

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

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16 **Summary**

- 17 • A recent study identified a fungal isolate from the Antarctic leafy liverwort *Cephaloziella*  
18 *varians* as the ericoid mycorrhizal associate *Rhizoscyphus ericae*. However, nothing is known  
19 about the wider Antarctic distribution of *R. ericae* in *C. varians*, and inoculation experiments  
20 confirming the ability of the fungus to form coils in the liverwort are lacking.
- 21 • Using direct isolation and baiting with *Vaccinium macrocarpon* seedlings, we isolated fungi  
22 from *C. varians* sampled from eight sites across a 1,875 km transect through sub- and maritime  
23 Antarctica, from Bird Island on South Georgia (54 °S, 38 °W) through to Alexander Island (71  
24 °S, 68 °W) on the western Antarctic Peninsula. We also tested the ability of an isolate to form  
25 coils in aseptically-grown *C. varians*.
- 26 • Fungi with 98-99% sequence identity to *R. ericae* internal transcribed spacer region and partial  
27 large subunit ribosomal (r)DNA sequences were frequently isolated from *C. varians* at all sites  
28 sampled. The EF4/Fung5 primer set did not amplify small subunit rDNA from three of five *R.*  
29 *ericae* isolates, probably accounting for the reported absence of the fungus from *C. varians* in a  
30 previous study. *R. ericae* was found to colonize aseptically-grown *C. varians* intracellularly,  
31 forming hyphal coils.
- 32 • This study shows that the association between *R. ericae* and *C. varians* is apparently widespread  
33 in Antarctica and confirms that *R. ericae* is at least in part responsible for the formation of the  
34 coils observed in rhizoids of field-collected *C. varians*.

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36  
37 **Key words:** *Cephaloziella varians*, dark septate endophyte, ericoid mycorrhiza, *Rhizoscyphus*  
38 *ericae*, liverwort, maritime and sub-Antarctica

39

40 **Introduction**

41 A range of leafy hepatics in the Jungermannopsida consistently form associations with  
42 ascomycetous fungi (Read *et al.*, 2000), forming ‘mycothalli’ (Boullard, 1988). In a study of British  
43 hepatics, species of the leafy liverwort genus *Cephaloziella* were found to possess fungal  
44 associations restricted to rhizoids, with over half of the rhizoid tips colonized (Duckett *et al.*, 1991).  
45 Fungal colonization of rhizoids is characterized by dense intracellular growth that appears  
46 analogous to ericoid mycorrhizal coils (Selosse, 2005). The functional nature of the relationship  
47 between liverworts and their endophytic fungi is not known, but the formation of structures similar  
48 to those seen in ericoid mycorrhizal roots suggests an active role in plant survival (Read *et al.*,  
49 2000).

50 *Cephaloziella varians* (Gottsche) Steph. is the most widespread species of liverwort in  
51 Antarctica, occurring in the maritime, continental and sub-Antarctic regions (Bednarek-Ochyra *et*  
52 *al.*, 2000). As in British *Cephaloziella*, hyphae and rudimentary coils of ascomycetous fungal  
53 endophytes are present in the rhizoids of this leafy liverwort collected from Botany Bay, Granite  
54 harbour (77 °S, 162 °E) and the Bailey Peninsula in the Windmill Islands (66 °S, 110 °E), both in  
55 eastern continental Antarctica (Williams *et al.*, 1994). Colonies of isolates from *C. varians* collected  
56 from these locations are slow-growing, waxy and become dark brown to black with age (Williams  
57 *et al.*, 1994). Using the ITS1/ITS4 primer set, Chambers *et al.* (1999) sequenced the internal  
58 transcribed spacer (ITS) region of an isolate from the Bailey Peninsula and showed it to have a high  
59 (99.4%) sequence identity to the type culture of the ericoid mycorrhizal fungus *Hymenoscyphus*  
60 *ericae*, recently renamed as *Rhizoscyphus ericae* (D.J. Read) W.H. Zhuang & Korf. (Zhang &  
61 Zhuang, 2004). In contrast, direct PCR analysis using the EF4/Fung5 primer set, which targeted a  
62 region of the small subunit (SSU) ribosomal (r)RNA gene, suggested that fungi bearing strong  
63 affinities to *R. ericae* are absent from *C. varians* at Rothera Point in the maritime Antarctic  
64 (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<sup>®</sup>; 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  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  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<sub>2</sub>O) 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<sub>2</sub>O 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*

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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<sub>2</sub>O, acidified in 10% HCl for 1 h, and  
111 transferred to 0.05% aniline blue (0.25 g aniline blue, 25 ml dH<sub>2</sub>O, 475 ml lactic acid) for 1 h and  
112 then to de-staining solution (25 ml dH<sub>2</sub>O, 475 ml lactic acid) for 2 h. Shoots were then mounted on  
113 microscope slides in lactoglycerol (14:1:1; lactic acid: glycerol: dH<sub>2</sub>O).

