Mycothalli of the hepatic *Barbilophozia hatcheri* in Antarctica: distribution and identities of mycobionts

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ABSTRACT

Forty-four herbarium specimens of the hepatic *Barbilophozia hatcheri*, collected from across the entire range of the species in Antarctica (54–68 °S), were examined for fungal colonisation. Mycothalli, consisting of hyaline hyphae in rhizoids or hyphal coils in rhizoid bases and stem medullary cells, were present in 42 specimens. The frequencies of mycothalli declined at higher latitudes and were associated positively with annual air temperatures. In contrast, the frequency of dark septate hyphae on stems increased at higher latitudes. Sequencing of ITS regions of fungal rRNA genes amplified from *B. hatcheri* from 54 °S, 61 °S and 68 °S indicated that *Cladophialophora* species colonised plants at higher latitudes, but that most fungi had >99% homology with members of Sebacinales clade B, with best matches to mycobionts of *Barbilophozia lycopodioides*, *Lophoziopsis excisa* and *Protolophozia crispata*. We propose the use of herbarium specimens of mycothalli as proxies for climate change in Antarctica.

*Keywords***:** Antarctica, *Barbilophozia hatcheri*, climate change, herbarium, latitudinal transect, mycothalli, Sebacinales clade B

Introduction

Leafy liverworts belonging to the order Jungermanniales are frequently colonised by ascomycete or basidiomycete fungi forming structures closely resembling mycorrhizas (Read *et al*. 2000; Selosse 2005). Associations between leafy liverworts and fungi, which are characterised by hyphae in the shafts of rhizoid cells and hyphal coils in the bases of rhizoids and adjacent medullary cells, are termed mycothalli, denoting symbioses with potentially mutualistic benefits (Boullard 1988). The ascomycetes forming these associations, which usually colonise liverworts in the Cephaloziaceae, Cephaloziellaceae, Lepidoziaceae, Calypogeiaceae and Schistochilaceae, are typically found to be *Rhizoscyphus ericae* or its allies. In contrast, the basidiomycetes, which usually colonise members of the Lophoziaceae, Arnelliaceae, Geocalycaceae and Scapaniaceae, are usually identified as members of Sebacinales clade B (Bidartondo & Duckett 2010; Pressel *et al*. 2010), fungi that also form endophytic associations and ericoid, orchid or cavendishioid mycorrhizas with a wide range of plant species (Weiβ *et al*. 2004). Although knowledge has accumulated about mycothalli in temperate and tropical habitats, comparatively little is known of the occurrence of these associations in polar regions, where bryophytes are usually the dominant plant form. Hitherto, there have been only three reports of mycothalli from the Antarctic, where the association has been found in two species of hepatic (Chambers *et al*. 1999; Upson *et al*. 2007; Newsham & Bridge 2010). Furthermore, little is known of the abiotic factors that control the frequencies of mycothalli, although the abundance of fungal structures in *Cephaloziella varians* is known to be associated with surface air temperature in the Antarctic (Newsham 2011).

Reporting the presence of fungi belonging to Sebacinales clade B in the hepatic *Lophoziopsis* (= *Lophozia*) *excisa* at Léonie Island in the southern maritime Antarctic, Newsham & Bridge (2010) advocated the examination of further leafy liverwort species from the continent, including *Barbilophozia hatcheri* (Evans) Loeske, for colonisation by these fungi. Here, we report a study that examined herbarium specimens of *B. hatcheri* collected from across the entire latitudinal range of the plant species in Antarctica (54–68 °S; Bednarek-Ochyra *et al*. 2000) for fungal colonisation. The aims of the study were to establish whether or not the frequencies of fungal structures alter with latitude and surface air temperature, and to determine the identities of the mycobionts present in the tissues of the liverwort.

Materials and methods

Forty-four air-dried specimens of *B. hatcheri* were sourced from the British Antarctic Survey's herbarium (AAS). The specimens had been collected from along a 2,312 km transect from South Georgia in the sub-Antarctic through to Léonie Island in the southern maritime Antarctic (Fig 1). Details of the locations from which the plants were sampled, and the years in which they were collected, are shown in Table 1.

Distribution of mycobionts

Specimens of *B. hatcheri* were sorted in water under a dissecting microscope, checking for the presence of underleaves and cilia at the bases of leaves. Any debris was removed from plants, which were then cleared for 24 h in 10% KOH solution. The KOH was removed and the plants were washed three times in water. They were then bleached in a solution of hydrogen peroxide (7.5%) and ammonium hydroxide (0.7%) for *c*. 3 min, after which they were washed several times in water and then acidified in 5% lactic acid for 1 h. The acid was drawn off and the plants were placed in 0.01% aniline blue in lactic acid for 24 h. They were then removed from the staining solution, excess stain was drawn off on absorbent tissue paper, and they were destained for at least 24 h in 80% lactic acid. Between 10 and 40 plants from each specimen were mounted on glass slides and were lightly squashed prior to observation under UV epifluorescence at × 400 magnification (BX51 microscope, Olympus UK Ltd). The line intersection method (McGonigle *et al*. 1990) was used to measure the percentages of stem length colonised by (i) hyphal coils in rhizoid bases and stem medullary cells, (ii) hyaline septate hyphae in stem medullary cells and (iii) dark septate hyphae on stem surfaces. The percentage of rhizoids colonised by hyaline hyphae was also recorded. Between 20 and 152 (mean 67) intersects and between 20 and 188 (mean 81) rhizoids were examined per specimen for colonisation. In total, 3,076 intersects and 3,725 rhizoids were examined for colonisation by fungal structures.

Linear regression analyses were used to test for associations between the frequencies of fungal structures in the tissues of *B. hatcheri* and latitude. Data from collection sites that were located ≤ 2 seconds of latitude (1.9 km) apart were grouped together in these analyses. Linear regressions were also used to test associations between the frequencies of fungal structures and the year in which each specimen had been collected, and the annual surface air temperatures recorded at eight research stations situated 0.1–110 km from the collection sites (Table 2). All air temperature data were extracted from the READER dataset (http://www.antarctica.ac.uk/met/READER/) except those from Palmer station, which were taken from Baker & Stammerjohn (1995). In addition, one way ANOVA was used to compare the frequencies of fungal colonisation in *B. hatcheri* tissues sampled from the same location on Signy Island (Factory Cove, a.k.a. 'the Backslope') in 1985 and 2011.

Identities of mycobionts

Specimens of *B. hatcheri* collected from South Georgia, Signy Island and Léonie Island in 2011 (Table 1) were selected for molecular analyses. Healthy apical parts of stems of *B. hatcheri* were cleaned of any debris and were bleached for 5 min in a 10% solution of commercial bleach (*c.* 0.5% sodium hypochlorite) prior to washing in 30 changes of sterile distilled water (10 ml) on a vortexer set to maximum speed. Between washes, each of which lasted for 5 min, water was drained from plant tissues on sterile mesh (1 mm). Tissues were homogenised with a TissueRuptor and DNA was extracted from the tissues using a DNeasy Plant Mini kit (both Qiagen, Hilden, Germany). PCR amplifications were carried out in 40 μl volumes, with concentrations of 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 units of Taq polymerase and 0.5 μM of each of the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes & Bruns 1993)/ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al*. 1990) and ITS1F/TW14 (5'-GCTATCCTGAGGGAAACTTC-3'; Cullings 1994). The PCR amplification programme for both primer sets consisted of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 3 min, followed by a final elongation step at 68 °C for 10 min (PTC-225 Peltier thermal cycler, MJ Research Inc., Watertown, MA, USA). The amplicons from the ITS1F/TW14 reactions were then used as templates in a second round of amplification using the primer set ITS3Seb (5'-TGAGTGTCATTGTAATCTCAC-3'/TW13 (5'-GGTCCGTGTTTCAAGACG-3'; White *et al*. 1990), which selectively amplifies the 5.8S–ITS2 and part of the 28S region of rRNA genes of fungi in the Sebacinales (Selosse *et al*. 2007). The PCR amplification program for this primer set consisted of denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 30 s, followed by a final elongation step at 72 °C for 10 min. Sub-samples of amplicons, stained with the nucleic acid stain GelRed (Biotum, Hayward, CA, USA), were visualised on 1.5% agarose gels. Negative controls, consisting of sterile water (1 μl) in place of template DNA, did not yield amplicons. All positive PCR amplicons were cloned and transformed using a TOPO TA Cloning Kit (Life Technologies, Paisley, UK) following the manufacturer's recommendations. The recombinant clones that were obtained were bidirectionally sequenced using forward and reverse M13 primers.

