

# Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort

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Many non-photosynthetic vascular plants in 10 diverse families obtain all of their carbon from fungi, but in most cases the fungi and the ultimate sources of carbon are unknown. In a few cases, such plants have been shown to be epiparasitic because they obtain carbon from neighbouring green plants through shared mycorrhizal fungi. In all such cases, the epiparasitic plants have been found to specialize upon narrow lineages of ecto- or arbuscular mycorrhizal fungi. Here we show that a non-vascular plant, the non-photosynthetic liverwort *Cryptothallus mirabilis*, is epiparasitic and is specialized on *Tulasnella* species that form ectomycorrhizae with surrounding trees at four locations in England, France and Portugal. By using microcosm experiments we show that the interaction with *Tulasnella* is necessary for growth of *Cryptothallus*, and by using labelling experiments we show that <sup>14</sup>CO<sub>2</sub> provided to birch seedlings is transferred to *Cryptothallus* by *Tulasnella*. This is one of the first documented cases of epiparasitism by a non-vascular plant and of ectomycorrhizal formation by *Tulasnella*. These results broaden the emerging association between epiparasitism and mycorrhizal specialization into a new class of plants and a new order of fungi.

**Keywords:** symbiosis; parasitism; mutualism; cheating; mycorrhizae

## 1. INTRODUCTION

The ghostwort, *Cryptothallus mirabilis*, is a non-photosynthetic and subterranean bryophyte. Plants such as these that have lost their ability to photosynthesize and have become dependent upon symbiotic fungi for their supplies of carbon are referred to as myco-heterotrophs (Leake 1994). A myco-heterotroph can be regarded as being epiparasitic upon neighbouring autotrophs if its fungal partners are at the same time forming mutualistic mycorrhizal associations with photosynthetic plants. Parasites of mutualisms, or cheaters, extract benefits normally exchanged exclusively between mutualists (Yu 2001). In the case of the mycorrhizal mutualism the autotroph supplies carbon to its fungal partners in exchange for mineral nutrients (Smith & Read 1997), and cheating of this symbiosis arises when the myco-heterotroph induces these same fungal partners to colonize it, thereby gaining access to photosynthate (Johnson *et al.* 1997).

Several myco-heterotrophic vascular plants have been shown to be epiparasitic upon neighbouring photosynthetic plants through shared ectomycorrhizal (Björkman 1960; Cullings *et al.* 1996; Taylor & Bruns 1997; Bidartondo & Bruns 2001; Selosse *et al.* 2002) or arbuscular mycorrhizal (Bidartondo *et al.* 2002) fungal symbionts. Although photosynthetic plants are generalists in their compatibility with fungal partners, the epiparasites examined so far display exceptional specificity towards narrow groups of closely related fungi (Cullings *et al.* 1996;

Taylor & Bruns 1997; Bidartondo & Bruns 2002; Bidartondo *et al.* 2002). Because *Cryptothallus* may be the only lineage of non-vascular plants to have achieved cheating, it is of considerable interest to identify its fungal symbionts and to determine the pathways and sources of its carbon supplies.

The genus *Cryptothallus* has a wide but patchy distribution: Costa Rica (Crum & Bruce 1996), Greenland (Petersen 1972), Britain (Paton 1999), Scandinavia (Dickson *et al.* 1975), Germany (Wiehle *et al.* 1989), France (Bates & Hodgetts 1995; Boudier *et al.* 1999) and Portugal (Sérgio & Séneca 1997). This thalloid liverwort forms dense aggregations underneath the moss and litter ground layers of forests of birch, pine, willow or oak. It is easily overlooked because of its subterranean habit, hence the full extent of its distribution remains uncertain (Sérgio & García 1999).

It has long been known that the thalli of *Cryptothallus* are heavily colonized by fungal hyphae that form tightly coiled aggregates (pelotons) within cortical cells (Denis 1919). The fungi associated with *Cryptothallus* are basidiomycetes with a unique ultrastructural characteristic: dolipore septa with continuous parentheses (Pocock & Duckett 1984; Ligrone *et al.* 1993; Read *et al.* 2000). This character is shared by clades of 'jelly fungi' and basal Homobasidiomycetes (Hibbett & Thorn 2001; Weiß & Oberwinkler 2001; Wells & Bandoni 2001). Currently, with the exception of some members of the Sebacinaceae (Selosse *et al.* 2002), these groups do not include known ectomycorrhizal lineages, though *Tulasnella* species (anamorph: *Epulorhiza*, Tulasnellales) are well known to form orchid mycorrhizas (Bernard 1909; Hadley

