Widespread association between the ericoid mycorrhizal fungus *Rhizoscyphus ericae* and a leafy liverwort in the maritime and sub-Antarctic

R. Upson[1, 2], D.J. Read[1] and K.K. Newsham[2]

[1]Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, S10 2TN, UK

[2]Biological Sciences Division, British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, UK

Author for correspondence: KK Newsham

E-mail: kne@bas.ac.uk

Tel: +44(01223 221400)

Fax: +44(01223 362616)

Summary

- A recent study identified a fungal isolate from the Antarctic leafy liverwort *Cephaloziella varians* as the ericoid mycorrhizal associate *Rhizoscyphus ericae*. However, nothing is known about the wider Antarctic distribution of *R. ericae* in *C. varians*, and inoculation experiments confirming the ability of the fungus to form coils in the liverwort are lacking.
- Using direct isolation and baiting with *Vaccinium macrocarpon* seedlings, we isolated fungi from *C. varians* sampled from eight sites across a 1,875 km transect through sub- and maritime Antarctica, from Bird Island on South Georgia (54 °S, 38 °W) through to Alexander Island (71 °S, 68 °W) on the western Antarctic Peninsula. We also tested the ability of an isolate to form coils in aseptically-grown *C. varians*.
- Fungi with 98-99% sequence identity to *R. ericae* internal transcribed spacer region and partial large subunit ribosomal (r)DNA sequences were frequently isolated from *C. varians* at all sites sampled. The EF4/Fung5 primer set did not amplify small subunit rDNA from three of five *R. ericae* isolates, probably accounting for the reported absence of the fungus from *C. varians* in a previous study. *R. ericae* was found to colonize aseptically-grown *C. varians* intracellularly, forming hyphal coils.
- This study shows that the association between *R. ericae* and *C. varians* is apparently widespread in Antarctica and confirms that *R. ericae* is at least in part responsible for the formation of the coils observed in rhizoids of field-collected *C. varians*.

Key words: *Cephaloziella varians*, dark septate endophyte, ericoid mycorrhiza, *Rhizoscyphus ericae,* liverwort, maritime and sub-Antarctica

Introduction

A range of leafy hepatics in the Jungermannopsida consistently form associations with ascomycetous fungi (Read *et al.,* 2000), forming 'mycothalli' (Boullard, 1988). In a study of British hepatics, species of the leafy liverwort genus *Cephaloziella* were found to possess fungal associations restricted to rhizoids, with over half of the rhizoid tips colonized (Duckett *et al.*, 1991). Fungal colonization of rhizoids is characterized by dense intracellular growth that appears analogous to ericoid mycorrhizal coils (Selosse, 2005). The functional nature of the relationship between liverworts and their endophytic fungi is not known, but the formation of structures similar to those seen in ericoid mycorrhizal roots suggests an active role in plant survival (Read *et al.,* 2000).

Cephaloziella varians (Gottsche) Steph. is the most widespread species of liverwort in Antarctica, occurring in the maritime, continental and sub-Antarctic regions (Bednarek-Ochyra *et al.,* 2000). As in British *Cephaloziella*, hyphae and rudimentary coils of ascomycetous fungal endophytes are present in the rhizoids of this leafy liverwort collected from Botany Bay, Granite harbour (77 °S, 162 °E) and the Bailey Peninsula in the Windmill Islands (66 °S, 110 °E), both in eastern continental Antarctica (Williams *et al.,* 1994). Colonies of isolates from *C. varians* collected from these locations are slow-growing, waxy and become dark brown to black with age (Williams *et al.,* 1994). Using the ITS1/ITS4 primer set, Chambers *et al.* (1999) sequenced the internal transcribed spacer (ITS) region of an isolate from the Bailey Peninsula and showed it to have a high (99.4%) sequence identity to the type culture of the ericoid mycorrhizal fungus *Hymenoscyphus ericae*, recently renamed as *Rhizoscyphus ericae* (D.J. Read) W.H. Zhuang & Korf. (Zhang & Zhuang, 2004). In contrast, direct PCR analysis using the EF4/Fung5 primer set, which targeted a region of the small subunit (SSU) ribosomal (r)RNA gene, suggested that fungi bearing strong affinities to *R. ericae* are absent from *C. varians* at Rothera Point in the maritime Antarctic (Jumpponen *et al.,* 2003).

65 Given the current uncertainty about the distribution of *R. ericae* in *C. varians* in Antarctica, we
66 addressed the question of whether or not *R. ericae* is consistently isolated from the tissues of the
67 liverwort collected from a wide range of locations in the maritime and sub-Antarctic. We also tested
68 whether the EF4/Fung5 primer set might not be appropriate for the amplification of *R. ericae* DNA,
69 possibly owing to the presence of introns in the SSU rDNA (Jumpponen *et al.,* 2003). A re-
70 inoculation experiment also determined whether or not *R. ericae* forms structures similar to those
71 observed in field-collected shoots. Finally, we tested the ability of Antarctic *R. ericae* to form
72 hyphal coils in the roots of an ericaceous plant species.
73
74 **Materials and Methods**
75 Field sampling
76
77 *Cephaloziella varians* was collected during the 2002 and 2005 austral summers from field sites at
78 Bird Island on South Georgia, Signy, Lynch and Coronation Islands in the South Orkneys, King
79 George and Livingston Islands in the South Shetlands, and Adelaide and Alexander Islands on the
80 western Antarctic Peninsula (Fig. 1; Table 1). Sites on the South Orkney, South Shetland and Bird
81 Islands were reached by helicopter or small boat from ships. The site on Adelaide Island was
82 reached on foot from the nearby British Antarctic Survey Rothera Research Station, and that at
83 Alexander Island was reached by fixed-wing aircraft fitted with skis, also from Rothera. At least
84 five samples of *C. varians* mat (up to 50 × 50 mm) were removed from each site with a knife wiped
85 with sterilant (Virkon®; Antec International Ltd., Sudbury, UK) between samples and placed in a
86 re-sealable polythene bag.
87
88 Treatment of plant material after sampling
89

90 Plants of *C. varians* sampled from the South Shetland Islands were initially stored for several days
91 at 4°C in cool boxes, then maintained for 12 wk in growth cabinets set to a 16 h cycle of light (300
92 μmol photons m^{-2} s^{-1} at 6°C) and 8 h of darkness (4°C). Those sampled from the South Orkney and
93 Adelaide Islands were maintained for 5 to 8 wk under the growth conditions described above and
94 were sprayed with sterile distilled water (dH_2O) to keep tissues moist. Plants sampled from
95 Alexander Island were stored for 4 h in a cool box prior to return to Rothera research station. Those
96 sampled from Bird Island were frozen within several hours of collection at –20°C for 8 wk.
97 All samples except those from Alexander Island were returned to the UK. On arrival,
98 samples from the South Orkney, South Shetland and Adelaide Islands were transported at 4°C to
99 the ANNEX growth room facility at the University of Sheffield. Plants from these locations were
100 transferred to sterile Petri dishes (100 mm × 100 mm; Bibby Sterilin, Stone, UK) and sprayed as
101 necessary with sterile dH_2O to keep tissues moist. Samples from different sites were kept in
102 separate closed dishes. Plants were maintained under the same growth conditions as those described
103 above for between 1 and 16 wk.
104
105 Microscopy analyses of field-collected *Cephaloziella varians*
106
107 Unstained or stained *C. varians* shoots, collected 6 wk previously from Rothera Point and
108 transferred back to the UK in a growth cabinet under the conditions described above, were
109 examined under bright field illumination (Olympus BX51; Olympus, Southall, UK). For staining,
110 shoots were cleared in 10% KOH for 24 h, washed in dH_2O, acidified in 10% HCl for 1 h, and
111 transferred to 0.05% aniline blue (0.25 g aniline blue, 25 ml dH_2O, 475 ml lactic acid) for 1 h and
112 then to de-staining solution (25 ml dH_2O, 475 ml lactic acid) for 2 h. Shoots were then mounted on
113 microscope slides in lactoglycerol (14:1:1; lactic acid: glycerol: dH_2O).
114
115 Isolation of fungi from *Cephaloziella varians*