Forward and reverse chromatograms from each sequenced clone were edited, then assembled, and vector and primer sequences were trimmed using Geneious v.6.1.6 (Drummond *et al*. 2011). The resulting assembly sequences were deposited in GenBank under the accession numbers KF636367– KF636410. MegaBLAST searches (word size: 16, maximum E value: 1×10^{-10}, maximum hits: 1000) of the GenBank database using the full length rRNA and ITS assembly sequences did not consistently recover the taxonomically closest ITS1 or ITS2 BLAST hits when these hits were short in length and the BLAST results were dominated by longer reads that included less variable rRNA genes from taxonomically distant accessions. Therefore, in order to optimise the likelihood of finding the most highly similar species matches in GenBank, we carried out further MegaBLAST searches, with the same settings as those given above, using only the ITS1 and ITS2 regions rather than the full length assembly. The resulting ITS-specific MegaBLAST GenBank matches were sorted into putative species groupings using BLASTclust analyses (http://toolkit.tuebingen.mpg.de/blastclust/). Parameters for the BLASTclust analyses were a sequence length coverage of 75% and a percentage identity threshold of 98% and 97% for ascomycetes and basidiomycetes, respectively (Blaalid *et al.* 2013).

Molecular phylogenies were estimated using the cloned sequences of the ITS1-5.8S-ITS2 region, generated from the ITS1F/ITS4 primer pair, together with GenBank matches to these sequences obtained from the MegaBLAST searches. The ITS1-5.8S-ITS2 region - the primary use of which was for DNA barcoding - was too variable to be of use in taxonomically broad phylogenies, and hence only the most similar GenBank matches were used. In order to select the most similar matches, we filtered the ITS1- and ITS2-specific MegaBLAST search results for DNA accessions that had both ITS1 and ITS2 BLAST hits with >75% coverage and >85% pairwise identity. The resulting DNA accessions were unambiguously aligned with the sequences from this study to generate ascomycete and basidiomycete DNA matrices consisting of 83 taxa by 519 characters and 67 taxa by 594 characters, respectively. Neither matrix showed evidence of nucleotide heterogeneity, as assessed using SeqVis v.1.5 software (Ho *et al.* 2006). Suitable DNA substitution models were hence selected using jModeltest v.2.1.1 (Darriba *et al.* 2012). A TIM2 model with equal base frequencies and a gamma variable sites parameter was selected for use with both matrices, which were subsequently analysed by maximum likelihood with GARLI v.0.96 (Zwickl 2006) and also by unweighted maximum parsimony with PAUP v.4.0b10 (Swofford 2002). Phylogeny support measures were generated using 1000 non-parametric bootstrap replicates.

Results

Distribution of mycobionts

Hyphal coils, which formed in stem medullary cells and the bases of rhizoid cells (Supplementary Fig S1), were more frequent in plants from lower latitudes. Coils were recorded in all specimens examined except that gathered from Lahille Island (Rils 4329). Lines of best fit from linear regression analyses indicated that the percentage of stem length colonised by hyphal coils declined significantly from 72% at 54 °S to 16% at 68 °S ($F_{1,13}$ = 10.8; r^2 adj. = 41%; P=0.006; slope -4.3%; Fig 2a). The hyphae that formed coils were continuous with hyaline septate hyphae (1.5–2.0 µm diam.) colonising the shafts of rhizoid cells (Supplementary Fig S1). These hyphae apparently entered the rhizoid cells through their tips and rarely branched within the shaft of the cell (Supplementary Fig S1). Hyaline septate hyphae were found in rhizoids in all specimens, except for one that had been gathered from Signy Island (Rils 5213). The percentage of rhizoids colonised by hyaline hyphae also declined significantly from 80% to 25% between 54 °S and 68 °S, respectively ($F_{1,13}$ = 9.6; r^2 adj. = 38%; P=0.008; slope -3.5%; Fig 2b). Broader (2–4 µm diam.) dark septate hyphae were frequently observed on the stem surfaces of plants collected from higher latitudes. Regression analyses indicated that the percentage of stem length colonised by these hyphae increased from 9% at 54 °S to 77% at 68 °S ($F_{1,13}$ = 8.7; r^2 adj. = 36%;

P=0.011; slope 5.1%; Fig 2c). The frequency of hyaline septate hyphae on the surfaces of stems and within epidermal cells, which were of the same diameter as those present in rhizoids, did not alter along the transect ($F_{1,13}$ = 1.8; r^2 adj. = 5%; P>0.05; data not shown). Regression analyses indicated that the frequencies of fungal colonisation of *B. hatcheri* tissues were not associated with the year of collection ($F_{1,18}$ = 0–3.8; r^2 adj. 0–13%; all P>0.05). ANOVA similarly indicated no differences between the frequencies of fungal structures in plants sampled in 1985 and 2011 from Factory Cove on Signy Island ($F_{1,8}$ = 0.02–1.28; r^2 adj. 0–3%; all P>0.05). In *B. hatcheri* samples from Factory Cove in 1985 and 2011 (n = 5 in both years), the mean stem lengths (± SEM) colonised by hyphal coils were 41.4% (11.5%) and 46.4% (11.5%), the frequencies of rhizoids colonised by hyaline hyphae were 46.8% (15.1%) and 49.3% (8.6%), and the stem lengths occupied by dark septate hyphae were 31.0% (10.3%) and 16.3% (7.8%), respectively (Fig 2a–c).

Regression analyses indicated that the frequencies of hyphal coils in stem and medullary cells and of hyaline hyphae in rhizoids were both positively associated with mean annual surface air temperatures (hyphal coils: $F_{1,6}$ = 6.4; r^2 adj. = 43%; P=0.045; slope 6.2%, hyphae in rhizoids: $F_{1,6}$ = 9.2; r^2 adj. = 54%; P=0.023; slope 6.3%; Fig 3a, b). The percentages of stem length colonised by hyaline or dark septate hyphae were not associated with mean annual air temperatures ($F_{1,6}$ = < 2.7; r^2 adj. < 20%; both P > 0.05; data not shown).