114

115 Isolation of fungi from *Cephaloziella varians*

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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<sub>2</sub>O in a Universal bottle. Shoots were  
122 serially washed in 20 changes of sterile dH<sub>2</sub>O for 5 min per wash on a wrist-action shaker at 7 beats  
123 s<sup>-1</sup>. 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<sub>2</sub>O, 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

142 each placed into 10 ml sterile dH<sub>2</sub>O, were serially washed as described above, and, after blotting,  
143 plated into 10% MMN and 1% MEA media. Between 10 and 15 dishes of each agar medium were  
144 prepared, incubated at 18°C and checked as above.

145

146 Characterization and quantification of fungi

147

148 Macroscopic and microscopic features of colonies were noted after growth for 20 d in the dark at  
149 18°C on 1% MEA medium. Sub-cultures of each were transferred to 5°C in an attempt to induce  
150 sporulation (Richard & Fortin, 1973). The type cultures of *Rhizoscyphus ericae* (Read 100 and 101)  
151 were used for comparison with fungal isolates. Isolation frequency was calculated as the number of  
152 colonies / the total number of liverwort or root segments plated × 100%.

153

154 Molecular characterization of dominant fungal morphotype

155

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

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

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

173

174 **Phylogenetic analyses.** Consensus sequences were produced for the ITS and LSU sequences  
175 obtained from this study using BioEdit (version 7.0.4.1; Hall, 1999), initially with the ClustalW  
176 option (Thompson *et al.*, 1994), and thereafter manually by visual inspection. NCBI BLAST  
177 (Altschul *et al.*, 1997) and Fasta searches (Fasta fungi and Fasta env) (Pearson & Lipman, 1988;  
178 <http://www.ebi.ac.uk/fasta33/nucleotide.html>) were carried out for each ITS and LSU consensus  
179 sequence. The exact positioning of the ITS1, 5.8S and ITS2 along sequences was determined using  
180 paired alignments with BLAST matches and information deposited in GenBank. ITS sequences  
181 were then aligned with the *Hymenoscyphus* ( $\equiv$  *Rhizoscyphus*) *ericae* aggregate (*sensu* Vrålstad *et*  
182 *al.*, 2000) alignment deposited in TreeBASE by Hambleton & Sigler (2005)  
183 (<http://www.treebase.org/treebase/>; study accession no. S1393). In order to align sequences from  
184 the current study with more distantly related taxa in the deposited alignment, the 5' partial SSU  
185 sequence and the first *c.* 23 bp of ITS1 were removed. ITS sequences were further shortened to 318-  
186 397 bp by removing the 3' partial LSU sequence and the last *c.* 61 bp of ITS2 to accommodate  
187 shorter sequences. Similarly, LSU sequences were shortened to 397 bp to align with those from a  
188 broad selection of Leotiomycetous taxa and selected Pezizomycetes. The aligned sequences were  
189 then subjected to phylogenetic analysis using the neighbour-joining (NJ) method (Saitou & Nei,  
190 1987) in MEGA version 3.1 (Kumar *et al.*, 2004) with the Kimura two-parameter model. Gaps or  
191 missing data were excluded from analyses and a uniform rate of mutation was assumed across sites.  
192 Robustness of phylogenetic trees was assayed using 1000 NJ bootstrap replications (Felsenstein,  
193 1985). Sequences were deposited in GenBank under accession numbers 000000-000000.



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195

196 **Comparison of ITS1F/ITS4 and EF4/Fung5 primer sets.** Three isolates of the dominant fungal  
197 morphotype from *C. varians* tissue and two from *V. macrocarpon* bait seedling roots were used for  
198 these analyses. Two *Phoma herbarum* isolates were used for comparison. All seven isolates  
199 originated from Rothera Point. DNA extraction from the isolates and PCR amplification were  
200 carried out as described above for the primer sets ITS1F/ITS4 and EF4/Fung5 (Smit *et al.*, 1999),  
201 except that the PCR cycle parameters used for the latter set were those used by Jumpponen *et al.*  
202 (2003). The PCR products were separated on 2% agarose gel.