116

117 Fungi were isolated directly from shoots collected from Bird, Signy, King George, Adelaide and
118 Alexander Islands. Samples from Bird Island were defrosted overnight at 4°C prior to isolations.
119 Fungi from Alexander Island were isolated immediately after return to Rothera research station.
120 From each of the five samples of *C. varians* collected from a given site, *c*. 25 mg (FW) of the
121 uppermost 10 mm of shoot was placed into 10 ml sterile dH_2O in a Universal bottle. Shoots were
122 serially washed in 20 changes of sterile dH_2O for 5 min per wash on a wrist-action shaker at 7 beats
123 s^{-1}. Shoots were blotted on sterile filter paper, cut into 1-2 mm length segments and plated into
124 either 10% modified Melin-Norkrans agar (MMN; acidified to pH 4·5 with 10% HCl) medium or
125 1% malt extract agar (MEA) medium in 90 mm Petri dishes. Each dish contained five segments of
126 shoot, plated equidistantly in the agar medium. Between 12 and 20 dishes of each agar medium
127 were prepared per site. Dishes were incubated in the dark at 18°C and checked daily for 14 d then
128 weekly for 10 wk. The number of fungal colonies present was recorded, and the most common
129 fungal morphotype sub-cultured onto 10% MMN and 1% MEA media and stored in the dark at
130 18°C.

131

132 Isolation of fungi from *Vaccinium macrocarpon* bait seedlings

133

134 Seeds of *V. macrocarpon* were sterilized for 10 min in calcium hypochlorite solution (1:28, w/v)
135 followed by three rinses in sterile dH_2O, and were germinated on 1.5% plant agar medium (Duchefa
136 Biochemie, Haarlem, The Netherlands). Five uncontaminated, two-week-old *V. macrocarpon*
137 seedlings were each planted into *C. varians* mat from Coronation, Lynch, Signy, King George,
138 Livingston and Adelaide Islands. The mats were placed into Petri dishes, which were sealed with
139 Parafilm and maintained under the same growth conditions as those described above for 8 wk.
140 Five 10 mm root segments were arbitrarily selected from each of the five *V. macrocarpon*
141 plants growing in *C. varians* collected from each site. Five of the 25 segments from each site were

each placed into 10 ml sterile dH_2O, were serially washed as described above, and, after blotting, plated into 10% MMN and 1% MEA media. Between 10 and 15 dishes of each agar medium were prepared, incubated at 18°C and checked as above.

Characterization and quantification of fungi

Macroscopic and microscopic features of colonies were noted after growth for 20 d in the dark at 18°C on 1% MEA medium. Sub-cultures of each were transferred to 5°C in an attempt to induce sporulation (Richard & Fortin, 1973). The type cultures of *Rhizoscyphus ericae* (Read 100 and 101) were used for comparison with fungal isolates. Isolation frequency was calculated as the number of colonies / the total number of liverwort or root segments plated × 100%.

Molecular characterization of dominant fungal morphotype

DNA extraction and sequencing. Using the method of Cubero *et al*. (1999), DNA was extracted from 23 isolates of the most frequent fungal morphotype from Bird, Signy, Lynch, Coronation, King George, Livingston, Adelaide and Alexander Islands. The fungal specific primer ITS1F (Gardes & Bruns, 1993) and the universal primer ITS4 (White *et al.,* 1990) were used to amplify the ITS region (ITS1-5.8S-ITS2) between the SSU and large subunit (LSU) rDNA. The primers NL5 and NL8 (Egger, 1995) were used to amplify a partial 5' section of the LSU, including the D1/D2 region, from three of the isolates.

PCR amplifications were carried out in 34 μl volumes, consisting of 30 μl ReddyMix PCR mix (Abgene, Epsom, UK), 2 μl template DNA and 1 μl of each primer, on a PTC-225 Peltier thermal cycler (MJ Research Inc., Watertown, MA, USA). Each amplification included a negative control in which 2 μl of sterile ddH_2O was substituted for the DNA template. Ice-cold reaction tubes were preheated for initial denaturation at 94°C for 5 min, followed by 30 cycles of

denaturation at 94°C for 1 min, annealing at 54°C for 1 min and synthesis at 72°C for 70 s, followed by a final extension step of 68°C for 10 min. Detectable PCR products were purified and target rDNA regions sequenced using a MegaBACE 1000 sequencer (Molecular Dynamics, Sunnyvale, CA, USA). The sequencing reactions were repeated for both forward and reverse primers.

Phylogenetic analyses. Consensus sequences were produced for the ITS and LSU sequences obtained from this study using BioEdit (version 7.0.4.1; Hall, 1999), initially with the ClustalW option (Thompson *et al.*, 1994), and thereafter manually by visual inspection. NCBI BLAST (Altschul *et al.*, 1997) and Fasta searches (Fasta fungi and Fasta env) (Pearson & Lipman, 1988; http://www.ebi.ac.uk/fasta33/nucleotide.html) were carried out for each ITS and LSU consensus sequence. The exact positioning of the ITS1, 5.8S and ITS2 along sequences was determined using paired alignments with BLAST matches and information deposited in GenBank. ITS sequences were then aligned with the *Hymenoscyphus* (≡ *Rhizoscyphus*) *ericae* aggregate (*sensu* Vrålstad *et al*., 2000) alignment deposited in TreeBASE by Hambleton & Sigler (2005) (http://www.treebase.org/treebase/; study accession no. S1393). In order to align sequences from the current study with more distantly related taxa in the deposited alignment, the 5' partial SSU sequence and the first *c*. 23 bp of ITS1 were removed. ITS sequences were further shortened to 318-397 bp by removing the 3' partial LSU sequence and the last *c.* 61 bp of ITS2 to accommodate shorter sequences. Similarly, LSU sequences were shortened to 397 bp to align with those from a broad selection of Leotiomycetous taxa and selected Pezizomycetes. The aligned sequences were then subjected to phylogenetic analysis using the neighbour-joining (NJ) method (Saitou & Nei, 1987) in MEGA version 3.1 (Kumar *et al.*, 2004) with the Kimura two-parameter model. Gaps or missing data were excluded from analyses and a uniform rate of mutation was assumed across sites. Robustness of phylogenetic trees was assayed using 1000 NJ bootstrap replications (Felsenstein, 1985). Sequences were deposited in GenBank under accession numbers 000000-000000.

Comparison of ITS1F/ITS4 and EF4/Fung5 primer sets. Three isolates of the dominant fungal morphotype from *C. varians* tissue and two from *V. macrocarpon* bait seedling roots were used for these analyses. Two *Phoma herbarum* isolates were used for comparison. All seven isolates originated from Rothera Point. DNA extraction from the isolates and PCR amplification were carried out as described above for the primer sets ITS1F/ITS4 and EF4/Fung5 (Smit *et al*., 1999), except that the PCR cycle parameters used for the latter set were those used by Jumpponen *et al*. (2003). The PCR products were separated on 2% agarose gel.

Synthesis experiments

Inoculation of *Cephaloziella varians.* Axenic gametophytes of *C. varians* were obtained from spores. Sporophytes were carefully removed from laboratory-grown *C. varians* using sterile fine forceps under a sterile flow hood. Several sporophytes were attached to the lid of a 90 mm Petri dish using sterile lanolin so that the sporophyte hung *c*. 5 mm above the surface of 1.5% plant agar medium. Dishes were sealed with Parafilm and maintained under the same growth conditions as those described above. A fresh lid was placed on the dish under a sterile flow hood when spores were seen on the agar medium surface. Ten gametophytes were each aseptically transferred to Petri dishes containing 1.5% plant agar medium when they reached *c*. 1 mm diameter. An 8 mm diameter 1.2% water agar medium plug, inoculated 14 d previously with an isolate of the most frequent fungal morphotype from Rothera Point, was positioned 20 mm from each of five *C. varians* gametophytes. An uninoculated 1.2% water agar plug was placed 20 mm from the each of the remaining gametophytes. Petri dishes were sealed with Parafilm, wrapped with aluminium foil and a hole was made in the foil above the gametophyte in order to admit light. Gametophytes were grown under the same conditions as those described above.

