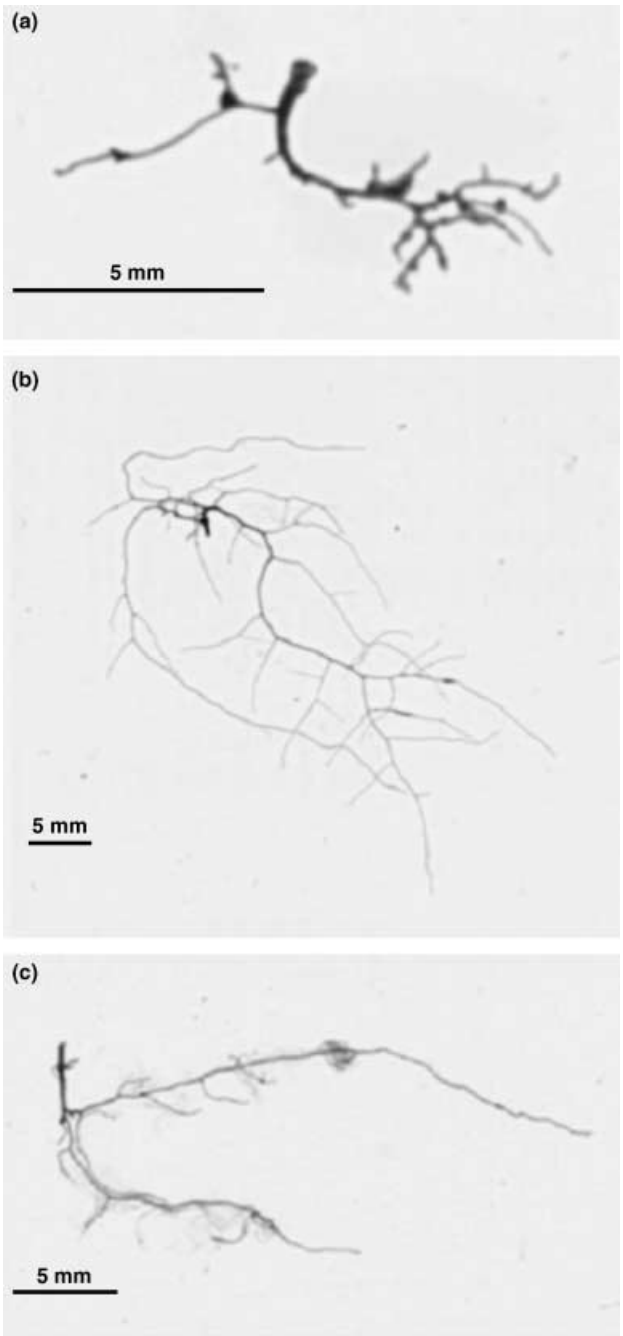


**Fig. 4** Root scanner images showing the effect of fungal isolate (LVR4069) on *Vaccinium myrtillus* hair root architecture. (a) Uninfected control; (b) infection from colonies on MISAG–cellophane; (c) infection from *Piceirhiza bicolorata*-like ectomycorrhizas.

in the presence of the fungus alone) and the number of hair root tips (sixfold increase in the presence of the fungus alone) (Fig. 4a–c). The effect of the fungus was in the same direction, but was of lesser magnitude when infection was by mycelia that were also connected to the ectomycorrhizas on Scots pine.

### Post-harvest fungal reisolation

Synthesized ectomycorrhiza and fragments of *V. myrtillus* hair roots gave rise to dark, slow-growing colonies on PDA, which appeared microscopically identical to the original isolate. The ITS sequence from synthesized ectomycorrhiza was 100% identical to that of the inoculated isolate.

## Discussion

### Systematic position of the isolate from *Piceirhiza bicolorata*-like mycorrhizas

Phylogenetic analysis of the *P. bicolorata*-like mycobiont (LVR4069) based on its ITS sequence grouped the isolate in clade IV of the *H. ericae* aggregate described by Vrålstad *et al.* (2002b). The majority of sequences in this clade come from fungi detected or isolated from the roots of ectomycorrhizal (ECM) hosts reported from Norway (Vrålstad *et al.*, 2002b), Sweden (Rosling *et al.*, 2003) and Finland (Wang & Wilcox, 1985), and include *C. finlandia* (AFO 11327).

In contrast, clade III of the phylogenetic tree is predominantly, but not exclusively, represented by fungi detected in the roots of ericaceous plants, and includes the isolates considered to be the putative teleomorph (*H. ericae*/UAMH6735) and anamorph (*Scytalidium vaccini*/UAMH5828) (Pearson & Read, 1973; Read, 1974; Egger & Sigler, 1993; Hambleton *et al.*, 1999). This distinction between the clades based on host, and hence ericoid mycorrhizal (ERM) or ECM, origin has been discussed previously (Vrålstad *et al.*, 2002b). This conclusion has been supported to some extent by data from resynthesis experiments using some isolates from the two clades with ERM and/or ECM hosts (Wilcox & Wang, 1987a, 1987b; Dalpé *et al.*, 1989; Monreal *et al.*, 1999; Vrålstad *et al.*, 2000, 2002a, 2002b). However, many more isolates need to be tested in dual and tripartite cross-infection experiments before any conclusion can be drawn on the separation of these clades based on mycorrhizal status. Jumpponen & Trappe (1998) pointed out that any outcome from pure culture synthesis needs to be interpreted with caution because the experimental conditions can influence the nature of the symbiotic response.