Identities of mycobionts

All PCRs produced positive amplicons. A total of 44 sequences were obtained from the cloned PCR products, with 29 and 15 derived from the ITS1F/ITS4 and ITS3Seb/TW13 primer sets, respectively. Results from the initial MegaBLAST analyses using full length sequences indicated that eight of the 29 queries from the ITS1F/ITS4 primer set were members of the Ascomycota. One of these eight sequences was derived from plants sampled from Signy Island and the remainder were obtained from Léonie Island (Supplementary Table S1). Ascomycete sequences were not obtained from specimens sampled from South Georgia. ITS1- and ITS2-specific MegaBLAST analyses of the ascomycete sequences yielded > 86.9% sequence identity matches to members of the Herpotrichiellaceae. Three sequences from Léonie Island (Léo2, Léo4 and Léo14) had 94.9% ITS1 and >96% ITS2 matches to accessions EU686075 and FN555433 (Supplementary Table S1), both of which are sequences of fungi associated with species of *Lophozia* or *Lophoziopsis* from Antarctica or North America. BLASTclust analysis suggested that these identities were insufficient for species determinations but were sufficient to indicate the same genus. Another three ascomycete sequences from the same island (Léo1, Léo13 and Léo15) had 100% ITS1 and >98.1% ITS2 matches to *Cladophialophora minutissima* from moss tissues in Canadian bogs (EF016376 and EF016382–4). The ITS1 and ITS2 BLASTclust groups for these sequences also included the ex-type strain of *Cladophialophora humicola* from a European arable soil (EU035408), with 98% and 93% matches to this sequence across the two regions, respectively, and two *Cladophialophora* clones from Arctic soils (HQ211827 and HQ211891). Two sequences (Sig1 and Léo16) had 86.9–94.2% identities to accessions of *C. minutissima* or to those of fungal clones from cold soils, montane rock surfaces or lichens. Maximum likelihood and maximum parsimony phylogenetic analyses of the ascomycete sequences generated the same suites of well-supported groups. Data from both methods were largely consistent with the BLASTclust groupings to *Cladophialophora* species, with the grouping of Léo2, Léo4 and Léo14 being supported in both analyses (Supplementary Fig S2). However, the grouping of sequence Léo15 with Léo1 and Léo13 was not statistically supported (Supplementary Fig S2), even though these three sequences formed a single BLASTclust group (Supplementary Table S1).

The remaining 36 query sequences were all found to be members of the Basidiomycota. MegaBLAST and BLASTclust analyses of these sequences indicated that they all matched closely (>99% ITS1 and ITS2 similarities) with those of fungi belonging to Sebacinales clade B (Supplementary Table S2). The single BLASTclust ITS1 group consisted of the accessions GQ907083, GQ907084, GQ907097 and GQ907138, members of Sebacinales clade B previously found in association with *Lophoziopsis excisa* and *Barbilophozia lycopodioides* from Switzerland, *L. excisa* from the UK and *Protolophozia crispata* from Chile, respectively. The ITS2 group contained the accessions FN555434, FN555435, GQ907083 and GQ907138 (Supplementary Table S2), the former two of which were associated with *L. excisa* from Léonie Island. The data strongly suggest that the basidiomycete sequences generated in the present study are all members of the same species in Sebacinales clade B (Supplementary Table S2). Data from maximum likelihood and maximum parsimony phylogenetic analyses produced similar phylogenies, and showed that the basidiomycete sequences from this study were resolved as a single well-supported group within Sebacinales clade B that contained the same accessions generated from the BLASTclust analysis, *viz*. GQ907083, GQ907084, GQ907097 and GQ907138 (Fig 4). Many of the most closely related fungi to the basidiomycetes present in Antarctic *B. hatcheri* were mycobionts of liverwort species from Northern Europe and Chile (Fig 4).

Discussion

The results reported here, showing reductions in the frequencies of colonised rhizoids and hyphal coils in the tissues of *Barbilophozia hatcheri* at higher latitudes, indicate that the distribution of a mycothallus alters significantly across the entire latitudinal range of the hepatic in Antarctica. These observations corroborate those of a previous study showing reductions in the frequencies of hyphal coils, but not of colonised rhizoids, in the tissues of the liverwort *Cephaloziella varians* at higher latitudes across a transect between South Georgia and south-eastern Alexander Island, 360 km south of Léonie Island (Newsham 2011). The slopes of these associations, derived from linear regression analyses, are similar for both hepatics, with changes of 3%–4% in the frequencies of hyphal coils per degree of latitude, and significant associations between hyphal coil frequencies and mean annual surface air temperatures in both cases. Associations were not recorded here between the frequencies of mycothalli and the year in which specimens had been collected. However, it should be noted that inaccuracies may have been introduced into the analyses by changes in surface air temperatures in the maritime Antarctic, estimated at 0.2–0.5 °C per decade (Turner *et al*. 2009), during the 34-year period over which the specimens were collected, particularly as the two end-members in the analyses (South Georgia and Léonie Island) were sampled the most recently. Nevertheless, the data here agree closely with the previous study on *C. varians*, in which specimens were collected over several years, and in which 4.6% increases in hyphal coils were recorded per degree Celsius rise in mean annual surface air temperature (Newsham 2011), compared with the 6.2% increase in coils per degree Celsius rise in temperature recorded here. These data support previous studies showing the important influence of air temperature, with which liquid water availability is strongly associated in polar environments, on fungal distributions at high latitudes (Wollan *et al*. 2008; Newsham *et al*. 2009). They also corroborate previous data showing reductions in the frequencies of arbuscular mycorrhizal fungi, the main fungal symbionts of herbaceous plant species, in roots sampled from higher latitudes in the Arctic and Antarctic (Väre *et al*. 1992; Olsson *et al*. 2004; Upson *et al*. 2008).

In the present study, hyaline septate hyphae were found to enter the rhizoids of *B. hatcheri* at their tips, from where they grew along rhizoid shafts and formed hyphal coils in the bases of rhizoid cells and adjacent medullary cells. These patterns of fungal colonisation are the same as those observed in *B. hatcheri* and related hepatics from Europe, *viz*. *Orthocaulis attenuatus* and *O. floerkei* (= *Barbilophozia attenuata* and *B. floerkei*), *Schljakovia kunzeana* (= *B. kunzeana*), *Schljakovianthus quadrilobus* (= *B. quadriloba*), *Barbilophozia lycopodioides* and *B. barbata* (Kottke *et al*. 2003; Duckett *et al*. 2006). Based on the presence of dolipores with imperforate parenthesomes, these patterns of colonisation were initially ascribed to the basidiomycetes, and specifically the Sebacinales (Kottke *et al*. 2003; Duckett *et al*. 2006). Subsequent sequencing data confirmed that the fungi associated with these hepatics from Northern Europe or Chile, along with those associated with *Diplophyllum*, *Lophozia*, *Scapania*, *Trilophozia*, *Nardia* and *Saccogyna* spp. from Europe, South America or Asia, are members of Sebacinales clade B (Bidartondo & Duckett 2010). The data reported here show 99%–100% homology between the ITS1 and ITS2 region sequences of the mycobionts in Antarctic *B. hatcheri* and these liverwort-associated members of Sebacinales clade B. Along with previous data showing the same fungi to be the mycobionts of *Lophoziopsis excisa* at Léonie Island (Newsham & Bridge 2010), they show the presence of a second mycothallus formed by members of the clade in Antarctica. Together with studies showing the presence of Sebacinales clade B in soils and the roots of plants sampled from North America, South America, Europe, Africa, Asia and Australia (Weiβ *et al*. 2011), they also indicate that members of the clade have a worldwide distribution.