220 Shoots were harvested 8 wk after hyphae had reached the gametophytes. They were viewed
221 unstained or stained with aniline blue under bright field or UV fluorescence.
222
223 **Inoculation of *Vaccinium macrocarpon.*** Polycarbonate Magenta® growth vessels (Sigma-Aldrich,
224 St Louis, MO, USA), fitted with 0.22 μm filter lids, were filled to a depth of 30 mm with 180 ml of
225 20% Rorison's nutrient solution (pH 5.4, with 0.5 g L^{-1} activated charcoal and 15 g L^{-1} agar).
226 Vessels and growth medium were sterilised at 121°C for 15 min and cooled to room temperature.
227 Five axenically-grown *V. macrocarpon* seedlings, germinated as described above, were
228 transferred to each of two growth vessels, and were inoculated with 1 ml macerate, consisting of
229 either uninoculated or inoculated pieces of 1.2% water agar (20 × 10 × 6 mm) in 15 ml sterile
230 dH_2O. Inoculated water agar had been cut under sterile conditions from the growing margin of the
231 same isolate from Rothera Point that was used to inoculate *C. varians*, which had been grown for 2
232 wk at 18°C in the dark. The growth vessels were sealed with Parafilm, transferred to the growth
233 room and maintained under the same growth conditions as those described above. Plants were
234 grown for 12 wk prior to harvest.
235 Upon harvest, *V. macrocarpon* roots were rinsed free of growth medium with sterile dH_2O
236 and were qualitatively assessed for the presence of ericoid coils in cortical cells. Roots from each
237 plant were observed unstained or stained with aniline blue under bright field and UV fluorescence,
238 as described above. For staining, *V. macrocarpon* roots were dipped in 10% HCl, transferred to
239 0.05% aniline blue for 10 min and rinsed in sterile dH_2O.
240
241 **Results**
242 Microscopy analyses of field-collected *Cephaloziella varians*
243
244 A network of dark septate 'runner' hyphae (*c*. 2 μm diameter) covered the caulid surfaces of *C.*
245 *varians* sampled from Rothera Point (Fig. 2a, b). Hyphae commonly entered caulid cells (Fig. 2b)

and colonised rhizoids (Fig. 2c), both in the apical and distal regions of the liverwort. Dark septate hyphae on caulid surfaces formed a continuum with intracellular pigmented, hyaline and aniline blue-staining septate hyphae, which were frequently observed to proliferate within cells, forming hyphal coils (Fig. 2d). The basal regions of rhizoids were also filled with hyphal coils (not shown). Pigmented, hyaline and aniline blue-staining septate hyphae were apparently able to pass from cell to cell by directly penetrating the cell wall, with no evidence of cell necrosis.

Characterization and quantification of fungi

A single morphotype, morphotype 1, was the most frequently-isolated fungus from *C. varians* tissue collected from Bird, Signy, King George, Adelaide and Alexander Islands (Table 2). Between one and three other morphotypes of dematiaceous fungi were isolated from *C. varians* from each of these locations (Table 2). Colonies of morphotype 1 were slow growing, with a mean radial extension rate of 3.0 (± 0.13) mm wk^{-1}, were often waxy and lacked dense aerial hyphae, except for in the centre of the colony, where ropes were formed. After 20 d, colonies of the morphotype were dark greenish grey from above with a white margin and were dark brown with a cream margin from below. They became dark brown with age. Sporulation structures were not observed in any cultures of morphotype 1, including those incubated at 5°C.

Colonies of morphotype 1 also dominated the fungal isolates from roots of *V. macrocarpon* bait seedlings grown in *C. varians* mats from Coronation, Lynch, Signy, King George, Livingston and Adelaide Islands (Table 2). It was the only morphotype isolated from *V. macrocarpon* roots grown in mats from Coronation, King George and Adelaide Islands. One or two other morphotypes of fungi were isolated from each of the other locations (Table 2). Fungi were not isolated from the roots of uninoculated *V. macrocarpon* bait seedlings.

Molecular characterization of dominant fungal morphotype

Phylogenetic analyses. Isolates of morphotype 1 produced single amplification products of *c*. 600 bp for both ITS and LSU sequences. Negative controls produced no amplification products. Consensus sequences of *c*. 425-610 bp were produced. Two isolates, Vm_ByP_S13 and Vm_MaP_S20 (see footnote to Table 3 for an explanation of isolate codes), were missing all or a large proportion of the ITS2 region and were therefore removed from all further analyses. These sequences matched *R. ericae* (AY394907) with 99% identity over their entire lengths. The sequence identity between all other morphotype 1 isolates was 97-100% over the entire ITS1-5.8S-ITS2 region. All morphotype 1 sequences retrieved *R. ericae* as the top taxonomically-identified BLAST match, showing 98-99% sequence identity over the entire ITS1-5.8S-ITS2 region (Table 3). Morphotype 1 isolates and the isolate from *C. varians* on the Bailey Peninsula in eastern Antarctica (AF069439) had 97-99% sequence identity over the ITS1-5.8S-ITS2 region. NJ analysis of morphotype 1 partial ITS sequences showed them to group with 99% bootstrap support within the *H. ericae* aggregate and with 81% bootstrap support within the *R. ericae* clade, alongside sequences obtained for the isolate from the Bailey Peninsula, and others from vascular plant species in Australia, Norway, Canada, the USA and the UK (Fig. 3).

The sequence identity between morphotype 1 isolates Cv_PoC_D3, Cv_RoP_D5 and Cv_MoV_M1 was 97-99% over the partial LSU region. BLAST searches retrieved *R. ericae* sequences as the best match for the three LSU sequences, showing 98-99% identity (Table 3). NJ analysis clustered the partial LSU sequences together in a 73% bootstrap-supported monophyletic group with *R. ericae* sequences (Fig. 4).

Comparison of ITS1F/ITS4 and EF4/Fung5 primer sets. All seven isolates produced amplification products of *c*. 600 bp for the ITS1F/ITS4 primer combination, whereas only the morphotype 1 isolates Vm_RoP_23 and Vm_RoP_22 and the two *Phoma herbarum* isolates produced amplification products with the EF4/Fung5 primer set (not shown). The amplification

298 products from the former two isolates, the first of which produced a very faint band, were *c.* 1000
299 bp and those from the latter two were *c*. 600 bp in length (not shown). The morphotype 1 isolates
300 Cv_RoP_D6, Cv_RoP_D5 and Cv_RoP_R07 failed to amplify with the EF4/Fung5 primer set.
301
302 Synthesis experiments
303
304 **Inoculation of *Cephaloziella varians.*** The patterns of colonization observed in the inoculated
305 plants of *C. varians* were similar to those seen in field-collected plants. The morphotype 1 isolate
306 Cv_RoP_D5 was found to colonize and form penetration structures on rhizoid and axial cells of *C.*
307 *varians* shoots. As in field-collected shoots, *R. ericae* hyphae grew within rhizoid (Fig. 5a) and
308 axial cells. Hyphae ramified throughout the entire shoot, forming a loose network of runner hyphae
309 over axial surfaces, and frequently formed intracellular hyaline septate hyphal coils (Fig. 5b). In
310 some rhizoids, hyphae branched into fan-like structures at the base of the cell, with hyphae directly
311 penetrating the cell wall and entering neighbouring cells. There was no fungal colonization of
312 uninoculated shoots.
313
314 **Inoculation of *Vaccinium macrocarpon*.** Well-defined intracellular hyphal coils frequently
315 developed in root epidermal cells of *V. macrocarpon* seedlings that had been inoculated with
316 Cv_RoP_D5 (not shown). There was no fungal colonization of uninoculated control plants.
317
318 **Discussion**
319 The current study has shown *R. ericae* to be present in *C. varians* from eight locations in the
320 Antarctic, spanning a 1,875 km southward transect from Bird Island on South Georgia through to
321 Moutonnée Valley on Alexander Island, at the southern limit of the maritime Antarctic. Direct
322 isolations showed the fungus to grow from as many as half of the segments of liverwort shoot
323 plated into agar media, with relatively few colonies of other morphotypes isolated. The study has

thus significantly extended the known range of the *Cephaloziella varians-Rhizoscyphus ericae* association in Antarctica, and, along with the data of Chambers *et al*. (1999), strongly suggests that the association is a consistent relationship in Antarctic plant communities. Other than the widely-documented occurrence of lichens in the Antarctic (e.g. Øvstedal & Smith, 2001), we are unaware of any other reports in the literature of consistent associations between autotrophs and fungi on the continent.