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1969; Currah *et al.* 1990). Orchid mycorrhizas differ fundamentally from ectomycorrhizas and arbuscular mycorrhizas in that there is no evidence of plant-to-fungus carbon flow (Purves & Hadley 1975; Alexander & Hadley 1985; Andersen & Rasmussen 1996) but the reverse direction of fungus-to-orchid carbon flow has been shown in protocorms (Smith 1967; Alexander & Hadley 1985) and in myco-heterotrophic species (McKendrick *et al.* 2000). The ultimate source of carbon for all *Tulasnella* species is widely considered to be soil organic matter (Smith & Read 1997; Roberts 1999) because they produce their reproductive structures on fallen decomposing wood (though they do not degrade lignin). While many orchid mycorrhizal fungi are aggressive necrotrophic parasites, *Tulasnella* species, in common with the fungi forming all other kinds of mycorrhiza, appear to lack necrotrophic properties (Roberts 1999). Pocock & Duckett (1984) noted striking morphological similarities between the fungal structures within *Cryptothallus* and orchid mycorrhizas (e.g. peloton formation). However, although most orchid fungi are fast growing and can be readily isolated from field-collected roots, the associates of *Cryptothallus* could not be isolated by these workers. Taken together with the close spatial association always observed between *Cryptothallus* and ectomycorrhizal roots, this contributed to the hypothesis that the fungi involved are ectomycorrhizal (Hadley 1986; Boullard 1988). The observation that some ectomycorrhizas of spruce were formed by unidentified fungi with continuous parentheses (Haug & Oberwinkler 1987) added weight to this possibility. However, Ligrone *et al.* (1993) could not find the type of septa of the fungi within *Cryptothallus* in adjacent field-collected birch (*Betula*) ectomycorrhizas, thus contradicting the shared mycorrhizal fungi hypothesis and leading them to conclude that the fungi are saprotrophic. Recently, Read *et al.* (2000) reported that a fungus associated with *Cryptothallus* could be isolated and could form ectomycorrhizas with birch seedlings in microcosms. Electron microscopy revealed that this fungus exhibits continuous parentheses both within thalli and birch roots. Unfortunately, reliance on culturability can introduce biases if the target fungi are difficult to isolate. In fact, recalcitrant fungi are often the sole mycorrhizal associates of non-photosynthetic mycorrhizal plants (Taylor & Bruns 1997; Bidartondo & Bruns 2001; Bidartondo *et al.* 2002).

Direct examination of field-collected thalli and neighbouring roots is needed to define the niche of the fungi that sustain *Cryptothallus*, but progress has been hindered by the unique features of some of the fungi potentially involved. *Tulasnella* is a diverse cosmopolitan group (*ca.* 50 described species) whose taxonomy is based exclusively on microscopic characters. Its nrDNA is highly divergent from other basidiomycete fungi and in many cases does not share priming sites with standard universal fungal oligonucleotides (Taylor 1997). In practice, this renders a hypothetically mycorrhizal *Tulasnella* undetectable because PCR-based mycorrhizal studies rely almost exclusively on nuclear ribosomal internal transcribed spacer (ITS) data (Horton & Bruns 2001). Further, molecular sequence data for *Tulasnella* are extremely scarce and thus sequences of unknowns can be difficult to identify as tulasnelloid or even fungal. In fact, tulasnelloid nrDNA data have been entirely excluded from molecular

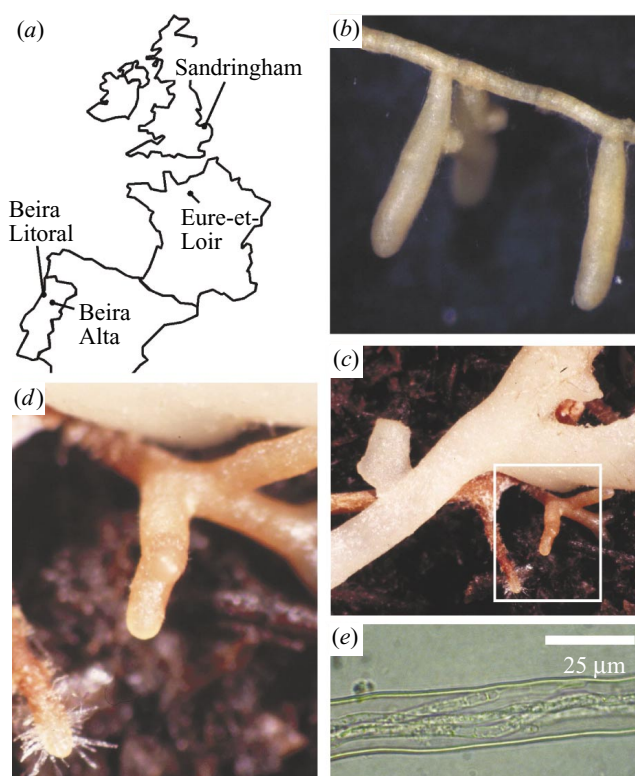


Figure 1. (a) Locations where we sampled *Cryptothallus mirabilis*. (b) Field-collected *Betula pendula*-*Tulasnella* sp. ectomycorrhizas (England). (c) *Cryptothallus* and *Pinus muricata* in a microcosm, 20 days after introduction of thalli, showing a *Pinus*-*Tulasnella* ectomycorrhiza in contact with a thallus and a non-mycorrhizal root with abundant root hairs. (d) Close-up of (c). (e) *Cryptothallus* rhizoids colonized by *Tulasnella* hyphae in a microcosm.