The occurrence of members of Sebacinales clade B is well documented in many families of higher plants, including the Ericaceae, Fabaceae, Lamiaceae, Orchidaceae and Poaceae (Weiβ *et al*. 2004; Setaro *et al*. 2006; Selosse *et al*. 2007, 2009; Weiβ *et al*. 2011). Given the frequent occurrence of these fungi in higher plants, it is notable that each of the best matches to the Sebacinales clade B sequences derived from the present study were to fungi previously found in association with the leafy liverworts *B. lycopodioides, L. excisa* and *Protolophozia crispata* (Bidartondo & Duckett 2010; Newsham & Bridge 2010). This observation supports the distinct clade of Sebacinales associated with the leafy liverworts *Pseudolophozia* (= *Lophozia*) *sudetica*, *Schistochilopsis* (= *Lophozia*) *incisa* and *Calypogeia muelleriana* found in a previous study (Weiβ *et al*. 2004). However, to test the idea that there may be a phylogenetically distinct clade of leafy liverwort-associated Sebacinales (Newsham & Bridge 2010), more DNA sequence data are required from genomic regions that are less variable than the ITS region used in this study in order to resolve deeper relationships among, and possibly within, this putative clade of fungi.

The data reported here indicate an increase in the frequency of dark septate hyphae on the stem surfaces of *B. hatcheri* at more southerly latitudes, corresponding with an increase in the frequency of fungi with ITS1 and ITS2 region sequences similar to those of the dematiaceous ascomycete *Cladophialophora*. The ITS1 regions of three sequences amplified from *B. hatcheri* from Léonie Island matched precisely with that of *C. minutissima* isolated from the tissues of the mosses *Polytrichum juniperinum* and *Aulacomnium palustre* inhabiting Canadian *Sphagnum* bogs (Davey & Currah 2007), and had >98% homology with the ex-type strain of *C. humicola*, from European arable soil (Crous *et al*. 2007). The data here confirm the presence of *Cladophialophora* and related fungi in the tissues of *L. excisa* from Léonie Island and a species of *Lophozia* from North America (Davis & Shaw 2008; Newsham & Bridge 2010) and support the view that members of the Chaetothyriales (such as *Cladophialophora*) become more frequent in disturbed or stressed environments (Lindahl *et al*. 2010). As for hyphal coils, the changes with latitude in the frequencies of dark septate hyphae on the stem surfaces of *B. hatcheri* were similar to those reported for *C. varians* (Newsham 2011), with 4%–5% increases in stem length occupied by these hyphae in both species for each degree of latitude further south. These findings support the view that fungal hyphae become increasingly melanised in cold and arid habitats in order to confer resistance to low temperatures and desiccation, broadening the ecological niches occupied by fungi (Jumpponen & Trappe 1998; Robinson 2001).

A previous study on arbuscular mycorrhizal fungi in the roots of Arctic plants suggested that archives of herbarium material represent untapped resources for determining the spatial and temporal responses of fungi to climatic change (Ormsby *et al*. 2007). The data reported here support this view. Recent estimates of the rates of climate change in the maritime Antarctic indicate a 0.2 °C decade^{-1} rise in annual surface air temperature since the 1950s at Orcados station on Laurie Island, 45 km to the east of Signy Island (Turner *et al*. 2009). Since 1985 there has hence been a rise in mean annual surface air temperature in the region of Signy Island of *c*. 0.5 °C, which, assuming increases reported here of 6.2%–6.3% in the frequencies of hyphal coils and colonised rhizoids for each degree Celsius change in surface air temperature, would have elicited increases of *c*. 3% in the frequencies of these structures in *B. hatcheri* at Signy Island over the last two and a half decades. This estimate agrees reasonably well with the observed data: in 1985 and 2011, the frequencies of hyphal coils in the tissues of *B. hatcheri* were 41.4% and 46.4%, and those of colonised rhizoids were 46.8% and 49.3%, respectively. There was considerable variation associated with these measurements, and hence no differences were found between 1985 and 2011 in the frequencies of mycothalli in *B. hatcheri* at Signy Island. Given this variation, and assuming the same level of replication as used here, it may be several decades before any statistically significant differences become apparent, relative to the latter decades of the 20th century, in the frequencies of mycothalli in maritime Antarctic hepatics. Nevertheless, with previous research similarly having shown significant associations between mean annual air temperatures and the frequency of hyphal coils in *Cephaloziella varians* (Newsham 2011), there is apparently potential for using herbarium specimens of mycothalli as proxies for decadal to centennial climate change impacts in Antarctica.

Acknowledgements

Funding was provided by the Natural Environment Research Council through the British Antarctic Survey’s Coastal and Terrestrial workpackage. Ron Lewis Smith, Ken Richard, Bill Hankinson, Alistair Moffat and Cora de Leeuw collected the specimens of *B. hatcheri*. The crew of RRS James Clark Ross assisted with transport to and from South Georgia and Signy Island, and Dave Hunt and Russ Ladkin assisted with fieldwork on Léonie Island. Magda Biszczuk drew Fig 1. Marc-André Selosse and an anonymous reviewer provided helpful comments on the manuscript. All are gratefully acknowledged.

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Figure legends

Fig 1 – map showing the locations of the sites from which the herbarium specimens were collected. Site numbers correspond to those shown in Tables 1 and 2 and Fig 3.

Fig 2 – (a) the percentage of stem length colonised by hyphal coils, (b) the percentage of rhizoids colonised by hyaline septate hyphae and (c) the percentage of stem length colonised by dark septate (DS) hyphae in the tissues of *B. hatcheri* as a function of latitude. Dotted lines are lines of best fit derived from linear regression analyses. Values on the *y*-axis are either single points or are means of 2–10 replicates ± SEM. Open and filled symbols at 61 °S represent values for specimens collected from Factory Cove on Signy Island in 1985 and 2011, respectively.

Fig 3 – (a) the percentage of stem length colonised by hyphal coils and (b) the percentage of rhizoids colonised by hyaline septate hyphae as a function of mean annual surface air temperature recorded at eight research stations. Dotted lines are lines of best fit derived from linear regression analyses. Values on the *x*-axis are means of 14–22 years ± SEM. Those on the *y*-axis are means of 2–14 replicates ± SEM. Numerals correspond to the site numbers shown in Tables 1 and 2.

Fig 4 – midpoint-rooted, maximum likelihood phylogram obtained from the alignment of ITS1-5.8S-ITS2 region sequences of basidiomycetes amplified from Antarctic *Barbilophozia hatcheri* with those of fungi in Sebacinales clade B having >75% ITS1 and ITS2 coverage and >85% pairwise identity with query sequences. GenBank accession numbers of reference sequences are shown, along with the plant species host or substrate and the country of origin in parentheses. Reference sequences marked in bold are accessions of fungi found in association with liverwort species. Symbols at branch nodes indicate bootstrap support at >50% (□) >80% (○) and at 100% (●) in both maximum parsimony and maximum likelihood analyses. Abbreviations: SG, South Georgia; Sig, Signy Island; Léo, Léonie Island.