The data reported here are at variance with those of Jumpponen *et al.* (2003), who found no evidence that *R. ericae* was present at Rothera Point, a location from which several isolates of the fungus were obtained in the current study. It is apparent from the data shown here that the EF4/Fung5 primer set used by Jumpponen *et al.* (2003) in their direct PCR study is not suitable for the amplification of *R. ericae* DNA: using this primer set, SSU region DNA from three of five *R. ericae* isolates from Rothera Point failed to amplify, and the *c.* 1000 bp product size of the other two isolates, compared with the *c*. 600 bp products of two *Phoma herbarum* isolates, implied the presence of a *c.* 400 bp insertion in the SSU region of the *R. ericae* DNA. As suggested by Jumpponen *et al.* (2003), it is possible that the presence of introns, which are frequent in the SSU rDNA of *R. ericae* (Perotto *et al*., 2000), could have interfered with PCR amplification in their study, leading to the absence of *R. ericae* sequences from clone libraries.

For the first time, the present study performed Koch's postulates for the *C. varians-R. ericae* association, inoculating axenically-grown liverwort with an isolate of the fungus from the plant. These experiments confirmed that the fungus is able to form hyphal coils similar to those observed in field-collected plants, indicating that the loose coil observed in the base of a *C. varians* rhizoid cell by Williams *et al.* (1994) was indeed most probably formed by *R. ericae*. We cannot, however, discount the fact that other fungi capable of forming hyphal coils may be present in the tissues of *C. varians*. We similarly cannot discount the possibility that the favourable conditions under which the liverwort was grown in cabinets account for the abundance of coils observed in shoots in the present

study. Further studies will therefore measure the frequency of coils in tissues of *C. varians* sampled directly from maritime and sub-Antarctic sites.

In contrast with the data of Williams *et al*. (1994), the entry points of *R. ericae* into *C. varians* tissues in the current study were not restricted to rhizoids, and hyphae were found to colonize the whole plant except for the apical meristem, frequently forming coils within axial cells. Williams *et al.* (1994) concluded that the fungal structures observed in *C. varians* resembled those described as mycorrhizas or mycothalli. The current study indicates that the latter term is the more appropriate, owing to the apparent systemic growth of the fungus and the fact that *C. varians* lacks flagelliform axes, which might be considered to function in an analogous way to roots (Duckett *et al.,* 1991). In other liverwort-fungal associations the fungal partner does not grow systemically, but proliferates in particular regions, such as in the rhizoids of other members of the Cephaloziaceae or in the inner stem region of members of the Lophoziaceae, Arnelliaceae and Scapaniaceae (Read *et al.,* 2000). Fungal hyphae within tissues of British *Cephaloziella* species similarly appear to be restricted to rhizoid cells and are not present in the walls between the contiguous bases of colonized rhizoids (Duckett *et al.,* 1991).

Previous work has shown ascomycetous associates of the liverwort genera *Cephalozia* and *Kurzia* to form ericoid mycorrhizas with axenically-grown plants of *Calluna*, *Erica* and *Vaccinium* spp., which co-occur with the liverworts in the same habitats (Duckett & Read, 1995). Despite the absence of ericaceous plant species from maritime and continental Antarctica, the current study similarly found that an isolate of *R. ericae* from Rothera Point was able to form coils in the root hair cells of *Vaccinium macrocarpon*. Although further experiments are required to assess the effects of Antarctic *R. ericae* isolates on ericaceous plants, this suggests that such isolates may not have lost their ability to form functional relationships with higher plant roots.

The formation of hyphal coil-like structures, which maximise the surface area of contact between symbionts in ericoid mycorrhizas (Smith & Read, 1997), is suggestive of active nutrient exchange between *C. varians* and *R. ericae*. However, whether or not *C. varians* benefits from the

well-documented saprotrophic capability of *R. ericae*, one of the major benefits imparted to plants forming ericoid mycorrhizas with the fungus (Smith & Read, 1997), is at present an open question. Despite the fact that certain features of the association are suggestive of a role in the growth and survival of the liverwort, the functional nature of the *C. varians*-*R. ericae* association remains unclear and should be a focus for future studies. Nevertheless, we can broadly conclude from the current study that there appears to be a widespread and consistent association between the liverwort and *R. ericae* in the maritime and sub-Antarctic, and that *R. ericae* is at least in part responsible for the formation of the coils observed in the rhizoids of field-collected *C. varians* in previous studies.

Acknowledgements

Thanks are due to Blair Lawley, David Pearce, Paul Bridge, Melody Clark and Elaine Fitzcharles for their guidance with molecular work. For support prior to and during fieldwork, we would like to thank Ron Lewis-Smith, Roger Worland, Mairi Nicolson, Kat Snell, Richard Burt, Rod Strachan, Sarah Lurcock, Jane Tanton and Pete Convey. The latter kindly collected samples from Bird Island. We would also like to thank Alan Meredith, the BAS Air Unit and Logistics Group and the crews of the RRS Ernest Shackleton and HMS Endurance for transportation to and from sampling sites. Thanks are also due to Irene Johnson and Peter Fretwell for technical assistance and to three anonymous referees for helpful comments. This research was supported by the Natural Environment Research Council through the Antarctic Funding Initiative (award no. NER/G/S/2000/00318) and by BAS CASE sponsorship.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Bednarek-Ochyra H, Vana J, Ochyra JVR, Smith RIL. 2000. *The liverwort flora of Antarctica*. Cracow, Poland: Polish Academy of Sciences, Institute of Botany, 236 pp.

Boullard B. 1988. Observations on the coevolution of fungi with hepatics. In: Pyrozynski KA, Hawksworth DL, eds. *Coevolution of fungi with plants and animals.* London, UK: Academic Press, 107-124.

Chambers SM, Williams PG, Seppelt RD, Cairney JWG. 1999. Molecular identification of *Hymenoscyphus* sp. from rhizoids of the leafy liverwort *Cephaloziella exiliflora* in Australia and Antarctica. *Mycological Research* **103**: 286-288.

Cubero OF, Crespo A, Fatehi J, Bridge PD. 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Plant Systematics and Evolution* **216**: 243-249.

Duckett JG, Renzaglia KS, Pell K. 1991. A light and electron microscope study of rhizoid ascomycete associations and flagelliform axes in British hepatics with observations on the effects of the fungi on host morphology. *New Phytologist* **118**: 233-257.

Duckett JG, Read DJ. 1995. Ericoid mycorrhizas and rhizoid-ascomycete associations in liverworts share the same mycobiont - isolation of the partners and resynthesis of the associations *in vitro*. *New Phytologist* **129**: 439-477.

Egger KN. 1995. Molecular analysis of ectomycorrhizal fungal communities. *Canadian Journal of Botany* **73**: 1415-1422.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.

Hall TA. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Bioedit (Biological Sequence Alignment Editor). *Nucleic Acids Symposium Series* **41:** 95-98

Hambleton S, Sigler L. 2005. *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (*Hymenoscyphus ericae*), Leotiomycetes. *Studies in Mycology* **53**: 1-27.

