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A cosmopolitan *Serendipita* forms mycothalli with sub-Antarctic leafy liverworts

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ABSTRACT

The occurrence of mycothalli, symbioses between liverworts and fungi, is poorly documented in sub-Antarctica, and biogeographical patterns in *Serendipita*, the main fungal genus forming the symbiosis, remain understudied. Here, 83 specimens of 16 leafy liverwort species were sampled from sub-Antarctic South Georgia and were examined for mycothalli. Microscopy was used to enumerate fungal structures in liverwort tissues, and sequencing of fungal ribosomal DNA was used to determine the taxonomic and biogeographical affinities of the fungi. Stained hyphal coils, a defining feature of the symbiosis, were found to be frequent (>40% of stem length colonised) in *Barbilophozia hatcheri*, *Cephaloziella varians* and *Lophoziopsis excisa*. A single species of *Serendipita*, based on a 3% cut-off for ITS2 region sequence divergence, was a frequent colonist of these liverworts. A further 18 basidiomycete and ascomycete taxa colonised other liverwort species. The presence of the *Serendipita* species was positively associated with the occurrence of stained hyphal coils in stem epidermal cells. Phylogenetic analyses, incorporating worldwide accessions from leafy liverwort-associated *Serendipita*, showed that the same species, which also occurs in Chile, mainland Europe and on Svalbard, is apparently the sole symbiont of sub- and maritime Antarctic leafy liverworts, and indicated much higher species richness of the genus outside Antarctica.

1. Introduction

Leafy liverworts in temperate, tropical and polar regions are frequently colonised by fungi (Read et al., 2000; Pressel et al., 2010; Newsham and Bridge, 2010; Newsham, 2021; Newsham and Goodall-Copestake, 2021; Chen and Nelson, 2022). Bearing structural similarities to mycorrhizas, the associations formed between liverworts and fungi are termed mycothalli, denoting potentially mutualistic symbioses (Boullard, 1988), the functional significance and biogeochemical impacts of which remain unresolved (Patiño et al., 2022). The liverwort thallus is typically anchored to its substrate by filamentous cells termed rhizoids that are often colonised by fungal hyphae, which grow along the shaft of the cell and form hyphal coils, a defining feature of mycothalli, in the base of the cell and in adjacent stem epidermal cells (Boullard, 1988; Pressel et al., 2010). Members of the Cephaloziellaceae and Cephaloziaceae are typically colonised by ascomycetes, usually *Hyaloscypha hepaticicola* (syn. *Rhizoscyphus ericae*; Fehrer et al., 2019) and related fungi (Duckett and Read, 1995; Chambers et al., 1999; Upson et al., 2007; Pressel et al., 2010; Kowal et al., 2018). In contrast, members of the leafy liverwort families Anastrophyllaceae, Lophoziaceae, Scapaniaceae, Arnelliaceae, Jungermanniaceae and Geocalycaceae are usually colonised by heterobasidiomycetes that were formerly placed in Sebacinales clade B (Read et al., 2000; Pressel et al., 2010) but which have now been assigned to the Serendipitaceae (Weiß et al., 2016).

The sub-Antarctic has a rich hepatic flora, with leafy liverworts being locally abundant in its terrestrial habitats (Bednarek-Ochyra et al., 2000). However, mycothalli have only been recorded in two liverwort species in the region, with previous studies on South Georgia and Bird Island finding *Serendipita* in *Barbilophozia hatcheri* (Anastrophyllaceae) and *H. hepaticicola* in *Cephaloziella varians* (Cephaloziellaceae) (Upson et al., 2007; Newsham et al., 2014). In view of the lack of information on the symbiosis in sub-Antarctica, here we report the occurrence of mycothalli in 16 leafy liverwort species, including *B. hatcheri* and *C. varians*, on South Georgia. Besides furthering current knowledge of mycothalli in sub-Antarctica, we aimed to determine if the occurrence of specific fungal taxa correlates with structures formed in liverwort tissues. We anticipated that, as in a previous study in the High Arctic, the

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presence of *Serendipita* would be associated with the formation of hyphal coils (Newsham and Goodall-Copestake, 2021). We also aimed to compare the phylogenetic relationships of *Serendipita* colonising leafy liverworts on South Georgia with those from maritime Antarctica, South America, Europe and the High Arctic. Although *Serendipita* has a global distribution, phylogeographic patterns within the genus and related taxa in the Sebacinales are poorly understood (Weiß et al., 2016). Previous studies indicate a high potential for regional and continental dispersal of these fungi (Selosse et al., 2007; Weiß et al., 2011; Setaro et al., 2012), suggesting the occurrence of globally distributed species in the Serendipitaceae (Weiß et al., 2016). We hence anticipated that *Serendipita* symbionts of sub-Antarctic leafy liverworts would be phylogenetically indistinguishable from those in other geographical regions, and that *Serendipita* species richness in sub-Antarctica would be comparable with that at lower latitudes.