203

204 Synthesis experiments

205

206 **Inoculation of *Cephaloziella varians*.** Axenic gametophytes of *C. varians* were obtained from  
207 spores. Sporophytes were carefully removed from laboratory-grown *C. varians* using sterile fine  
208 forceps under a sterile flow hood. Several sporophytes were attached to the lid of a 90 mm Petri  
209 dish using sterile lanolin so that the sporophyte hung *c.* 5 mm above the surface of 1.5% plant agar  
210 medium. Dishes were sealed with Parafilm and maintained under the same growth conditions as  
211 those described above. A fresh lid was placed on the dish under a sterile flow hood when spores  
212 were seen on the agar medium surface. Ten gametophytes were each aseptically transferred to Petri  
213 dishes containing 1.5% plant agar medium when they reached *c.* 1 mm diameter. An 8 mm diameter  
214 1.2% water agar medium plug, inoculated 14 d previously with an isolate of the most frequent  
215 fungal morphotype from Rothera Point, was positioned 20 mm from each of five *C. varians*  
216 gametophytes. An uninoculated 1.2% water agar plug was placed 20 mm from the each of the  
217 remaining gametophytes. Petri dishes were sealed with Parafilm, wrapped with aluminium foil and  
218 a hole was made in the foil above the gametophyte in order to admit light. Gametophytes were  
219 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<sup>®</sup> growth vessels (Sigma-Aldrich,  
224 St Louis, MO, USA), fitted with 0.22  $\mu\text{m}$  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<sup>-1</sup> activated charcoal and 15 g L<sup>-1</sup> 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<sub>2</sub>O. 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<sub>2</sub>O  
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<sub>2</sub>O.

240

## 241 **Results**

242 Microscopy analyses of field-collected *Cephaloziella varians*

243

244 A network of dark septate 'runner' hyphae (*c.* 2  $\mu\text{m}$  diameter) covered the caulid surfaces of *C.*  
245 *variens* sampled from Rothera Point (Fig. 2a, b). Hyphae commonly entered caulid cells (Fig. 2b)

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

252

253 Characterization and quantification of fungi

254

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

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

270

271 Molecular characterization of dominant fungal morphotype

272

273 **Phylogenetic analyses.** Isolates of morphotype 1 produced single amplification products of *c.* 600  
274 bp for both ITS and LSU sequences. Negative controls produced no amplification products.  
275 Consensus sequences of *c.* 425-610 bp were produced. Two isolates, Vm\_ByP\_S13 and  
276 Vm\_MaP\_S20 (see footnote to Table 3 for an explanation of isolate codes), were missing all or a  
277 large proportion of the ITS2 region and were therefore removed from all further analyses. These  
278 sequences matched *R. ericae* (AY394907) with 99% identity over their entire lengths. The sequence  
279 identity between all other morphotype 1 isolates was 97-100% over the entire ITS1-5.8S-ITS2  
280 region. All morphotype 1 sequences retrieved *R. ericae* as the top taxonomically-identified BLAST  
281 match, showing 98-99% sequence identity over the entire ITS1-5.8S-ITS2 region (Table 3).  
282 Morphotype 1 isolates and the isolate from *C. varians* on the Bailey Peninsula in eastern Antarctica  
283 (AF069439) had 97-99% sequence identity over the ITS1-5.8S-ITS2 region. NJ analysis of  
284 morphotype 1 partial ITS sequences showed them to group with 99% bootstrap support within the  
285 *H. ericae* aggregate and with 81% bootstrap support within the *R. ericae* clade, alongside sequences  
286 obtained for the isolate from the Bailey Peninsula, and others from vascular plant species in  
287 Australia, Norway, Canada, the USA and the UK (Fig. 3).

288 The sequence identity between morphotype 1 isolates Cv\_PoC\_D3, Cv\_RoP\_D5 and  
289 Cv\_MoV\_M1 was 97-99% over the partial LSU region. BLAST searches retrieved *R. ericae*  
290 sequences as the best match for the three LSU sequences, showing 98-99% identity (Table 3). NJ  
291 analysis clustered the partial LSU sequences together in a 73% bootstrap-supported monophyletic  
292 group with *R. ericae* sequences (Fig. 4).