phylogenetic studies owing to excessive alignment ambiguity (Blanz & Gottschalk 1986; Weiß & Oberwinkler 2001). For these reasons, here we use a broad array of sequence data (mtLSU, nrITS, nrLSU and nrSSU) obtained from: (i) thalli, to unambiguously identify the fungi associated with *Cryptothallus* and assess their diversity among distant locations, and (ii) roots of neighbouring autotrophs to test whether these fungi form ectomycorrhizas in nature. In addition, we used  $^{14}\text{C}$  pulse labelling to test for carbon transfer from autotrophs to thalli via fungal connections.

## 2. MATERIAL AND METHODS

### (a) Field collection

Thalli of *Cryptothallus mirabilis* were obtained from four different locations: a *Betula pendula* forest at Sandringham Estate in Norfolk (England), the *Betula pubescens*, *Alnus glutinosa*, *Salix cinerea*, *S. aurita* forest of Senonches in Eure-et-Loir (France) (described by Boudier *et al.* (1999)), a *Pinus pinaster* plantation in Beira Alta, São João da Lourosa (Portugal) and a *P. pinaster* forest in Beira Litoral, Gafanha da Encarnação (Portugal) (localities 2 and 4, respectively, by Sérgio & Garcia (1999)) (figure 1). Roots adjacent to thalli (less than 10 cm away) were also collected. All samples were maintained cool and moist until arrival in the laboratory 1–5 days after field collection, where they were cleaned with distilled water and fine tweezers to remove soil, sand and organic debris. Individual thalli (*ca.* 10

and ectomycorrhizal roots (*ca.* 20) from each location were selected for analysis with stereo microscopes. In the English and Portuguese collections, we biased our sampling towards ectomycorrhizae that coarsely resembled those observed by Read *et al.* (2000) in microcosms inoculated with *Cryptothallus*. In the French collection, which contained diminutive *Salix* ectomycorrhizae, we sampled ectomycorrhizae arbitrarily.

### (b) Molecular characterization

DNA was extracted from fresh or lyophilized tissue of field or microcosm samples, by using single *ca.* 0.5 mm sections from thalli, excised rhizoids, or single root tips. The DNA extraction (Gardes & Bruns 1993) was modified to use a silica matrix (Q-BIOgene) for binding genomic DNA. The extracts provided templates for PCR reactions using the following primer combinations (target specificities listed between parentheses): ML5/ML6 (fungal mitochondrial large subunit, mtLSU), ITS1/ITS4-Tul (*Tulasnella* ITS), ITS1F/ITS4B (basidiomycete nuclear ribosomal ITS), ITS1F/ITS4 (fungal ITS), ITS1/TW14 (eukaryotic 5' nuclear ribosomal large subunit, nrLSU) and NS1/ITS4-Tul (*Tulasnella* nuclear ribosomal small subunit, nrSSU and ITS). Oligonucleotide sequences and PCR results are included as electronic Appendices A and B available on The Royal Society's Publications Web site. PCR was carried out on 96-well plates in Eppendorf Mastercycler. For restriction fragment length polymorphisms we used the enzyme *Hinf*I. Most PCR products could be directly sequenced bi-directionally, but problematical products were cloned by using a TOPO TA Kit for Sequencing (Invitrogen, Inc.). Products obtained with NS1/ITS4-Tul were sequenced by using primers NS1, NS3, NS4, NS8, ITS1 and ITS4-Tul. PCR products were purified with QIAquick kits (Qiagen). DNA sequencing was performed on an ABI 3100 Genetic Analyser by using BigDye v. 3 chemistry (Applied Biosystems). All DNA sequences obtained were compared with those available in GenBank by using BLAST. Whenever possible, sequences were then aligned manually to DNA sequence databases, new (*Tulasnella* ITS) or pre-existing (basidiomycete mtLSU (Bruns *et al.* 1998), and *Tulasnella* SSU and LSU nrDNA; M. Weiß, unpublished data), and analysed by neighbour-joining with Kimura 2-parameter distances and branch lengths estimated with maximum parsimony as implemented in PAUP\* v. 4.0beta10 (Swofford 2002). Ambiguous alignment regions were excluded. ITS sequences could be unambiguously aligned only with those obtained in this study. Regions of tulasnelloid nrLSU sequences that were highly divergent were recoded as missing data. GenBank accession numbers are AY192440–AY192525.