Table 1**.** Herbarium specimens of *Barbilophozia hatcheri* examined in the study

Site no.[*]	Location	Latitude and longitude	Year collected	AAS accession no.
1	South Georgia	54° 17’ S; 36° 31’ W	2011	KN 1–KN 5
2	Signy Island	60° 42’ S; 45° 36’ W	1985	Rils 5209–Rils 5213
	Signy Island	60° 42’ S; 45° 36’ W	2011	KN 143–KN 147
3	King George Island	62° 06’ S; 58° 29’ W	1980	Ochyra 1758
4	King George Island	62° 07’ S; 58° 24’ W	1980	Ochyra 1757
5	King George Island	62° 13’ S; 58° 25’ W	1980	Ochyra 1730
6	Livingston Island	62° 37’ S; 61° 09’ W	1991	Richard 44
7	Livingston Island	62° 39’ S; 61° 10’ W	1991	Richard 28, Richard 29
8	Brabant Island	64° 02’ S; 62° 35’ W	1985	Moffat 61, Moffat 76
9	Brabant Island	64° 17’ S; 62° 20’ W	1984	Hankinson 189
10	Omega Island	64° 20’ S; 62° 56’ W	1981	Rils 4126
11	Gamma Island	64° 20’ S; 63° 00’ W	1981	Rils 4153
12	Hunt Island	64° 20’ S; 62° 09’ W	1985	Moffat 51
13	Cuverville Island	64° 41’ S; 62° 38’ W	1981	Rils 4212
	Cuverville Island	64° 41’ S; 62° 38’ W	1993	de Leeuw 12, de Leeuw 13
	Cuverville Island	64° 41’ S; 62° 38’ W	2005	Rils 11938, Rils 11953
14	Danco Coast	64° 44’ S; 62° 33’ W	1981	Rils 4225
15	Joubin Island	64° 47’ S; 64° 26’ W	1977	Rils 1990, Rils 1998
16	Uruguay Island	65° 14’ S; 64° 14’ W	1981	Rils 4274, Rils 4275
17	Rasmussen Island	65° 15’ S; 64° 06’ W	1977	Rils 1926
18	Lahille Island	65° 32’ S; 64° 22’ W	1981	Rils 4329
19	Takaki Point	65° 32’ S; 64° 14’ W	1981	Rils 4349, Rils 4391
20	Léonie Island	67° 36’ S; 68° 20’ W	2011	KN 148–KN 150

[*] See Fig 1. Further details of the specimens and the habitats from which they were collected can be accessed at http://apex.nerc-bas.ac.uk/f?p=252:1

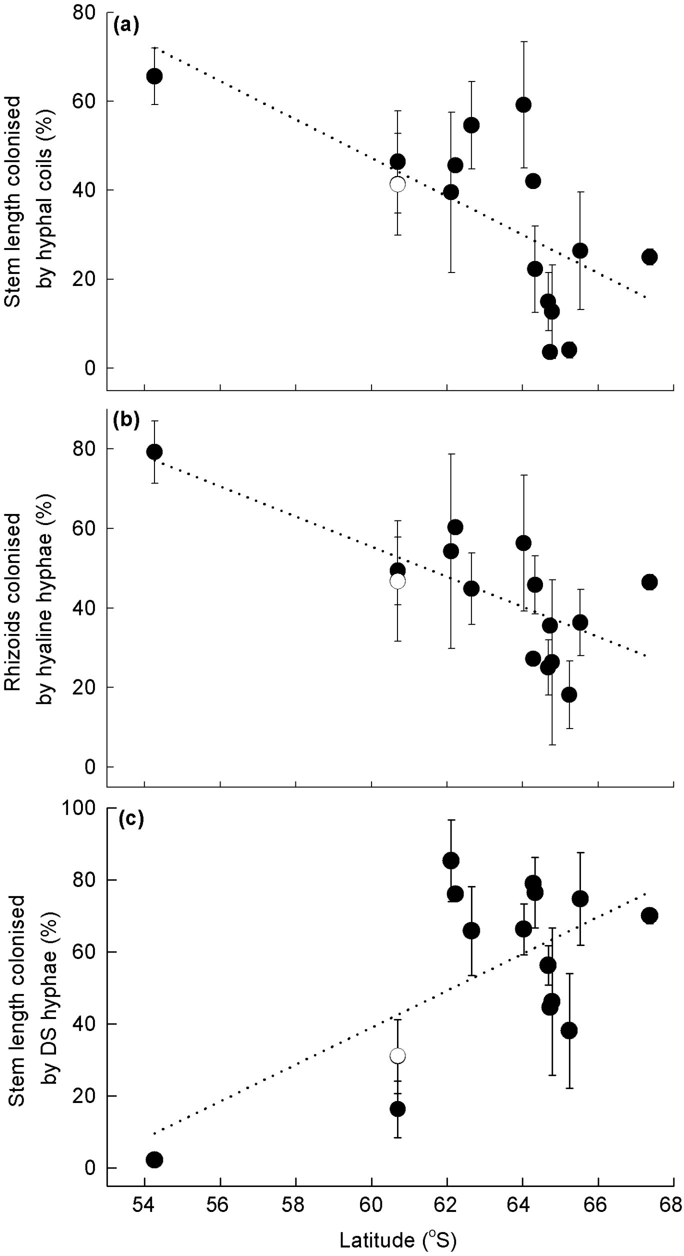
‘KN’ and ‘Rils’ denote collections by K. Newsham and R.I.L. Smith, respectively.

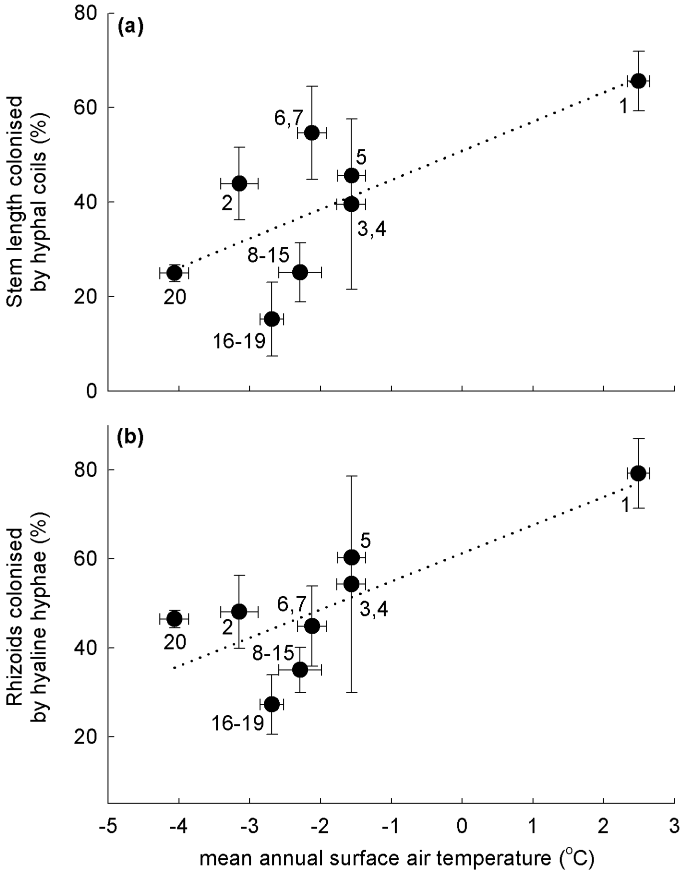
Table 2**.** Research stations at which surface air temperature data were recorded and their distances from collection sites