293

294 **Comparison of ITS1F/ITS4 and EF4/Fung5 primer sets.** All seven isolates produced  
295 amplification products of *c.* 600 bp for the ITS1F/ITS4 primer combination, whereas only the  
296 morphotype 1 isolates Vm\_RoP\_23 and Vm\_RoP\_22 and the two *Phoma herbarum* isolates  
297 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 *variens* 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

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

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

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

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

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

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

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

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

383

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395



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

497

498 **Fig. 2** Bright field micrographs of *Cephaloziella varians* tissues collected from Rothera Point, Adelaide

499 Island. (a) Runner hyphae (arrows) forming loose network on the surface of shoot. Bar, 50  $\mu\text{m}$ . (b)

500 Runner hyphae forming strands (black arrow) and entering caulid cells at intervals along the main axis

501 (white arrows). Bar, 50  $\mu\text{m}$ . (c) Dark septate hypha (arrow) colonizing rhizoid cell. Bar, 50  $\mu\text{m}$ . (d)

502 Hyaline septate hyphae colonizing axial cells intracellularly and forming hyphal coils (arrows). Bar, 10

503  $\mu\text{m}$ .

504

505 **Fig. 3** Bootstrap consensus NJ tree obtained from the alignment of partial ITS1 (last 137 bp), 5.8S and

506 partial ITS2 (first 82 bp) sequences of morphotype 1 isolates with sequences from the *Hymenoscyphus*

507 *ericae* aggregate and other alignable taxa. The Kimura two-parameter model was used for pair-wise

508 distance measurement. Bootstrap replication frequencies above 50% are indicated (1000 replications).

509 The scale bar indicates two base changes per 100 nucleotide positions.

510

511 **Fig. 4** Bootstrap consensus neighbour-joining tree obtained from the LSU sequence alignment of

512 morphotype 1 isolates with sequences from the Leotiomycetes. Selected *Pezizomycete* taxa were used to

513 root the tree. The Kimura two-parameter model was used for pair-wise distance measurement. Bootstrap

514 replication frequencies above 50% are indicated (1000 replications). The scale bar indicates two base

515 changes per 100 nucleotide positions.

516

517 **Fig. 5** Images of *Rhizoscyphus ericae* (isolate Cv\_RoP\_D5) in cells of laboratory-grown *Cephaloziella*

518 *variens*. (a) Bright field micrograph of hyphae (arrows) in rhizoid cell. Bar, 10  $\mu\text{m}$ . (b) Fluorescence UV

519 image of aniline blue-stained hyphal coils in the base of a rhizoid cell. Bar, 10  $\mu\text{m}$ .

520 **Table 1** Descriptions of sampling sites

521

Location	Site name	Site abbreviation	Latitude and longitude	Altitude (m a.s.l.)	Aspect
<i>South Georgia</i>					
Bird Island	Stejneger Peak	StP	54° 00' S, 38° 04' W	100	north west
<i>South Orkney Islands</i>					
Coronation Island	Mansfield Point	MaP	60° 39' S, 45° 42' W	15	north west
Lynch Island	no name <sup>1</sup>	Lyn	60° 39' S, 45° 36' W	10	north west
Signy Island	Berntsen Point	BeP	60° 43' S, 45° 36' W	15	north east
<i>South Shetland Islands</i>					
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
<i>western Antarctic Peninsula</i>					
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

<sup>1</sup>north west side of island, close to helicopter landing site

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**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	
<i>Cephaloziella varians</i>	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
<i>Vaccinium macrocarpon</i>	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

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

Isolate code <sup>1</sup>	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 <i>Rhizoscyphus ericae</i>	Helotiaceae, Helotiales, Leotiomycetidae	98	0.0
Cv_PoC_D4	EF658742	ITS	27-187	188-345	346-489	534	AY394907 <i>R. ericae</i>	...	98	0.0
Cv_RoP_D5	EF658743	ITS	32-192	193-350	351-494	530	AY394907 <i>R. ericae</i>	...	98	0.0
Cv_RoP_D6	EF658744	ITS	31-191	192-349	350-493	538	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_PoC_S1	EF658747	ITS	64-224	225-382	383-526	531	AY762620 <i>R. ericae</i>	...	98	0.0
Vm_BeP_S2	EF658745	ITS	44-204	205-362	363-506	554	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_Lyn_S10	EF658756	ITS	193-353	354-511	512-610 <sup>2</sup>	610	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_Lyn_S11	EF658755	ITS	213-373	374-531	532-610 <sup>2</sup>	610	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_ByP_S13	EF658749	ITS	229-389	390-545 <sup>2</sup>	-	545	AY394907 <i>R. ericae</i>	...	99	0.0
Vm_PoC_S14	EF658750	ITS	63-223	224-281	382-525	577	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_Lyn_S15	EF658746	ITS	43-203	204-361	362-489 <sup>2</sup>	489	AY762620 <i>R. ericae</i>	...	99	0.0
Vm_MaP_S20	EF658751	ITS	131-291	292-449	450-518 <sup>2</sup>	518	AY394907 <i>R. ericae</i>	...	99	0.0
Vm_RoP_S22	EF658754	ITS	51-211	212-369	370-513	565	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_RoP_S23	EF658753	ITS	56-216	217-374	375-518	555	AY394907 <i>R. ericae</i>	...	98	0.0
Vm_BeP_S26	EF658752	ITS	58-218	219-376	377-520	554	AY762620 <i>R. ericae</i>	...	98	0.0
Vm_PoC_S28	EF658748	ITS	56-216	217-374	375-517 <sup>2</sup>	517	AY762620 <i>R. ericae</i>	...	98	0.0
Cv_StP_G06	EF658761	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	99	0.0
Cv_StP_G07	EF658760	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	99	0.0
Cv_StP_G08	EF658762	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	99	0.0
Cv_StP_G09	EF658759	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	99	0.0
Cv_StP_G10	EF658758	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	98	0.0
Cv_StP_G11	EF658757	ITS	1-161	162-319	320-463	467	AF069439 <i>R. ericae</i>	...	99	0.0
Cv_PoC_D3	EF658763	LSU	-	-	-	459	AY394907 <i>R. ericae</i>	...	98	0.0
Cv_RoP_D5	EF658764	LSU	-	-	-	425	AY394907 <i>R. ericae</i>	...	99	0.0
Cv_MoV_M1	EF658765	LSU	-	-	-	577	AY394907 <i>R. ericae</i>	...	99	0.0