### (c) Axenic fungal isolation

A subset of thalli from the field and from microcosms was used for axenic isolations. We surface-sterilized with 15% H<sub>2</sub>O<sub>2</sub> or 1% Ca(OCl)<sub>2</sub> with 0.5% Tween 80 for 30 s, sectioned (less than 0.5 mm wide), and plated 250 sections on modified Melin-Norkrans (Marx 1969) agar media supplemented with 1.25 g l<sup>-1</sup> glucose and 5 g l<sup>-1</sup> malt extract, or Basal Salts with 1% glucose (Caldwell *et al.* 1991) agar media supplemented with 0.05 g l<sup>-1</sup> Novobiocin (Sigma). Inoculated agar plates were maintained in the dark at room temperature for up to four months and examined weekly.

### (d) Tripartite microcosms

Microcosms using non-mycorrhizal *B. pendula* or *Pinus muricata* seedlings were constructed from 15 cm × 15 cm × 0.3 cm sheets

of transparent Perspex for *Betula* or 20 cm × 20 cm × 0.5 cm for *Pinus*. The basic set-up has been described elsewhere (Bending & Read 1996). *Pinus* microcosms were used for long-term study and *Betula* microcosms were used for short-term carbon transfer experiments. The *Betula* microcosms were transferred to a controlled environment chamber where they were incubated with the emerging shoots exposed to a photosynthetic photon flux density of 120 μmol m<sup>-2</sup> s<sup>-1</sup> for four weeks allowing the root systems to occupy the surface over the top third of each microcosm. Photoperiods consisted of an 18 L : 6 D cycle at 15/10 °C, respectively. At four weeks, the *Betula* microcosms were opened and onto the peat surface of each of six of them we placed four thalli of *C. mirabilis* from the English *B. pendula* forest, at a distance of *ca.* 5 cm from the advancing roots and *ca.* 2 cm apart from each other. Two microcosms received no thalli and were retained as controls to determine whether the peat contained any mycorrhizal inoculum. All *Betula* microcosms were incubated for a further 12 weeks. In *Pinus* microcosms, four to six thalli from each field location sampled were placed in direct contact with young roots or more than 10 cm away from any root and maintained in environmental chambers on an 18 L : 6 D cycle at 18/16 °C, respectively, where only the shoots received light. Four *Pinus* microcosms were retained as controls. Microcosms were inspected weekly to determine whether mycorrhizal formation had occurred.

### (e) Carbon transfer

After 12 weeks, two control manipulations were done in each microcosm. In the first of these (control 1), which was applied to all six *Betula* microcosms, mycelial connections between two of the thalli and roots were broken by temporarily raising the thalli from the peat surface with forceps and replacing them in their original positions. The second control manipulation (control 2) involved the addition to each microcosm of two new thalli. Shoots of *B. pendula* growing in the six microcosms were each placed into small (100 ml) chambers with gas-tight transparent lids. Into each was injected 1.0 MBq <sup>14</sup>C as sodium bicarbonate solution (NaH <sup>14</sup>CO<sub>3</sub>—specific activity 2.1 GBq mmol) from which <sup>14</sup>CO<sub>2</sub> was released by adding 10% lactic acid. The microcosms were then returned to the environmental chambers and incubated for 24 h, during which all the <sup>14</sup>CO<sub>2</sub> was assimilated by *Betula*. The chambers were then opened and the labelled plants were incubated for a further 21 days. This period was selected as being adequate to enable distribution and accumulation of radioactivity in 'sink' tissues while being sufficiently short to prevent the re-establishment (control 1) or establishment (control 2) of hyphal connections between the thalli and *Betula* roots. All thalli from each <sup>14</sup>C-fed microcosm were individually removed, lightly washed to remove adhering peat, dried and weighed before combustion in a Packard 307 Sample Oxidizer (Packard Instrument). The released <sup>14</sup>C was trapped in a scintillation cocktail containing 8 ml Carbosorb E and 10 ml Permafluor E (Packard Bioscience). Radioactivity was counted on a Packard Tricarb 1600TR liquid scintillation analyser and corrected for quench and background.

## 3. RESULTS AND DISCUSSION

Most *Cryptothallus thalli* were consistently found to harbour *Tulasnella* (32 of the 35 thalli examined). They consistently produced fungal mtLSU sequences that are most closely related to *Tulasnella* sequences (figure 2). No other fungi were detected in thalli by this approach. Lack of