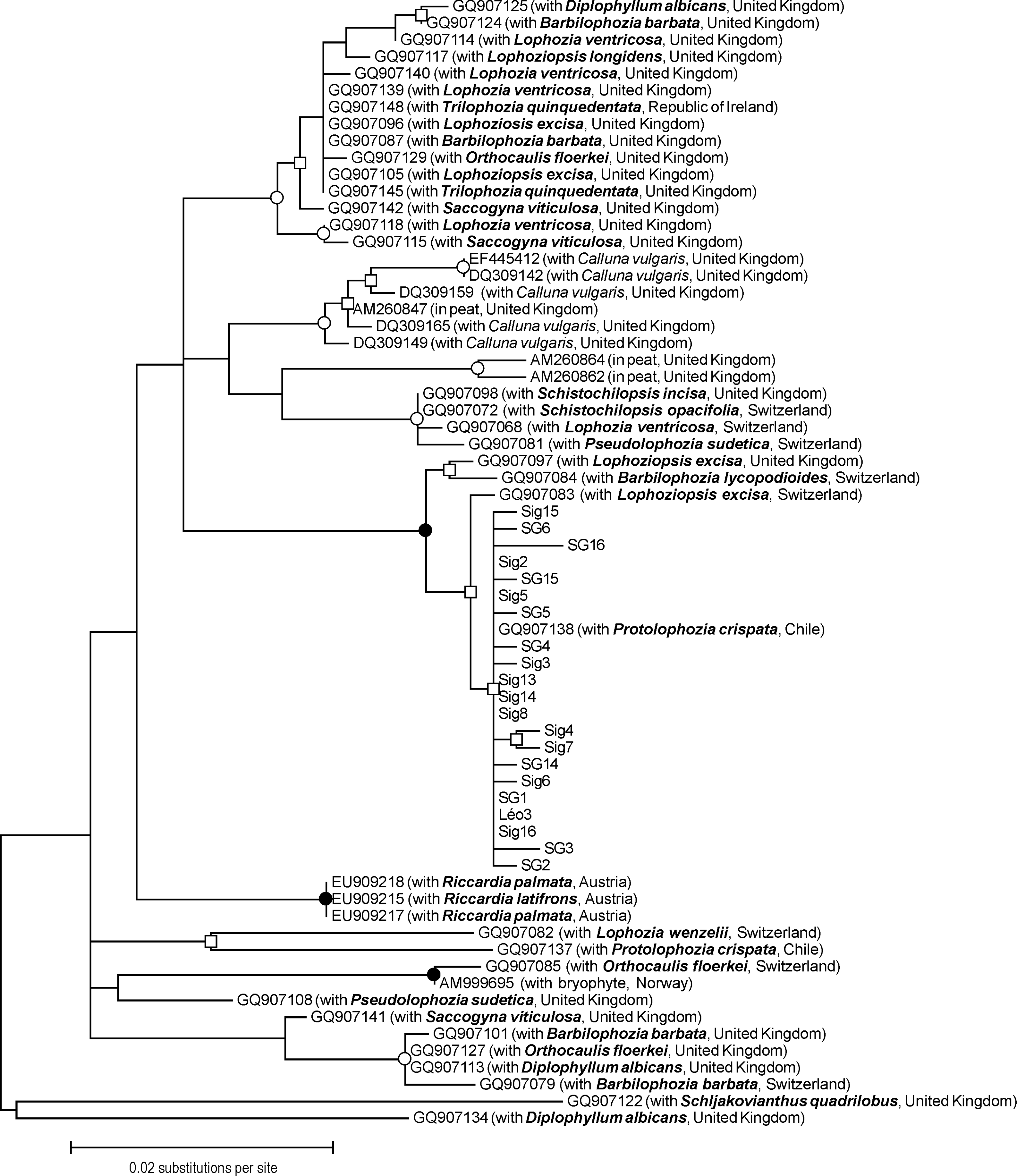
Station	Island	Latitude and longitude	Years in which data were recorded	Site no.[*]	Distance from station to site (km)
Grytviken	South Georgia	54° 17’ S; 36° 31’ W	1985–1988; 2002–2011	1	1
Signy	Signy Island	60° 42’ S; 45° 36’ W	1982–1995	2	0.1
Comandante Ferraz	King George Island	62° 05’ S; 58° 23’ W	1992–2009	3, 4	4, 5, 6[a]
Jubany	King George Island	62° 14’ S; 58° 40’ W	1988–2001; 2005–2008	5	13
Capitan Arturo Prat	Greenwich Island	62° 30’ S; 59° 41’ W	1980–1990; 1993–2003	6, 7	78
Palmer	Anvers Island	64° 46’ S; 64° 05’ W	1974–1996	8–15	12, 70, 110[a]
Vernadsky	Galindez Island	65° 15’ S; 64° 15’ W	1990–2011	16–19	5, 20, 38[a]
Rothera	Léonie Island	67° 34’ S; 68° 08’ W	1990–2011	20	10

[*] See Fig 1 and Table 1. [a]Values are minimum, mean and maximum distances


60°W
50°W
40°W
South Georgia
1
55°S
0
250
500
Km
Signy Island
2
60°S
King George Island
3,4
5
Livingston Island
6,7
8-19
Brabant Island
8
11
10
9
12
Anvers Island
13
14
15
16
17
18
19
65°S
20
Léonie Island


(a)
Stem length colonised by hyphal coils (%)
0
20
40
60
80
(b)
Rhizoids colonised by hyaline hyphae (%)
0
20
40
60
80
(c)
Stem length colonised by DS hyphae (%)
0
20
40
60
80
100
54
56
58
60
62
64
66
68
Latitude (°S)


(a)
Stem length colonised by hyphal coils (%)
0
20
40
60
80
1
2
3,4
5
6,7
8-15
16-19
20
(b)
Rhizoids colonised by hyaline hyphae (%)
20
40
60
80
1
2
3,4
5
6,7
8-15
16-19
20
-5
-4
-3
-2
-1
0
1
2
3
mean annual surface air temperature (°C)


GQ907125 (with **Diplophyllum albicans**, United Kingdom)
GQ907124 (with **Barbilophozia barbata**, United Kingdom)
GQ907114 (with **Lophozia ventricosa**, United Kingdom)
GQ907117 (with **Lophoziopsis longidens**, United Kingdom)
GQ907140 (with **Lophozia ventricosa**, United Kingdom)
GQ907139 (with **Lophozia ventricosa**, United Kingdom)
GQ907148 (with **Trilophozia quinquedentata**, Republic of Ireland)
GQ907096 (with **Lophoziosis excisa**, United Kingdom)
GQ907087 (with **Barbilophozia barbata**, United Kingdom)
GQ907129 (with **Orthocaulis floerkei**, United Kingdom)
GQ907105 (with **Lophoziopsis excisa**, United Kingdom)
GQ907145 (with **Trilophozia quinquedentata**, United Kingdom)
GQ907142 (with **Saccogyna viticulosa**, United Kingdom)
GQ907118 (with **Lophozia ventricosa**, United Kingdom)
GQ907115 (with **Saccogyna viticulosa**, United Kingdom)
EF445412 (with *Calluna vulgaris*, United Kingdom)
DQ309142 (with *Calluna vulgaris*, United Kingdom)
DQ309159 (with *Calluna vulgaris*, United Kingdom)
AM260847 (in peat, United Kingdom)
DQ309165 (with *Calluna vulgaris*, United Kingdom)
DQ309149 (with *Calluna vulgaris*, United Kingdom)
AM260864 (in peat, United Kingdom)
AM260862 (in peat, United Kingdom)
GQ907098 (with **Schistochilopsis incisa**, United Kingdom)
GQ907072 (with **Schistochilopsis opacifolia**, Switzerland)
GQ907068 (with **Lophozia ventricosa**, Switzerland)
GQ907081 (with **Pseudolophozia sudetica**, Switzerland)
GQ907097 (with **Lophoziopsis excisa**, United Kingdom)
GQ907084 (with **Barbilophozia lycopodioides**, Switzerland)
GQ907083 (with **Lophoziopsis excisa**, Switzerland)
Sig15
SG6
SG16
Sig2
SG15
Sig5
SG5
GQ907138 (with **Protolophozia crispata**, Chile)
SG4
Sig3
Sig13
Sig14
Sig8
Sig4
Sig7
SG14
Sig6
SG1
Léo3
Sig16
SG3
SG2
EU909218 (with **Riccardia palmata**, Austria)
EU909215 (with **Riccardia latifrons**, Austria)
EU909217 (with **Riccardia palmata**, Austria)
GQ907082 (with **Lophozia wenzelii**, Switzerland)
GQ907137 (with **Protolophozia crispata**, Chile)
GQ907085 (with **Orthocaulis floerkei**, Switzerland)
AM999695 (with bryophyte, Norway)
GQ907108 (with **Pseudolophozia sudetica**, United Kingdom)
GQ907141 (with **Saccogyna viticulosa**, United Kingdom)
GQ907101 (with **Barbilophozia barbata**, United Kingdom)
GQ907127 (with **Orthocaulis floerkei**, United Kingdom)
GQ907113 (with **Diplophyllum albicans**, United Kingdom)
GQ907079 (with **Barbilophozia barbata**, Switzerland)
GQ907122 (with **Schljakovianthus quadrilobus**, United Kingdom)
GQ907134 (with **Diplophyllum albicans**, United Kingdom)
0.02 substitutions per site