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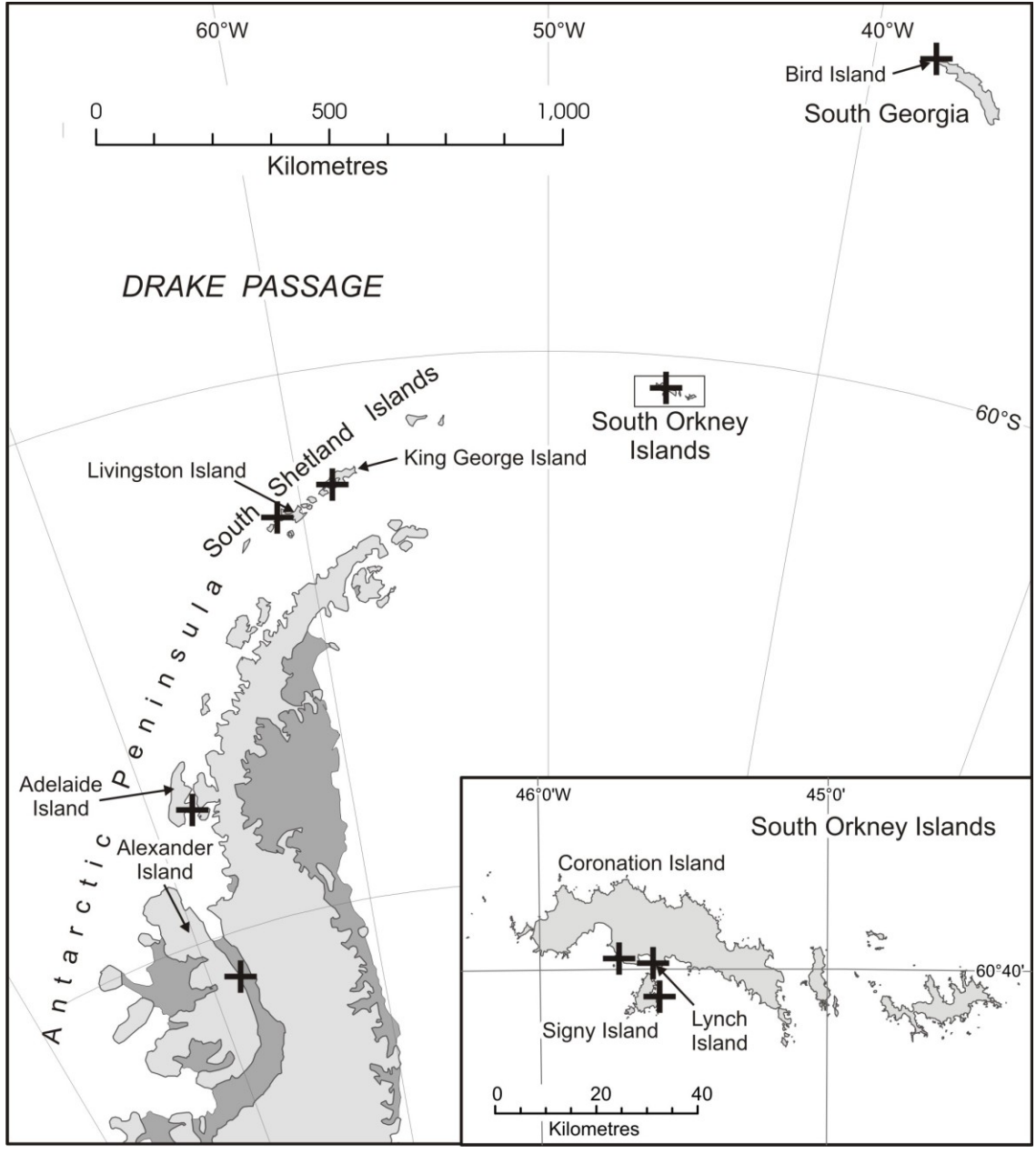
<sup>1</sup>The first two letters in each code indicate the plant species from which the isolate was derived (Cv = *Cephaloziella varians*, Vm = *Vaccinium macrocarpon* bait seedling), the second set of letters indicates the site from which the isolate came (see Table 1 for site abbreviations), and the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