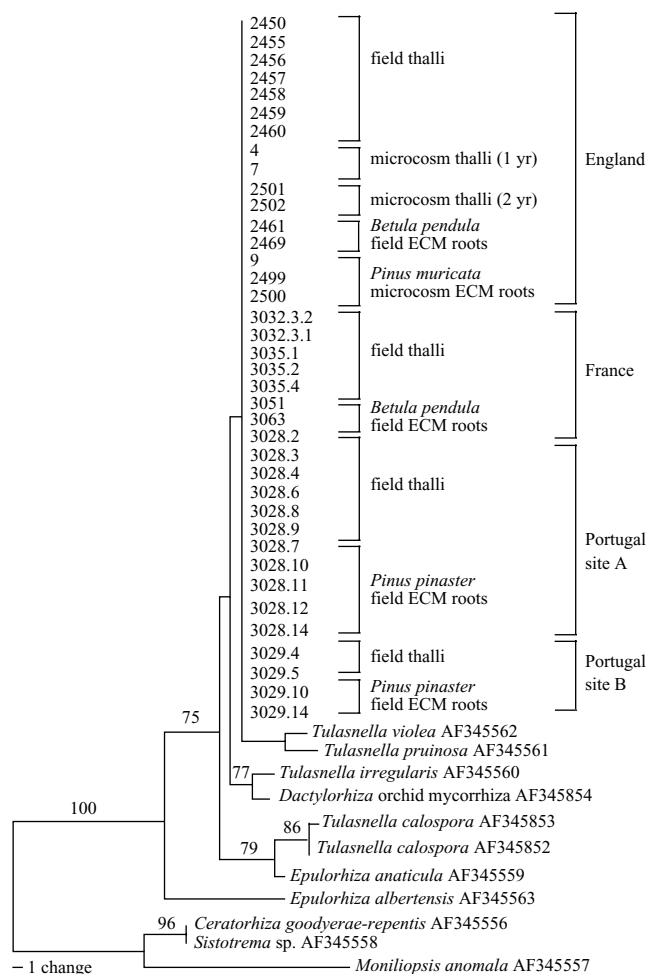


Figure 2. Phylogenetic placement of *Cryptothallus*-associated fungi using partial mtLSU sequences of *Tulasnella* (and its anamorph *Epulorhiza*) available in GenBank. The origin of *Cryptothallus*-associated fungal sequences is shown on the right. Analysis was performed by neighbour-joining with 1000 bootstrap replicates (values of more than 70% are shown above branches). *Ceratorhiza*, *Sistotrema* and *Moniliopsis* were used as outgroup taxa.

amplification in three samples may reflect the fact that fungal colonization is absent in regions such as meristems, the upper parenchyma of immature thalli and reproductive structures (Read *et al.* 2000). Because the priming sites used to target part of the fungal mtLSU locus are shared by most basidiomycetes tested (Bruns *et al.* 1998), the consistency observed constitutes strong evidence that thalli are predominantly occupied by a tulasnelloid symbiont. However, a few groups of basidiomycetes contain introns that preclude PCR amplification of their mtLSU with the primers used here. Thus, we chose to target additional loci.

The ITS is widely used in ectomycorrhizal molecular ecology because its priming sites are universally conserved in fungi with only rare exceptions. Because available basidiomycete- and fungal-specific ITS primer sets fail to amplify *Tulasnella*, we used tulasnelloid-specific primers to target the ITS. This approach consistently produced amplification (32 of 35 thalli), in agreement with the mtLSU data. The ITS sequences were similar across locations (figure 3), but showed differences between the fungi associated with *Betula* in England and France, and