Jumpponen A, Newsham KK, Neises DJ. 2003. Filamentous ascomycetes inhabiting the rhizoid environment of the liverwort *Cephaloziella varians* in Antarctica are assessed by direct PCR and cloning. *Mycologia* **95**: 457-466.

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150-163.

Pearson WR, Lipman DJ. 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences of the USA* **85**: 2444-2448.

Perotto S, Nepote-Fus P, Saletta L, Bandi C, Young JPW. 2000. A diverse population of introns in the nuclear ribosomal genes of ericoid mycorrhizal fungi includes elements with sequence similarity to endonuclease-coding genes. *Molecular Biology and Evolution* **17**: 44-59.

Read DJ, Duckett JG, Francis R, Ligrone R, Russell A. 2000. Symbiotic fungal associations in 'lower' land plants. *Philosophical Transactions of the Royal Society of London, B* **355**: 815-831.

Richard C, Fortin JA. 1973. The identification of *Mycelium radicis atrovirens*. *Canadian Journal of Botany* **51**: 2247-2248.

Saitou N, Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Selosse M-A. 2005. Are liverworts imitating mycorrhizas? *New Phytologist* **165**: 345-349.

Smit E, Leeflang P, Glandorf B, Dirk J, van Elsas JD, Wernars K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* **65**: 2614-2621.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis*. 2nd ed. London, UK: Academic Press.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Vrålstad T, Fossheim T, Schumacher T. 2000. *Piceirhiza bicolorata* - the ectomycorrhizal expression of the *Hymenoscyphus ericae* aggregate? *New Phytologist* **145**: 549–563.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego: Academic Press, 315-322.

Williams PG, Roser DJ, Seppelt RD. 1994. Mycorrhizas of hepatics in continental Antarctica. *Mycological Research* **98**: 34-36.

Zhang YH, Zhuang WY, 2004. Phylogenetic relationships of some members in the genus *Hymenoscyphus* (Ascomycetes, Helotiales). *Nova Hedwigia* **78**: 475-484.

Øvstedal DO, Smith RIL, 2001. *Lichens of Antarctica and South Georgia: a guide to their identification and ecology*. Studies in Polar Research. Cambridge, UK: Cambridge University Press.

Fig. 1 Map showing the locations of the sampling sites (plus symbols). See Table 1 for further details.

Fig. 2 Bright field micrographs of *Cephaloziella varians* tissues collected from Rothera Point, Adelaide Island. (a) Runner hyphae (arrows) forming loose network on the surface of shoot. Bar, 50 μm. (b) Runner hyphae forming strands (black arrow) and entering caulid cells at intervals along the main axis (white arrows). Bar, 50 μm. (c) Dark septate hypha (arrow) colonizing rhizoid cell. Bar, 50 μm. (d) Hyaline septate hyphae colonizing axial cells intracellularly and forming hyphal coils (arrows). Bar, 10 μm.

Fig. 3 Bootstrap consensus NJ tree obtained from the alignment of partial ITS1 (last 137 bp), 5.8S and partial ITS2 (first 82 bp) sequences of morphotype 1 isolates with sequences from the *Hymenoscyphus ericae* aggregate and other alignable taxa. The Kimura two-parameter model was used for pair-wise distance measurement. Bootstrap replication frequencies above 50% are indicated (1000 replications). The scale bar indicates two base changes per 100 nucleotide positions.

Fig. 4 Bootstrap consensus neighbour-joining tree obtained from the LSU sequence alignment of morphotype 1 isolates with sequences from the Leotiomycetes. Selected *Pezizomycete* taxa were used to root the tree. The Kimura two-parameter model was used for pair-wise distance measurement. Bootstrap replication frequencies above 50% are indicated (1000 replications). The scale bar indicates two base changes per 100 nucleotide positions.

Fig. 5 Images of *Rhizoscyphus ericae* (isolate Cv_RoP_D5) in cells of laboratory-grown *Cephaloziella varians*. (a) Bright field micrograph of hyphae (arrows) in rhizoid cell. Bar, 10 μm. (b) Fluorescence UV image of aniline blue-stained hyphal coils in the base of a rhizoid cell. Bar, 10 μm.

520 **Table 1** Descriptions of sampling sites

521

Location	Site name	Site abbreviation	Latitude and longitude	Altitude (m a.s.l.)	Aspect
South Georgia					
Bird Island	Stejneger Peak	StP	54° 00’ S, 38° 04’ W	100	north west
South Orkney Islands					
Coronation Island	Mansfield Point	MaP	60° 39’ S, 45° 42’ W	15	north west
Lynch Island	no name[1]	Lyn	60° 39’ S, 45° 36’ W	10	north west
Signy Island	Berntsen Point	BeP	60° 43’ S, 45° 36’ W	15	north east
South Shetland Islands					
King George Island	Potter Cove	PoC	62° 14’ S, 58° 41’ W	5	level
Livingston Island	Byers Peninsula	ByP	62° 40’ S, 61° 08’ W	5	level
western Antarctic Peninsula					
Adelaide Island	Rothera Point	RoP	67° 34’ S, 68° 07’ W	5	level
Alexander Island	Moutonnée Valley	MoV	70° 55’ S, 68° 20’ W	60	north

522 [1]north west side of island, close to helicopter landing site
523

524 **Table 2** Morphotypes of dematiaceous fungi isolated from *Cephaloziella varians* and *Vaccinium macrocarpon* bait seedlings

	Location	Isolation frequency (%)		No. morphotypes isolated
		morphotype 1	other morphotypes	
Cephaloziella varians	Bird Island	51.3	2.0	4
	Signy Island	28.5	2.5	2
	King George Island	26.2	4.0	2
	Adelaide Island	15.0	0.5	2
	Alexander Island	41.2	1.0	3
Vaccinium macrocarpon	Coronation Island	1.7	0	1
	Lynch Island	14.8	1.5	3
	Signy Island	22.2	5.2	3
	King George Island	11.0	0	1
	Livingston Island	7.4	3.5	2
	Adelaide Island	1.3	0	1