<sup>2</sup>partial sequence



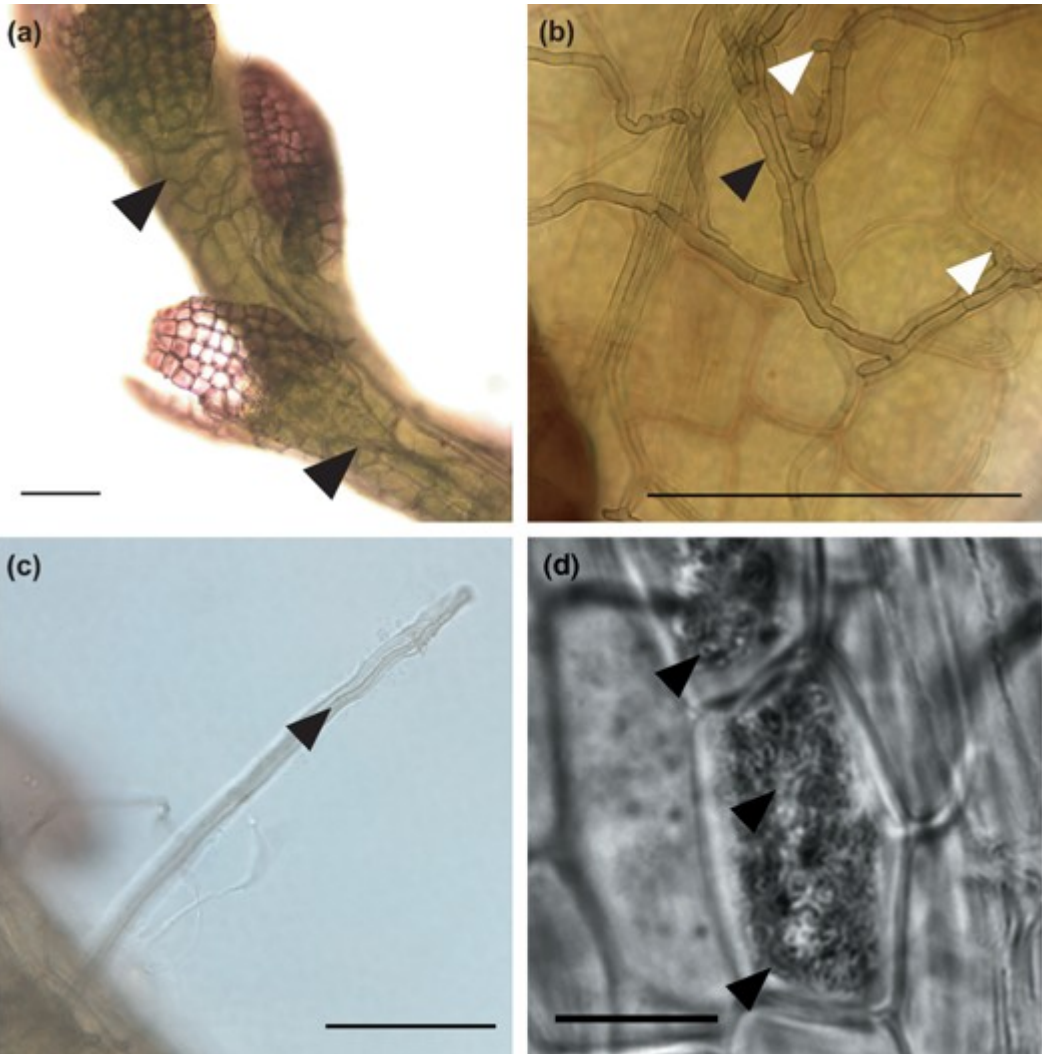
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**Fig. 1**