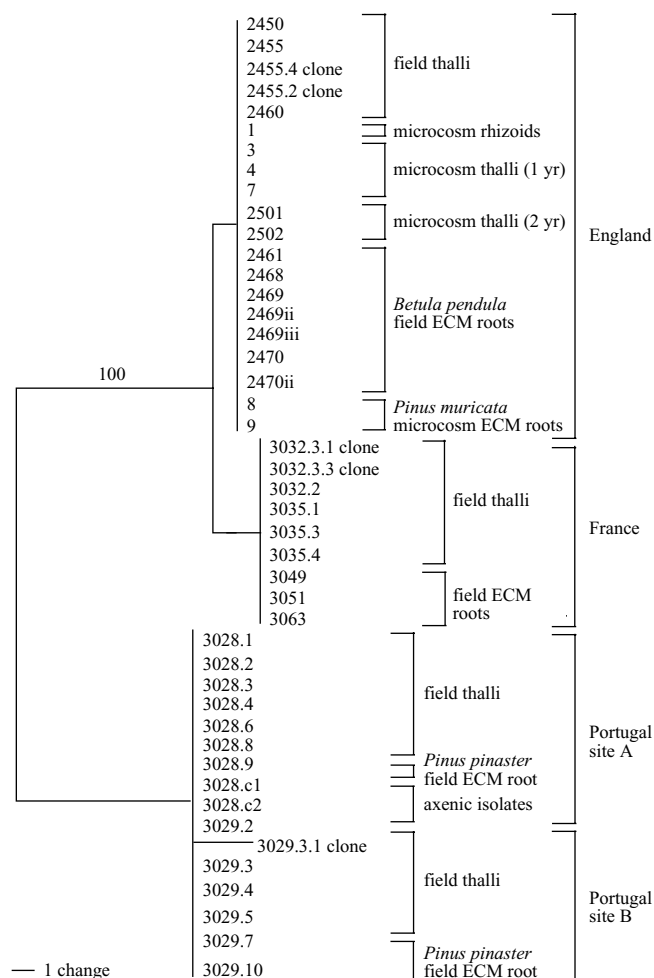


Figure 3. Phylogram depicting nrITS distances among *Cryptothallus* fungal symbionts. The origin of sequences is shown to the right. All sequences from Portugal obtained directly from PCR products shared three heterozygotic nucleotide sites in the ITS 2. One of these variants was cloned and sequenced (3029.3.1). Analysis was performed by neighbour-joining with 1000 bootstrap replicates (values of more than 70% are shown above branches). Midpoint rooting was used.

those associated with *Pinus* in Portugal. However, there is no absolute specificity among these clades because fungi from thalli from *Betula* forests were able to associate with *Pinus* in microcosms.

No ITS sequences are currently available from *Tulasnella* in GenBank, and no close relatives of our sequences have yet been obtained in this diverse group (P. Roberts and P. Bridge, personal communication; M. Weiß, unpublished data). The otherwise universally conserved 5.8S ribosomal gene, flanked by the ITS, when amplified and sequenced from *Tulasnella*, does not show detectable similarity to any DNA sequence currently available in GenBank. To confirm that the ITS sequences discussed above were *Tulasnella* ITS sequences, we amplified, cloned and sequenced the nrSSU and nrLSU located directly downstream and upstream, respectively, from the ITS for representatives of the two major ITS clades. In both cases, the ITS was part of the amplicons obtained, and was also sequenced to infer the phylogenetic affinities of the ITS sequences obtained previously. *Tulasnella* forms a monophyletic clade for the nrLSU, albeit one with

remarkable sequence heterogeneity (figure 4). This clade includes the fungal sequences obtained from *Cryptothallus* and *Tulasnella asymmetrica* (*T. pinicola* in Roberts 1999), an orchid mycorrhizal associate (Warcup & Talbot 1967). The nrSSU fungal sequences from *Cryptothallus* are also most closely related to *Tulasnella* sequences.

Basidiomycete- and fungal-specific primer sets failed to consistently produce amplicons, and if they did they only produced weak amplifications. This is the expected result given the divergent priming sites of *Tulasnella* (e.g. three mismatches in ITS1F, two in ITS4, seven in ITS4B, and absent ITS2 and ITS3 sites); weak amplifications probably reflect sub-optimal annealing to *Tulasnella*, or non-specific annealing to other fungal or plant loci. On a few occasions (6 in 31 thalli for ITS1F/ITS4, 1 in 24 for ITS1F/ITS4B), multiple differently sized PCR products were observed; these probably reflect the presence of fungi whose ITS is co-amplified. Most such amplifications occurred with universal fungal primers. We suspect many of these are soil fungi present as spores or hyphae in the surface of thalli (which were sampled from wet organic soil), or saprobic fungi decomposing the older senescing end of each thallus (a necrotic area which supported abundant ascomycete sporulation just one week after placement in microcosms). Lack of amplification (28 in 55 PCR reactions) indicates that very few fungi occupy portions of many thalli, at least any fungi that can be detected with the basidiomycete- and/or fungal-specific primer sets. In particular, basidiomycete-specific primers generally failed to produce amplicons (12 in 24 thalli), or did so only weakly (10 in 24 thalli). We identified only two fungi with these two primer sets, a russuloid and a *Hymenoscyphus* sp., both in thalli that were also found to be predominantly occupied by a tulasnelloid fungus according to the mtLSU data.

As previously reported, ectomycorrhizal roots were abundant near *Cryptothallus* (Pocock & Duckett 1984; Ligrone *et al.* 1993; Read *et al.* 2000). Those studies are in conflict regarding whether the fungi colonizing *Cryptothallus* are also associated with vascular plant roots. We used the same molecular approach described above to screen ectomycorrhizal roots that were collected near thalli in the field, and also *P. muricata* roots from microcosms inoculated with thalli of English *Cryptothallus*. In the case of *B. pendula* roots at the English site, 10 out of 31 individual ectomycorrhizal root tips were found to harbour *Tulasnella*, as determined by mtLSU and/or tulasnelloid-specific ITS-PCR. One root produced a cantharelloid (a group of ectomycorrhizal fungi) mtLSU sequence and a *Tulasnella* ITS sequence, in addition to another ITS (likely to be cantharelloid). Another root harboured an unknown basidiomycete (universal fungal ITS-PCR) in addition to *Tulasnella* (mtLSU and *Tulasnella*-specific ITS-PCR). These latter roots may have been infected by two ectomycorrhizal fungi, or by a saprobic fungus in addition to an ectomycorrhizal fungus. At the French site, which had several tree species, three of 27 roots produced *Tulasnella* sequences. Two of those produced exclusively *Tulasnella* sequences and were *B. pubescens* roots as determined by sequencing of the plastid *trn* L intron and *trn* L-F intergenic spacer regions from the same root DNA extracts used for fungal identification. An additional root belonging to an unidentified plant produced a russuloid