525

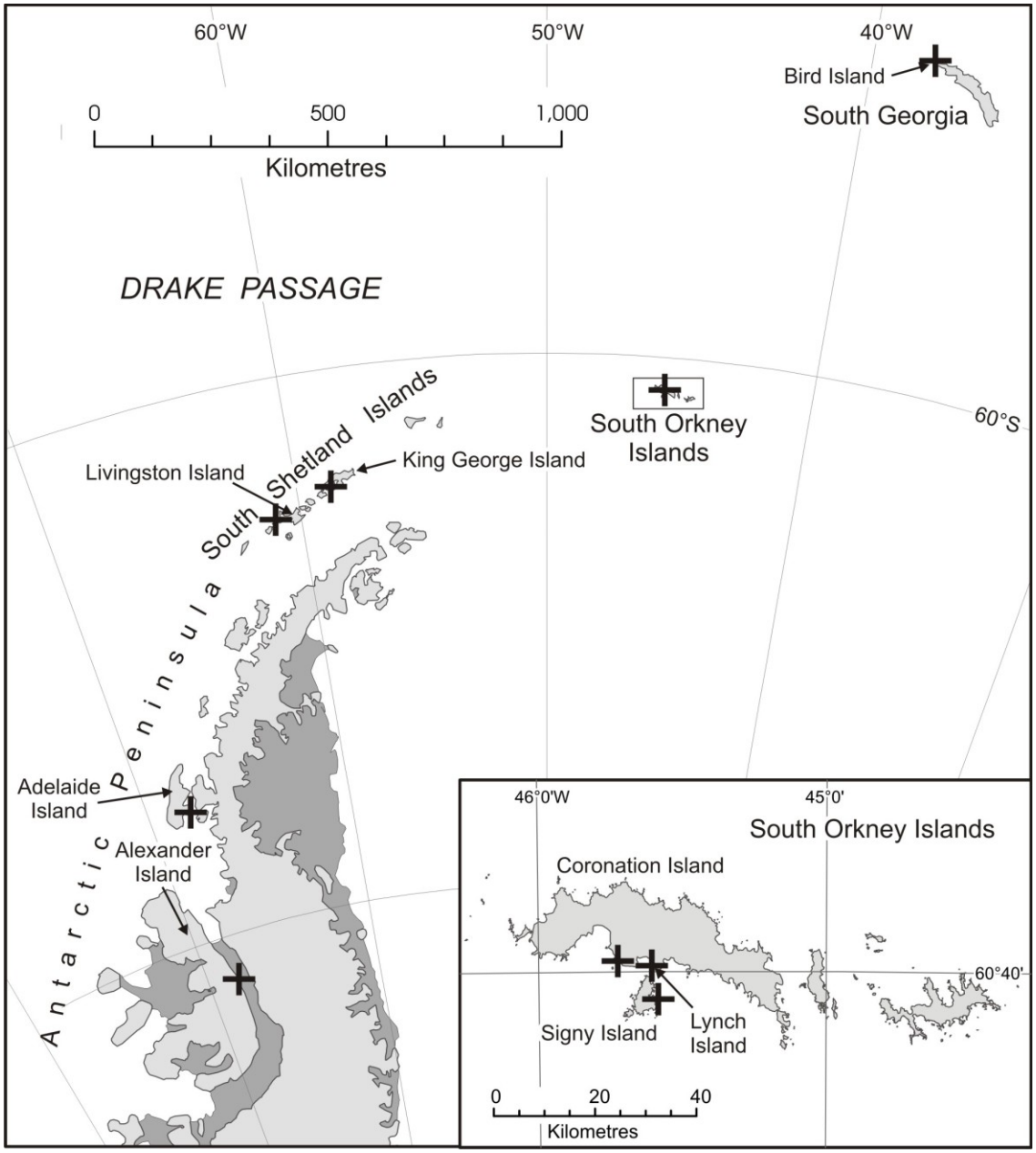
Table 3 ITS1-5.8S-ITS2 and partial LSU sequences and top taxonomically-identified BLAST matches of morphotype 1 isolates

Isolate code[1]	Accession no.	Target DNA region	ITS1 position (bp)	5.8S position (bp)	ITS2 position (bp)	Total sequence length (bp)	Top taxonomically-identified BLAST match	Lineage	Identity (%)	e-value
Cv_PoC_D3	EF658741	ITS	36-196	197-354	355-498	498	AY762620 *Rhizoscyphus ericae*	Helotiaceae, Helotiales, Leotiomycetidae	98	0.0
Cv_PoC_D4	EF658742	ITS	27-187	188-345	346-489	534	AY394907 *R. ericae*	...	98	0.0
Cv_RoP_D5	EF658743	ITS	32-192	193-350	351-494	530	AY394907 *R. ericae*	...	98	0.0
Cv_RoP_D6	EF658744	ITS	31-191	192-349	350-493	538	AY394907 *R. ericae*	...	98	0.0
Vm_PoC_S1	EF658747	ITS	64-224	225-382	383-526	531	AY762620 *R. ericae*	...	98	0.0
Vm_BeP_S2	EF658745	ITS	44-204	205-362	363-506	554	AY394907 *R. ericae*	...	98	0.0
Vm_Lyn_S10	EF658756	ITS	193-353	354-511	512-610[2]	610	AY394907 *R. ericae*	...	98	0.0
Vm_Lyn_S11	EF658755	ITS	213-373	374-531	532-610[2]	610	AY394907 *R. ericae*	...	98	0.0
Vm_ByP_S13	EF658749	ITS	229-389	390-545[2]	-	545	AY394907 *R. ericae*	...	99	0.0
Vm_PoC_S14	EF658750	ITS	63-223	224-281	382-525	577	AY394907 *R. ericae*	...	98	0.0
Vm_Lyn_S15	EF658746	ITS	43-203	204-361	362-489[2]	489	AY762620 *R. ericae*	...	99	0.0
Vm_MaP_S20	EF658751	ITS	131-291	292-449	450-518[2]	518	AY394907 *R. ericae*	…	99	0.0
Vm_RoP_S22	EF658754	ITS	51-211	212-369	370-513	565	AY394907 *R. ericae*	...	98	0.0
Vm_RoP_S23	EF658753	ITS	56-216	217-374	375-518	555	AY394907 *R. ericae*	...	98	0.0
Vm_BeP_S26	EF658752	ITS	58-218	219-376	377-520	554	AY762620 *R. ericae*	...	98	0.0
Vm_PoC_S28	EF658748	ITS	56-216	217-374	375-517[2]	517	AY762620 *R. ericae*	...	98	0.0
Cv_StP_G06	EF658761	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	99	0.0
Cv_StP_G07	EF658760	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	99	0.0
Cv_StP_G08	EF658762	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	99	0.0
Cv_StP_G09	EF658759	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	99	0.0
Cv_StP_G10	EF658758	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	98	0.0
Cv_StP_G11	EF658757	ITS	1-161	162-319	320-463	467	AF069439 *R. ericae*	...	99	0.0
Cv_PoC_D3	EF658763	LSU	-	-	-	459	AY394907 *R. ericae*	...	98	0.0
Cv_RoP_D5	EF658764	LSU	-	-	-	425	AY394907 *R. ericae*	...	99	0.0
Cv_MoV_M1	EF658765	LSU	-	-	-	577	AY394907 *R. ericae*	...	99	0.0

[1]The first two letters in each code indicate the plant species from which the isolate was derived (Cv = *Cephaloziella varians*, Vm = *Vaccinium macrocarpon* bait seedling), the second set of letters indicates the site from which the isolate came (see Table 1 for site abbreviations), and the last set of letters and numbers refer to the specific strain of the isolate

[2]partial sequence

Fig. 1


60°W
50°W
40°W
0
500
1,000
Kilometres
Bird Island
South Georgia
DRAKE PASSAGE
South Shetland Islands
South Orkney Islands
60°S
Livingston Island
King George Island
Antarctic Peninsula
Adelaide Island
Alexander Island
46°0'W
45°0'
South Orkney Islands
Coronation Island
60°40'
Signy Island
Lynch Island
0
20
40
Kilometres