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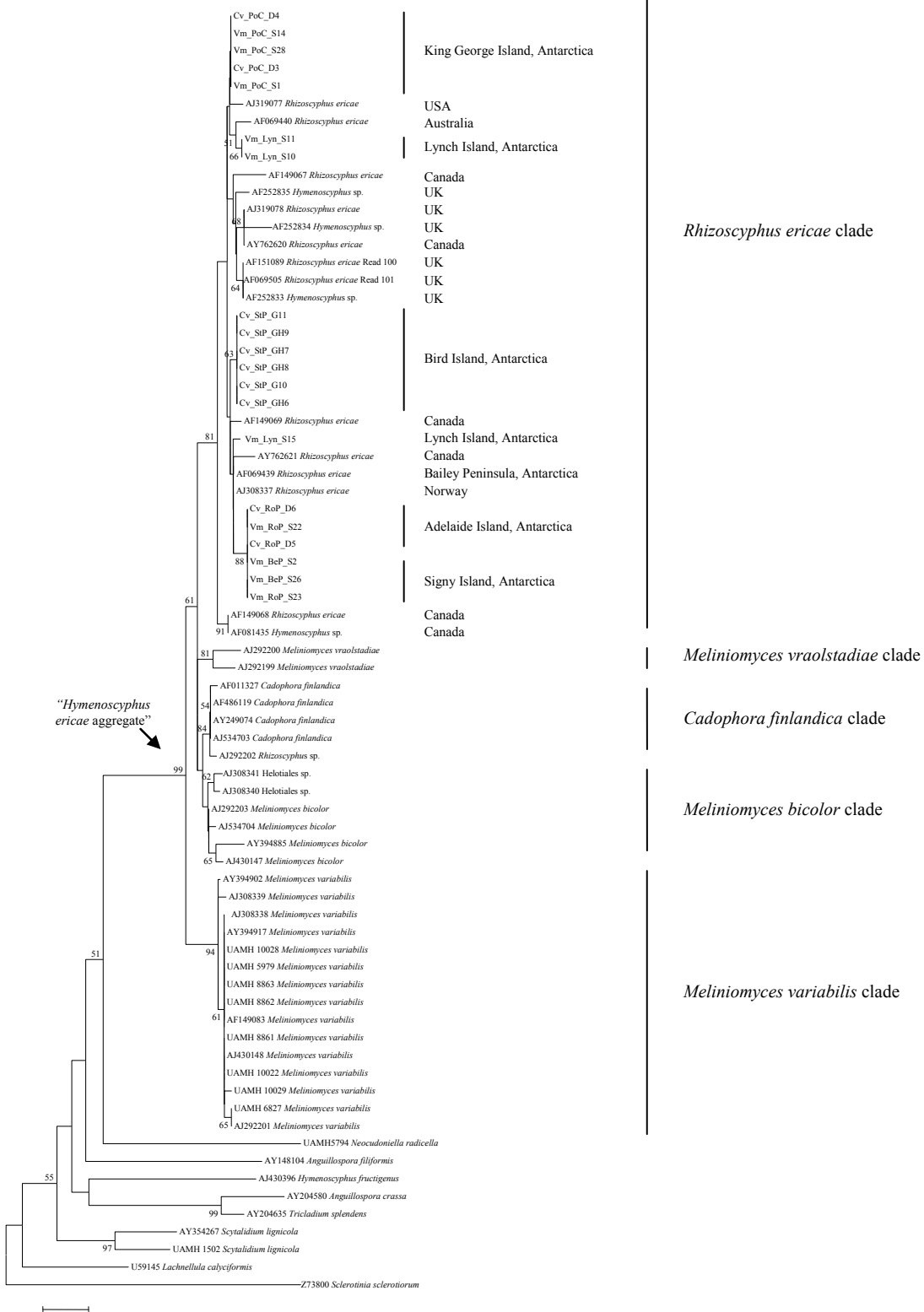