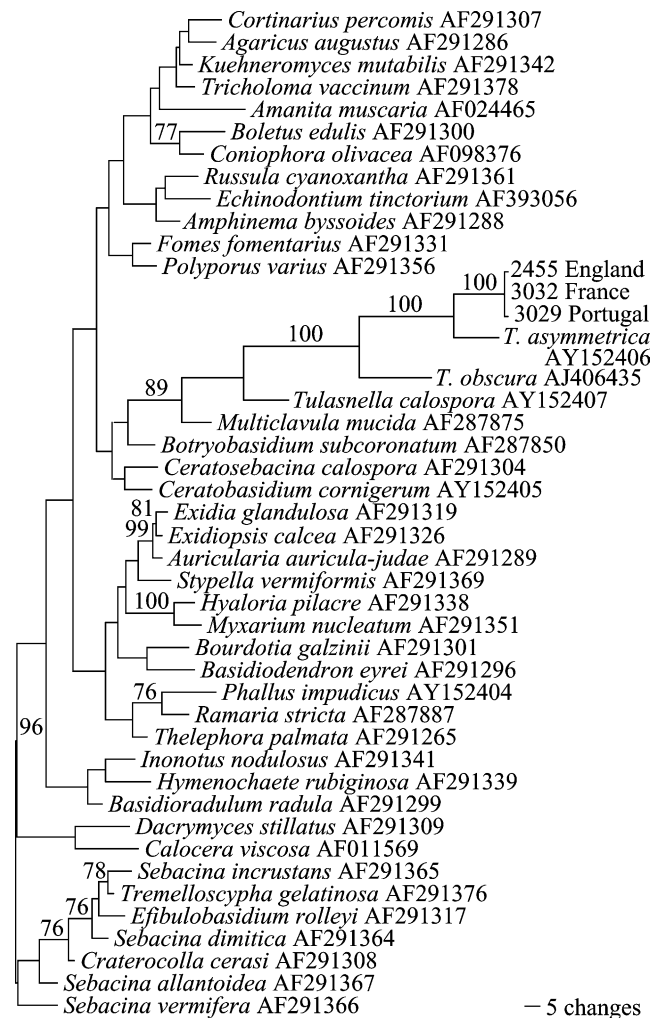


Figure 4. Phylogenetic placement of *Cryptothallus*-associated fungi using nrLSU sequences of main groups of Hymenomycetes obtained from GenBank. Analysis was performed by neighbour-joining with 1000 bootstrap replicates (values of more than 70% are shown above branches). The Sebacinaceae was used as outgroup.

sequence (a lineage of ectomycorrhizal fungi) in addition to a *Tulasnella* sequence. In *P. pinaster* from Portugal, 15 out of 18 individual ectomycorrhizal root tips were found to harbour the *Tulasnella* previously detected in neighbouring thalli. One of those roots also harboured a *Pseudotomentella* sp. (an ectomycorrhizal genus) and two also harboured unidentified fungi (no significant similarity to any GenBank sequences).

The fungi found within thalli are connected to the surrounding soil by rhizoids that emanate from the epidermis of the thalli (Pocock & Duckett 1984; Ligrone *et al.* 1993). The *Tulasnella* detected within English thalli was also detected in rhizoids emanating from thalli in microcosms. There are two kinds of rhizoid formed in microcosms. Short younger rhizoids are translucent and do not contain hyphae. Long older rhizoids are golden and contain abundant hyphae that can be observed at 400× magnification without staining (figure 1e). Branching and septation are evident, but no clamp connections were observed. Rhizoids were used for DNA extraction and PCR-amplification after observation under the microscope. Single rhizoids (young or old) failed to amplify, as did sets of

multiple young rhizoids (data not shown). Of three sets of older rhizoids (each rhizoid *ca.* 2 mm long, 3–6 individual rhizoids per DNA extraction), one produced both mtLSU and *Tulasnella*-specific ITS-PCR products and one produced only *Tulasnella*-specific ITS-PCR products.

We obtained axenic cultures of *Tulasnella* from thalli from the field and from microcosms by using 1% Ca(OCl)<sub>2</sub> and Basal Salts medium. Fast-growing, sporulating ascomycetes were common. One isolate that initially grew slowly (growth was first visible one week after inoculation) and tightly pressed against the agar surface, with hyaline hyphae and no clamp connections, was confirmed to be *Tulasnella* by sequencing ITS-PCR products obtained with *Tulasnella*-specific ITS primers.