### Dual infection of *Vaccinium* and *Pinus* roots

This study has demonstrated for the first time that an isolate from the *H. ericae* aggregate obtained from wild *P. bicolorata*-like (*sensu* Brand *et al.*, 1992) mycorrhizas on Scots pine from native ecosystems can be used to synthesize ectomycorrhizas *in vitro* on *P. sylvestris*. We also show that the same isolate in dual and tripartite culture enters the hair root epidermal cells of *V. myrtillus* and forms hyphal coils, similar to the typical ericoid mycorrhizal structures described by Bonfante & Gianinazzi-Pearson (1979) and Read (1983). Under our experimental conditions, colonization of *V. myrtillus* induced a substantial

growth response in the root system. It must be emphasized that our designation of these structures as putative ericoid mycorrhizas is based on the presence of fungal coils and the positive growth effects seen in *Vaccinium* growing in our experimental system. Much more detailed experimentation will be required to satisfy fully the criteria of Read (2000) and to demonstrate that mutually beneficial metabolite transfer takes place between the partners.

Previous resynthesis attempts have produced contradictory evidence about the ability of individual isolates from the *H. ericae* aggregate to form both ecto- and ericoid mycorrhizas (Monreal *et al*., 1999; Bergero *et al*., 2000; Vrålstad *et al*., 2002a). Monreal *et al*. (1999) suggested (but presented no evidence) that an ectendomycorrhizal isolate of *C. finlandia* could form ERM with *Gaultheria*. In the same context, Bergero *et al*. (2000) demonstrated that identical fungal genets were present in ECM and ERM hosts in Mediterranean ecosystems, but did not demonstrate ECM or ERM formation by the same mycobiont. Finally Vrålstad *et al*. (2000, 2002b) were able to demonstrate that, on the one hand, there is strong phylogenetic relationship between *H. ericae* (ERM) and *C. finlandia* (ECM) as well as the presence of identical or nearly identical ITS genotypes in ECM and ERM hosts; but on the other hand, none of the ECM-forming strains produced ERM on *Vaccinium*.

### Positive response of *Vaccinium* to fungal presence

Under the experimental conditions used, infection of epidermal hair roots cells of *V. myrtillus* in dual culture led to a significant increase in root length and number of root tips, suggesting a benefit to the host. This could be a response to improved nutrition, as has been demonstrated in ericoid mycorrhizal associations (Mitchell & Read, 1981). Alternatively, Berta & Gianinazzi-Pearson (1986) suggested stimulation of plant hormone production or the production of indole-3-acetic acid (IAA) by the fungus as a possible cause of the substantial change in root length and number of hair roots in *C. vulgaris* seedlings infected with *H. ericae*. IAA has been identified in culture filtrates from *H. ericae* strains (Gay & Debaud, 1986) and recently in a '*Phialophora* sp.' (Rommert *et al*., 2002) subsequently identified as *Phialocephala fortinii* based on molecular evidence (B. Schulz, Technical University of Braunschweig, Germany, personal communication). Rommert *et al*. (2002) also reported a positive effect on the growth of *Larix decidua* plants and an increase in root system branching, both when their *P. fortinii* isolate was applied as a fungal extract and when it colonized the roots. It should be noted that the production of IAA was demonstrated only in the presence of tryptophane (Gay & Debaud, 1986), which was not added to our culture media.

The effect of the fungus on *Vaccinium* hair root growth was greater when the infection arose from colonies on MISAG–cellophane rather than from pine mycorrhizas. The reasons for this are unclear, but were not related to the extent of infection of the hair roots, which was the same in both cases. The fungus appeared more vigorous when growing from colonies on MISAG–cellophane, and this might affect the production of IAA.

### Ecological significance

The success of ericaceous plants in heathland ecosystems is the result of the ability of the plant/fungal symbiosis to succeed in conditions of extreme mineral N and P limitation and accumulation of recalcitrant organic matter (Cairney & Meharg, 2003; Read & Pérez-Moreno, 2003). In addition, boreal and mediterranean biomes support ericaceous plants as understoreys of ectomycorrhizal conifer or broadleaf forests that have been interpreted as 'relict populations' trapped by recurrent glaciation events (Read, 1991; Rendell & Ennos, 2002). In these two situations, environmental and edaphic conditions may operate in a different fashion, and can be expected to produce different selection pressures on both photo- and mycobionts along a gradient of increasing organic matter decomposability from open moorlands to woodlands (Cornelissen *et al*., 2001; Wilson & Puri, 2001). Soil fungal community structure derived from ITS- denaturing gradient gel electrophoresis (DGGE) profiles shows a distinct shift along a moorland–pine forest environmental gradient (Anderson *et al*., 2003), as do soil physical, chemical and biological properties (Chapman *et al*., 2001, 2003). Our observations support the notion that members of the well supported clade III of the *H. ericae* agreggate may have evolved as strict ericoid endophytes in moorlands, but that members of clade IV (including *C. finlandia*) are primarily ECM fungi, but may have the potential to be ERM symbionts in woodlands containing both ericaceous and ectomycorrhizal plants. This raises the intriguing possibility that understorey and overstorey plants are linked by a common mycorrhizal fungal mycelium. Identifying the functional significance, if any, of such linkages will require careful experimentation in the field.

Brundrett (2002) suggested that the evolution of mycorrhizas in Ericales is represented by a shift from arbuscular mycorrhizal ancestors towards ericoid systems (and subsequently to 'exploitative' ECM in Monotropoidae). He claims that the important event in the evolution of ericoid mycorrhiza was a 'switch to a new fungal lineage' in which the first ericoid endophyte evolved from an ECM fungus, probably from the *H. ericae* aggregate. Our results support this notion in as much as we have demonstrated that a strain from the *H. ericae* aggregate has the potential to produce both ectomycorrhizas and what appear to be ericoid mycorrhizas.

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