Supplementary Table S1. MegaBLAST hits and BLASTclust groups for ITS1 and ITS2 ascomycete sequences generated using the ITS1F/ITS4 primer pair

Sequences	ITS1 MegaBLAST top hit(s)*	ITS1 BLASTclust group	ITS2 MegaBLAST top hit(s)*	ITS2 BLASTclust group	Species inference
Sig1	92.7%, 100%, 2.39e-64 to AY843170, EF016376, EF016382–4, FJ553294 and JN032522	Orphan sequence[a]	Unique ITS2, 94.2%, 96.9%, 2.14e-59 to: AY843167, EF016376, EU725709	Orphan sequence[a]	Ascomycota; Herpotrichiellaceae sp.
Léo2, Léo4 and Léo14	94.9%, 100%, 1.82e-70 to EU686075	Orphan sequences[a]	96.3%–96.9%, 99.9%–100% 9.91e-68–2.14e-69 to FN555433	Grouped together[b]	Ascomycota; Herpotrichiellaceae sp.
Léo1, Léo13 and Léo15	100%, 100%, 5.02e-86 to AY843170, EF016376, EF016382–4, FJ553294 and JN032522	AY843164–6, AY843168, AY843170, AY843198, EF016376, EF016382–5, EU035408, EU725686, EU725691, FJ553294, FR682153, HQ211827, HQ211891, JN032522 and JX031869	98.1%–98.8%, 96.9%–100%, 5.94e-70–2.11e-74 to EU516794, AY843164, AY843166, AY843168, AY843198, AY843170, EF016385, FJ553294 and FR682153	FJ197877, EU516794, AY843164–8, AY843170, AY843198, EF016376–86, FJ552708, FJ552814, FJ553294, FR682153, EU035408, EU725686, EU725691, EU725709, HQ211827 and HQ211891	Ascomycota; Herpotrichiellaceae; *Cladophialophora minutissima* / *C. humicola*
Léo16	86.9%, 98.8%, 5.18e-46 to EF433988, EU292482 and JN889864	Orphan sequence[a]	91.6%, 100%, 4.78e-56 to EU139143 and EU139144	Orphan sequence[a]	Ascomycota; Herpotrichiellaceae sp.

*Data under MegaBLAST top hits are ranges or absolute values of pairwise identities, query sequence coverages and E-values, respectively. [a]Orphan sequences were not grouped with any other sequences in BLASTclust analyses. [b]These sequences grouped with each other but did not match with any sequences in GenBank.

Supplementary Table S2. MegaBLAST hits and BLASTclust groups for ITS1 and ITS2 basidiomycete sequences generated using the ITS1F/ITS4 and ITS3Seb/TW13 primer pairs

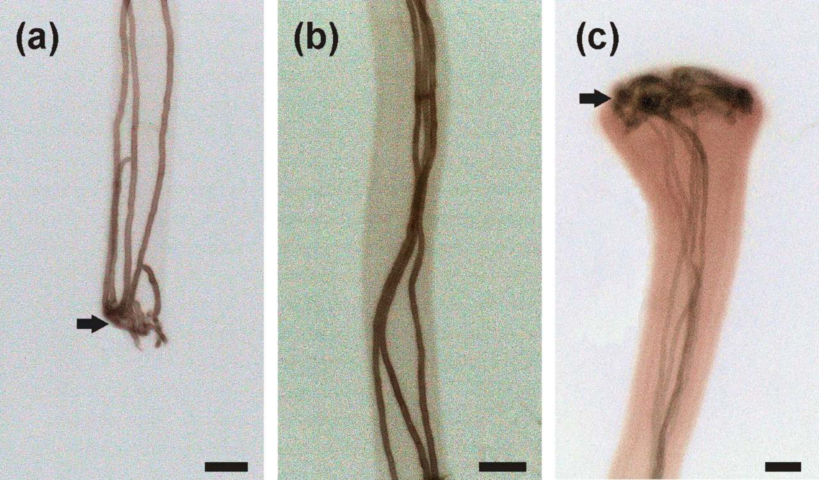
Sequences	Primer pair	ITS1 MegaBLAST top hit(s)*	ITS1 BLASTclust group	ITS2 MegaBLAST top hit(s)*	ITS2 BLASTclust group	Species inference
SG1–SG6, SG14–SG16, Sig2–Sig8, Sig13–Sig16 and Léo3	ITS1F/ ITS4	99.5%–100%, 99.5%–100%, 9.31e-94–1.98e-95 to GQ907084 and GQ907097	GQ907083, GQ907084, GQ907097 and GQ907138	99.5%–100%, 100%, 1.05e-108–2.26e-110 to GQ907138	FN555434, FN555435, GQ907083 and GQ907138	Basidiomycota; Sebacinales clade B
SG7–12, Sig9–Sig12, Léo5, Léo7–Léo9 and Léo12	ITS3Seb /TW13	No ITS1 sequence	m.d.	99.1%–100%, 100%, 4.87e-107–2.26e-110 to GQ907138	FN555434, FN555435, GQ907083 and GQ907138	