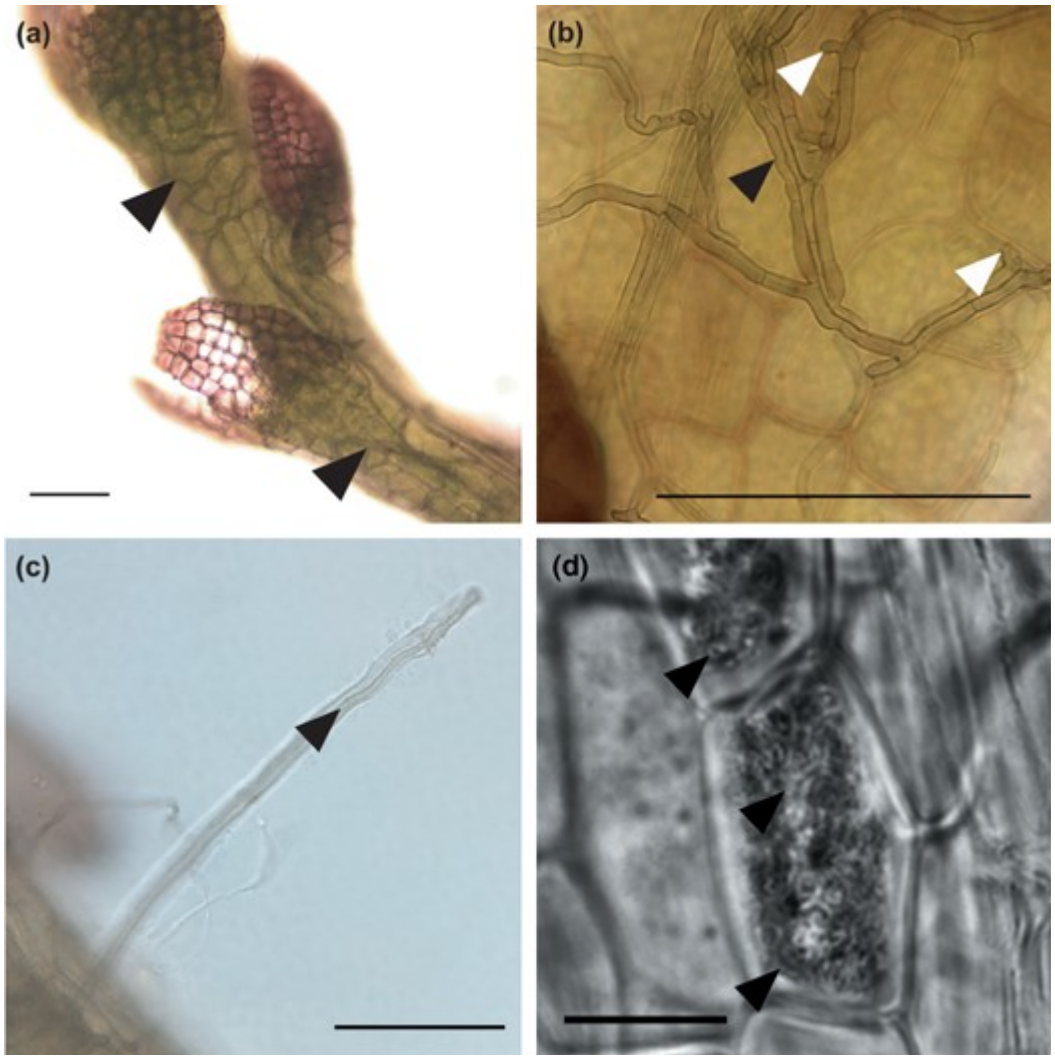


Fig. 2

Fig. 3

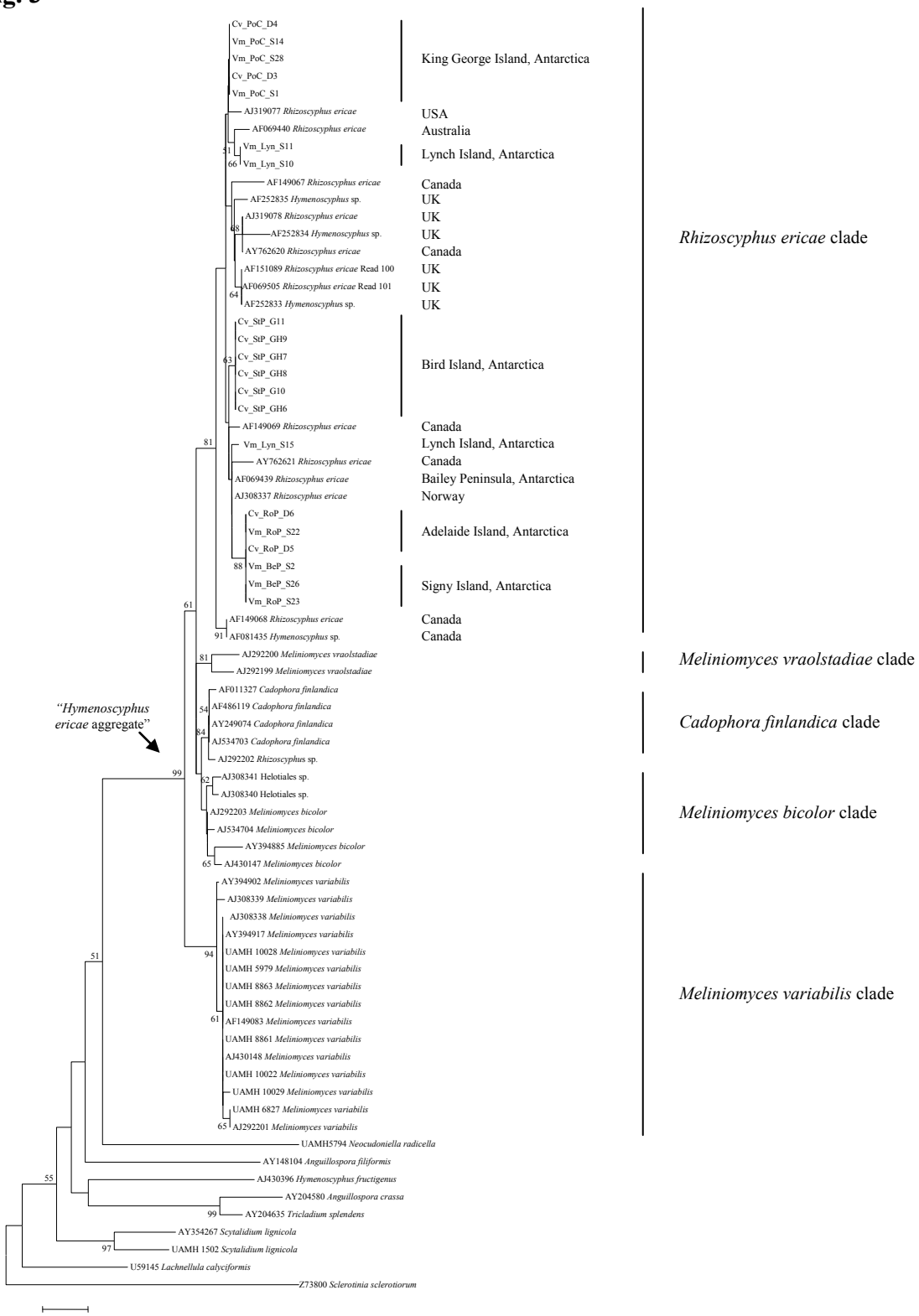
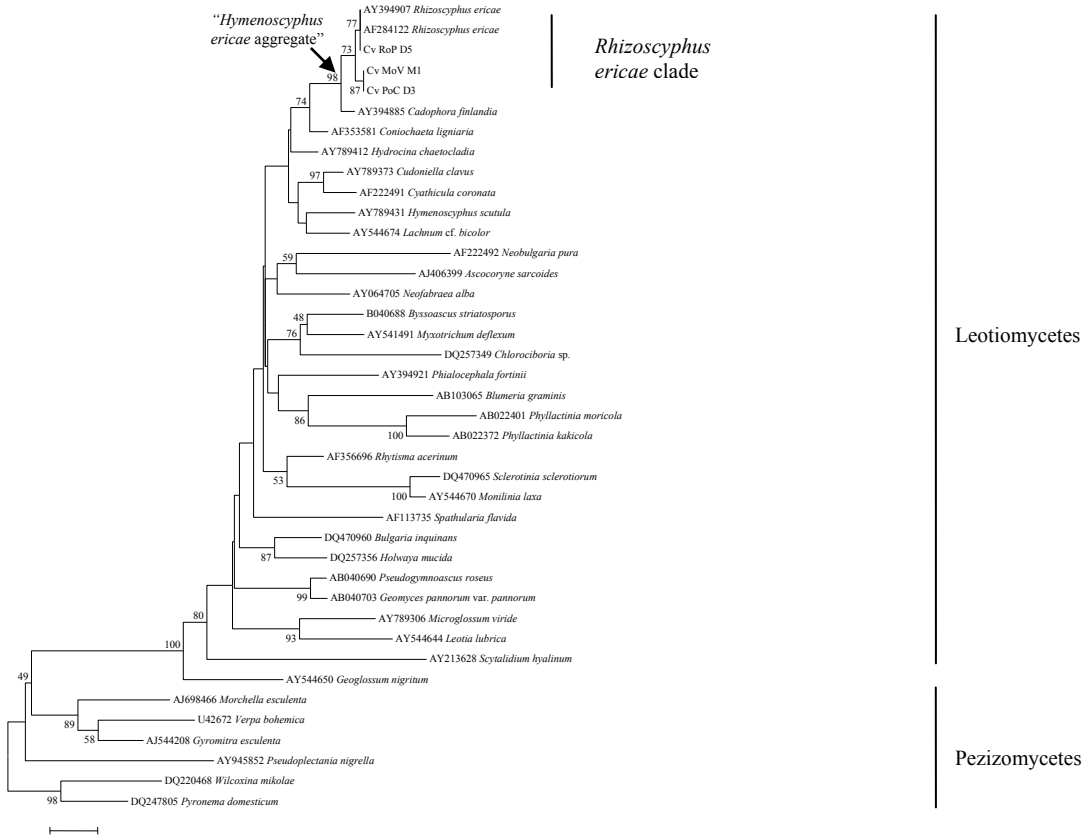
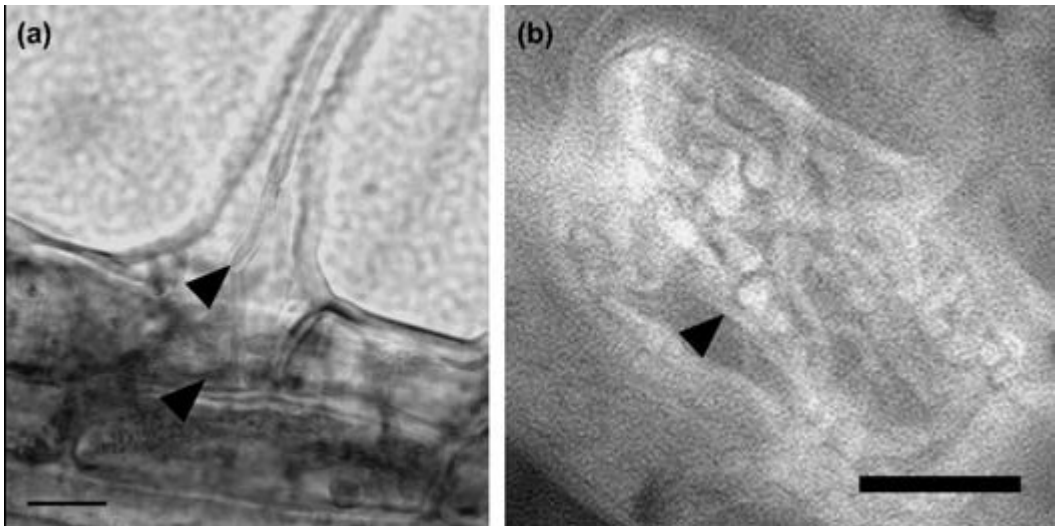