The thalli placed in microcosms directly in contact with young roots survived and grew for at least 2 years. Ectomycorrhiza formation by the *Tulasnella* present in thalli occurred as early as 20 days in *Pinus* microcosms (figure 1*c,d*) and 35 days in *Betula* microcosms as evidenced by loss of root hairs, swelling, mantle and Hartig net formation, and DNA sequence confirmation. The first roots to become colonized were those in the immediate proximity of thalli (figure 1*d*); subsequently, infection spread to secondary laterals over most of the root system. No mycorrhizal colonization was observed in microcosms lacking thalli. The thalli placed more than 10 cm away from *Pinus* roots had senesced completely within a month. However, thalli maintained submerged in distilled H<sub>2</sub>O at 4 °C for six weeks were able to resume growth when placed in contact with young non-mycorrhizal roots in microcosms. The ability of *Tulasnella* to colonize new roots rapidly from within colonized thalli is likely to contribute to its competitiveness within diverse ectomycorrhizal communities, and to its ability to survive soil disturbances such as compaction, grazing, flooding, drought and frost.

Significant amounts of carbon were transferred from *Betula* in all microcosms but only to those thalli which had been the source of inoculum for the *Betula* roots and had retained their hyphal connections intact through the incubation period (figure 5). Carbon accumulation in the controls was below background in all cases. In intact thalli, the quantities of <sup>14</sup>C accumulating over the 21 day incubation ranged between 0.2 and 0.5% of that originally released as a pulse into the chambers. This is a minimum estimate because respiratory loss was not measured. Although the relatively large autotroph, with the extensive mycelial system of the fungus, retain the bulk of the carbon fixed during the <sup>14</sup>C pulse, it is evident that the intact mycelium provides a pathway for transfer of carbon into thalli.

In conclusion, we have demonstrated that *Cryptothallus* associates with a narrow clade of *Tulasnella* and that these same fungi have the ability to form ectomycorrhizas on *Betula* and *Pinus*. It was then shown that *Tulasnella* provides the pathways through which fixed carbon flows from one of its autotrophic co-hosts to *Cryptothallus*. For the first time, to our knowledge, this study provides identification of the mycorrhizal associate of this unique myco-heterotrophic liverwort and evidence of the ability of *Tulasnella* to form ectomycorrhizas. It also provides the first unequivocal demonstration that the source of carbon that sustains the liverwort is the autotrophic co-host of the fungus rather than the soil. The mycorrhizal specificity of

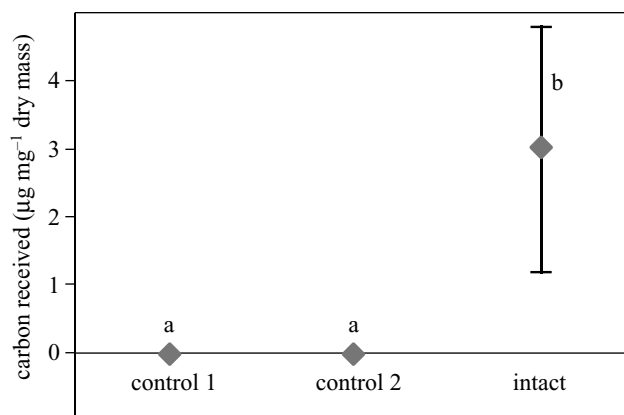


Figure 5. The concentration of carbon received 21 days after a <sup>14</sup>CO<sub>2</sub> pulse, by individual thalli of *Cryptothallus* grown with *Betula pendula*. The thalli had *Tulasnella* hyphal connections to the roots of *Betula* which were forming ectomycorrhizas. In control 1, hyphal connections between thalli and roots were severed immediately before <sup>14</sup>CO<sub>2</sub> exposure. In control 2, thalli were introduced to the microcosms immediately before <sup>14</sup>CO<sub>2</sub> exposure. Bars show standard error of the mean. Different letters indicate significant differences ( $p = 0.05$ , Tukey test).

*Cryptothallus* reported here is the most evolutionarily distant example of the epiparasitic syndrome; so far there are no exceptions to the pattern of epiparasitic plants specializing on phylogenetically narrow clades of fungi. Specialized mycorrhizal cheating is remarkable for several reasons: (i) it exploits one of the most widespread mutualisms in terrestrial ecosystems; (ii) it is one of the few non-animal examples supporting the view that parasites are more specialized than mutualists (Price 1980); (iii) it represents one extreme of the mycorrhizal continuum where plant–fungus interactions range from parasitism of either partner at the ends to mutualism at the centre (Johnson *et al.* 1997); (iv) it demonstrates that for some plants the high biodiversity of mycorrhizal communities lacks redundancy (Bruns 1995; Bidartondo *et al.* 2000); and (v) it shows that mycorrhizal networks provide conduits for net fluxes of carbon from plants to fungi to plants. The latter two points have direct implications for myco-heterotrophic plant conservation strategies: specific soil fungi and photosynthetic plants must be taken into account.

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