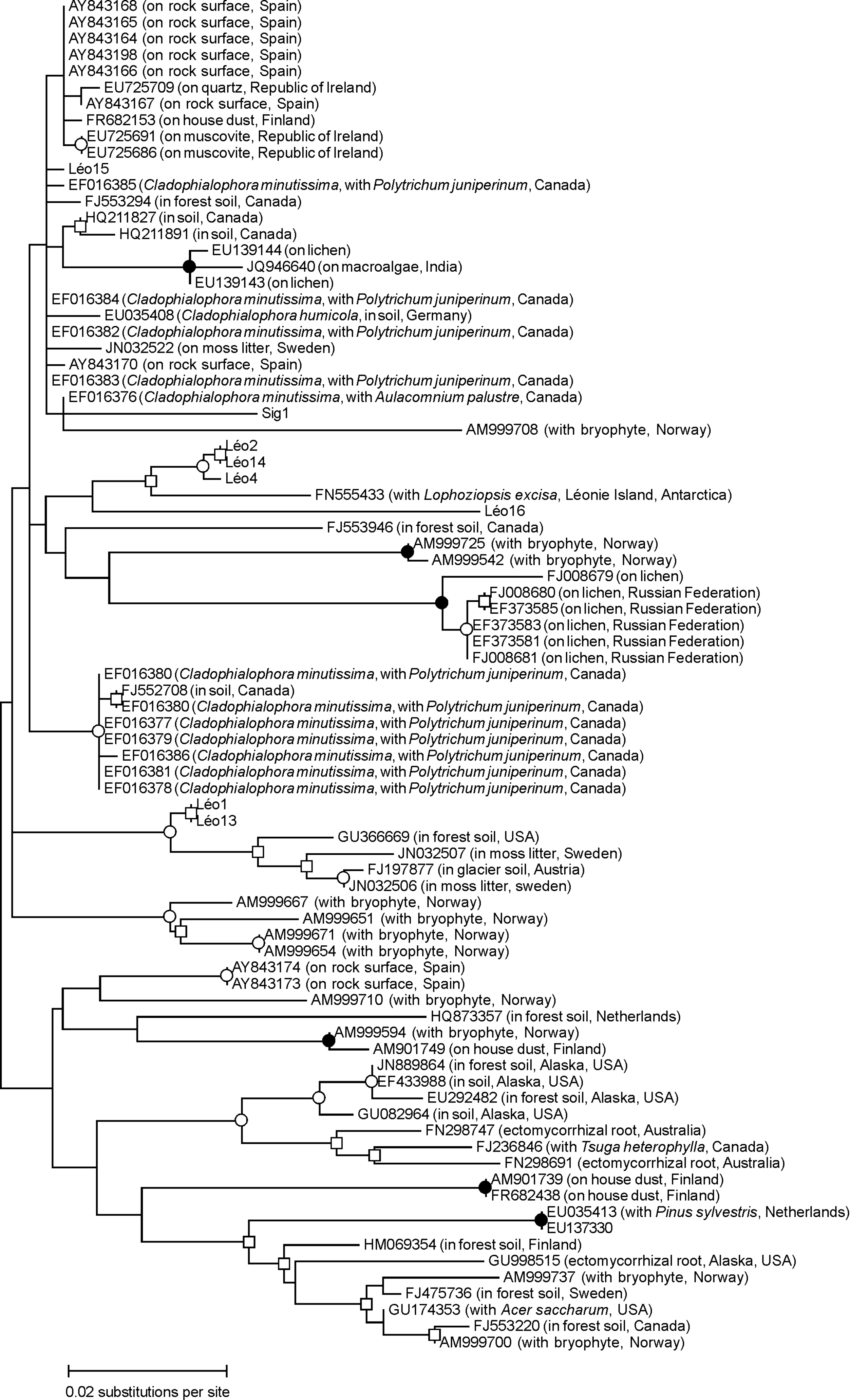
*Data under MegaBLAST hits are ranges or absolute values of pairwise identities, query sequence coverages and E-values, respectively. *Abbreviation*: m.d., missing data

Supplementary Figure legends

Supplementary Fig S1 – mycothalli in rhizoid cells of *Barbilophozia hatcheri* from South Georgia. Images are of hyphae that have (a) penetrated the tip of a rhizoid (arrowed), (b) grown within the shaft of the cell and (c) formed a hyphal coil in its base (arrowed). Scale bars in each panel are 10 µm in length. For clarity, the images are negatives of the originals, captured under UV epifluorescence at × 400 magnification.

Supplementary Fig S2 – midpoint-rooted, maximum likelihood phylogram obtained from the alignment of ITS1-5.8S- ITS2 region sequences of ascomycetes amplified from Antarctic *Barbilophozia hatcheri* with those of fungi having >75% ITS1 and ITS2 coverage and >85% pairwise identity with query sequences. GenBank accession numbers of reference sequences are shown, along with unambiguously named fungal taxa, the plant species host or substrate and the country of origin in parentheses. Symbols at branch nodes indicate support at >50% (□) >80% (○) and at 100% (●) in maximum parsimony and maximum likelihood analyses. Abbreviations: SG, South Georgia; Sig, Signy Island; Léo, Léonie Island.


(a)
(b)
(c)


AY843168 (on rock surface, Spain)
AY843165 (on rock surface, Spain)
AY843164 (on rock surface, Spain)
AY843198 (on rock surface, Spain)
AY843166 (on rock surface, Spain)
EU725709 (on quartz, Republic of Ireland)
AY843167 (on rock surface, Spain)
FR682153 (on house dust, Finland)
EU725691 (on muscovite, Republic of Ireland)
EU725686 (on muscovite, Republic of Ireland)
Léo15
EF016385 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
FJ553294 (in forest soil, Canada)
HQ211827 (in soil, Canada)
HQ211891 (in soil, Canada)
EU139144 (on lichen)
JQ946640 (on macroalgae, India)
EU139143 (on lichen)
EF016384 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EU035408 (*Cladophialophora humicola*, in soil, Germany)
EF016382 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
JN032522 (on moss litter, Sweden)
AY843170 (on rock surface, Spain)
EF016383 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016376 (*Cladophialophora minutissima*, with *Aulacomnium palustre*, Canada)
Sig1
AM999708 (with bryophyte, Norway)
Léo2
Léo14
Léo4
FN555433 (with *Lophoziopsis excisa*, Léonie Island, Antarctica)
Léo16
FJ553946 (in forest soil, Canada)
AM999725 (with bryophyte, Norway)
AM999542 (with bryophyte, Norway)
FJ008679 (on lichen)
FJ008680 (on lichen, Russian Federation)
EF373585 (on lichen, Russian Federation)
EF373583 (on lichen, Russian Federation)
EF373581 (on lichen, Russian Federation)
FJ008681 (on lichen, Russian Federation)
EF016380 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
FJ552708 (in soil, Canada)
EF016380 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016377 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016379 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016386 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016381 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
EF016378 (*Cladophialophora minutissima*, with *Polytrichum juniperinum*, Canada)
Léo1
Léo13
GU366669 (in forest soil, USA)
JN032507 (in moss litter, Sweden)
FJ197877 (in glacier soil, Austria)
JN032506 (in moss litter, sweden)
AM999667 (with bryophyte, Norway)
AM999651 (with bryophyte, Norway)
AM999671 (with bryophyte, Norway)
AM999654 (with bryophyte, Norway)
AY843174 (on rock surface, Spain)
AY843173 (on rock surface, Spain)
AM999710 (with bryophyte, Norway)
HQ873357 (in forest soil, Netherlands)
AM999594 (with bryophyte, Norway)
AM901749 (on house dust, Finland)
JN889864 (in forest soil, Alaska, USA)
EF433988 (in soil, Alaska, USA)
EU292482 (in forest soil, Alaska, USA)
GU082964 (in soil, Alaska, USA)
FN298747 (ectomycorrhizal root, Australia)
FJ236846 (with *Tsuga heterophylla*, Canada)
FN298691 (ectomycorrhizal root, Australia)
AM901739 (on house dust, Finland)
FR682438 (on house dust, Finland)
EU035413 (with *Pinus sylvestris*, Netherlands)
EU137330
HM069354 (in forest soil, Finland)
GU998515 (ectomycorrhizal root, Alaska, USA)
AM999737 (with bryophyte, Norway)
FJ475736 (in forest soil, Sweden)
GU174353 (with *Acer saccharum*, USA)
FJ553220 (in forest soil, Canada)
AM999700 (with bryophyte, Norway)
0.02 substitutions per site