Cv_PoC_D4
Vm_PoC_S14
Vm_PoC_S28
Cv_PoC_D3
Vm_PoC_S1
King George Island, Antarctica
AJ319077 Rhizoscyphus ericae
USA
AF069440 Rhizoscyphus ericae
Australia
Vm_Lyn_S11
Vm_Lyn_S10
Lynch Island, Antarctica
AF149067 Rhizoscyphus ericae
Canada
AF252835 Hymenoscyphus sp.
UK
AJ319078 Rhizoscyphus ericae
UK
AF252834 Hymenoscyphus sp.
UK
AY762620 Rhizoscyphus ericae
Canada
AF151089 Rhizoscyphus ericae Read 100
UK
AF069505 Rhizoscyphus ericae Read 101
UK
AF252833 Hymenoscyphus sp.
UK
Rhizoscyphus ericae clade
Cv_StP_G11
Cv_StP_GH9
Cv_StP_GH7
Cv_StP_GH8
Cv_StP_G10
Cv_StP_GH6
Bird Island, Antarctica
AF149069 Rhizoscyphus ericae
Canada
Vm_Lyn_S15
Lynch Island, Antarctica
AY762621 Rhizoscyphus ericae
Canada
AF069439 Rhizoscyphus ericae
Bailey Peninsula, Antarctica
AJ308337 Rhizoscyphus ericae
Norway
Cv_RoP_D6
Vm_RoP_S22
Cv_RoP_D5
Adelaide Island, Antarctica
Vm_BeP_S2
Vm_BeP_S26
Vm_RoP_S23
Signy Island, Antarctica
AF149068 Rhizoscyphus ericae
Canada
AF081435 Hymenoscyphus sp.
Canada
AJ292200 Meliniomyces vraolstadiae
AJ292199 Meliniomyces vraolstadiae
Meliniomyces vraolstadiae clade
AF011327 Cadophora finlandica
AF486119 Cadophora finlandica
AY249074 Cadophora finlandica
AJ534703 Cadophora finlandica
AJ292202 Rhizoscyphus sp.
Cadophora finlandica clade
"Hymenoscyphus ericae aggregate"
AJ308341 Helotiales sp.
AJ308340 Helotiales sp.
AJ292203 Meliniomyces bicolor
AJ534704 Meliniomyces bicolor
AY394885 Meliniomyces bicolor
AJ430147 Meliniomyces bicolor
Meliniomyces bicolor clade
AY394902 Meliniomyces variabilis
AJ308339 Meliniomyces variabilis
AJ308338 Meliniomyces variabilis
AY394917 Meliniomyces variabilis
UAMH 10028 Meliniomyces variabilis
UAMH 5979 Meliniomyces variabilis
UAMH 8863 Meliniomyces variabilis
UAMH 8862 Meliniomyces variabilis
AF149083 Meliniomyces variabilis
UAMH 8861 Meliniomyces variabilis
AJ430148 Meliniomyces variabilis
UAMH 10022 Meliniomyces variabilis
UAMH 10029 Meliniomyces variabilis
UAMH 6827 Meliniomyces variabilis
AJ292201 Meliniomyces variabilis
Meliniomyces variabilis clade
UAMH5794 Neocudoniella radicella
AY148104 Anguillospora filiformis
AJ430396 Hymenoscyphus fructigenus
AY204580 Anguillospora crassa
AY204635 Tricladium splendens
AY354267 Scytalidium lignicola
UAMH 1502 Scytalidium lignicola
U59145 Lachnellula calyciformis
Z73800 Sclerotinia sclerotiorum

Fig. 4


"Hymenoscyphus ericae aggregate"
AY394907 Rhizoscyphus ericae
AF284122 Rhizoscyphus ericae
Cv RoP D5
Cv MoV M1
Cv PoC D3
Rhizoscyphus ericae clade
AY394885 Cadophora finlandia
AF353581 Coniochaeta ligniaria
AY789412 Hydrocina chaetocladia
AY789373 Cudoniella clavus
AF222491 Cyathicula coronata
AY789431 Hymenoscyphus scutula
AY544674 Lachnum cf. bicolor
AF222492 Neobulgaria pura
AJ406399 Ascocoryne sarcoides
AY064705 Neofabraea alba
B040688 Byssoascus striatosporus
AY541491 Myxotrichum deflexum
DQ257349 Chlorociboria sp.
AY394921 Phialocephala fortinii
AB103065 Blumeria graminis
AB022401 Phyllactinia moricola
AB022372 Phyllactinia kakicola
AF356696 Rhytisma acerinum
DQ470965 Sclerotinia sclerotiorum
AY544670 Monilinia laxa
AF113735 Spathularia flavida
DQ470960 Bulgaria inquinans
DQ257356 Holwaya mucida
AB040690 Pseudogymnoascus roseus
AB040703 Geomyces pannorum var. pannorum
AY789306 Microglossum viride
AY544644 Leotia lubrica
AY213628 Scytalidium hyalinum
AY544650 Geoglossum nigritum
AJ698466 Morchella esculenta
U42672 Verpa bohemica
AJ544208 Gyromitra esculenta
AY945852 Pseudoplectania nigrella
DQ220468 Wilcoxina mikolae
DQ247805 Pyronema domesticum
Leotiomycetes
Pezizomycetes

580 **Fig. 5**



581
582
583
584
585
586