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**Fig. 2**

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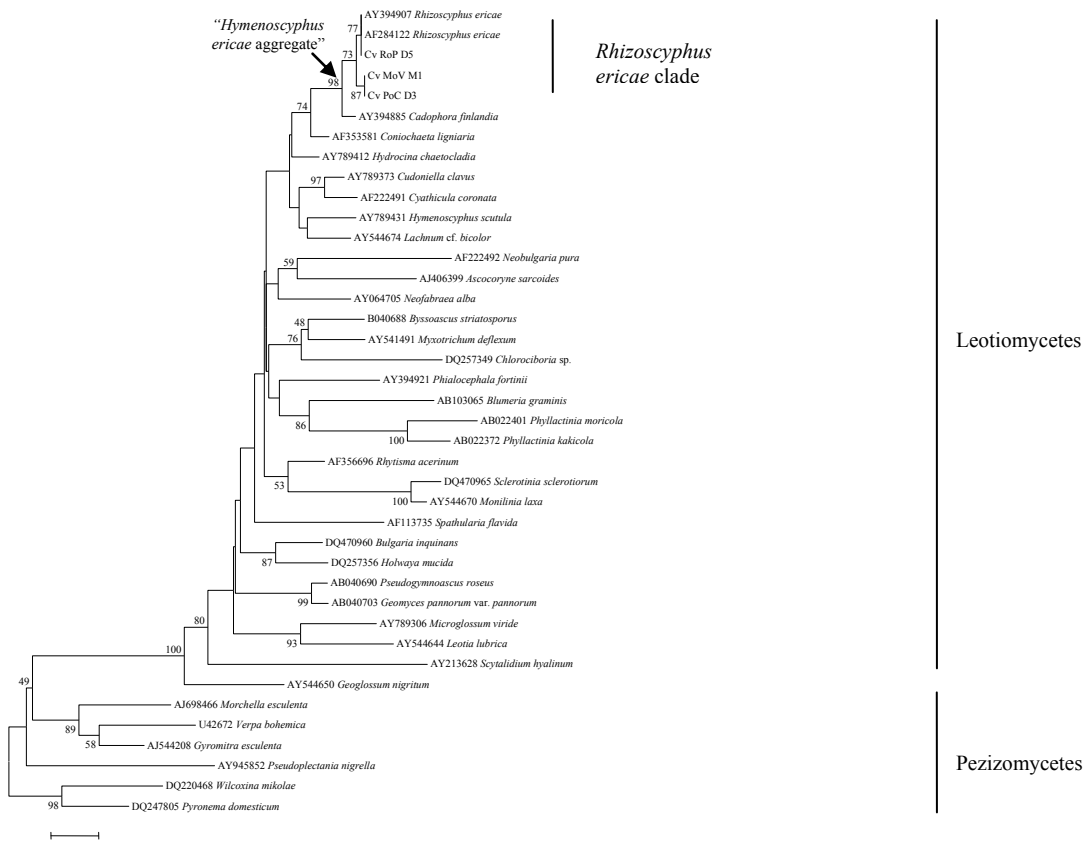
Fig. 3



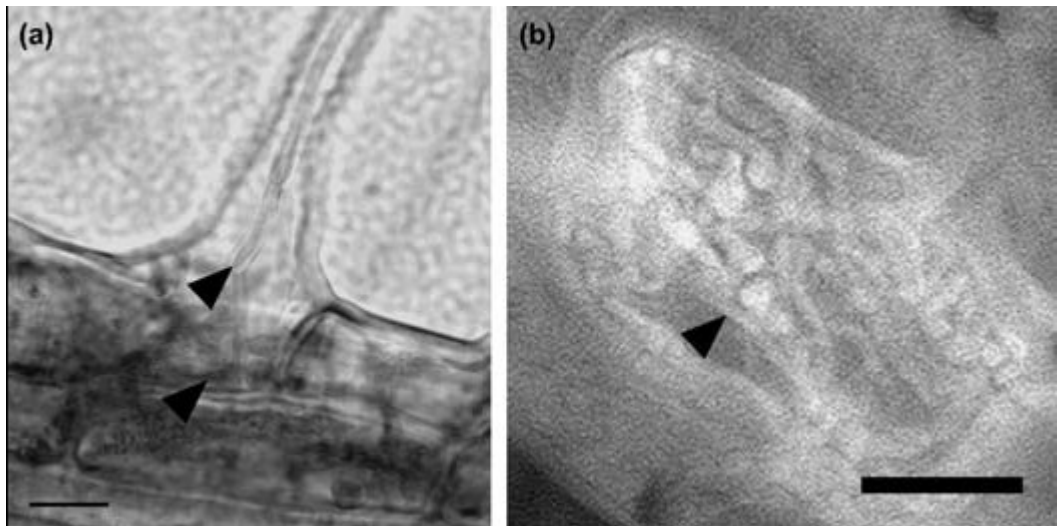
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**Fig. 4**



580 **Fig. 5**



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