2. Materials and methods

2.1. Sampling and sites

In October–November 2011 and January–February 2016, 83 specimens of 16 leafy liverwort species in the families Adelanthaceae, Anastrophyllaceae, Cephaloziaceae, Cephaloziellaceae, Lophoziaceae, Lophocoleaceae, Plagiochilaceae and Schistochilaceae (Table 1) were collected from 12 sites on the Thatcher Peninsula on South Georgia (Fig. 1). Between three and 12 specimens of each species were collected (Table 1), with 1–5 specimens of each species being gathered from each site (Newsham, 2022). The specimens, which were air dried at room temperature in clean paper packets a few hours after collection, have been deposited in the British Antarctic Survey herbarium (AAS; see https://data.bas.ac.uk/metadata.php?id=GB/NERC/BAS/AEDC/00023).

Table 1
Leafy liverwort taxa sampled from South Georgia.

Family[a]	Species[a]
Adelanthaceae	*Adelanthus integerrimus* (4)
	Syzygiella jacquinotii (12)
	Syzygiella spegazziniana (3)
Anastrophyllaceae	*Barbilophozia hatcheri* (8)
Cephaloziaceae	*Cephalozia badia* (6)
Cephaloziellaceae	*Cephaloziella varians* (4)
Lophoziaceae	*Lophoziopsis excisa* (6)
Lophocoleaceae	*Chiloscyphus koeppensis* (3)
	Clasmatocolea humilis (3)
	Clasmatocolea rigens (4)
	Cryptolophocolea chiloscyphoidea (4)
	Leptoscyphus antarcticus (9)
	Leptoscyphus chilensis (4)
	Pachyglossa spegazziniana (3)
Plagiochilaceae	*Plagiochila molliuscula* (4)
Schistochilaceae	*Pachyschistochila splachnophylla* (6)

Values in parentheses indicate the numbers of specimens of each species examined by microscopy. The locations from which the samples were gathered are shown in Fig. 1 and Supplementary Fig. 1.
[a] Taxonomy follows Söderström et al. (2016).

Images and details of the sampling sites on the Thatcher Peninsula are shown in Supplementary Fig. 1 and Supplementary Table 1, respectively. Since 2002, mean annual air temperatures at Grytviken on the peninsula have ranged between 2.2 °C and 3.4 °C, with maximum and minimum monthly mean temperatures of 6.5 °C (February) and −1.3 °C (July) over this period (see https://legacy.bas.ac.uk/met/READER/data.html). Total annual precipitation on South Georgia is approximately 1500 mm, with frequent rain during summer and up to several metres of snowcover during winter. The snow-free period at sea level typically lasts for 20–25 weeks (Øvstedal and Smith, 2001).

2.2. Microscopy

Fungal structures in leafy liverwort tissues were stained with aniline blue following previously described methods (Newsham and Goodall-Copestake, 2021). Between 10 and 20 stems of each species, and at least 40 stems of *Cephalozia badia*, *Cephaloziella varians* and *Clasmatocolea humilis*, were lightly squashed in 80 % (v/v) lactic acid on glass slides prior to observation under UV epifluorescence at × 400 magnification using an Olympus BX51 microscope equipped with a UPlanApo × 40 objective lens, a 100-Watt mercury short arc lamp and an ultraviolet fluorescence filter cube (U-MWU2, consisting of a BP 330–385 excitation filter, a DM 400 dichromatic mirror and an LP 420 emission filter; Olympus Life Science, Tokyo, Japan). The line intersection method of McGonigle et al. (1990) was used to calculate the percentages of stem length colonised (SLC) by (i) stained hyphal coils in rhizoid bases and stem epidermal cells, (ii) stained septate hyphae in stem epidermal cells and (iii) dark septate (DS) hyphae on stem surfaces. A total of 4442 intersections were examined, with up to 119 intersections being scored in each sample. The percentage of at least 30 rhizoids in each specimen that were colonised by hyphae was also recorded, with a total of 2541 rhizoids being examined. Insufficient rhizoids were observed on the stems of *Chiloscyphus koeppensis* or *Pachyglossa spegazziniana* to derive means for rhizoid colonisation.

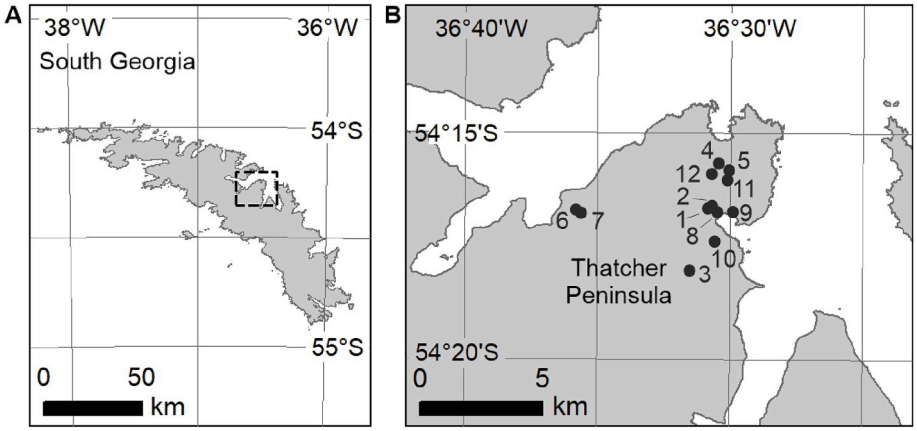

Fig. 1. Maps showing the positions of (**A**) South Georgia and (**B**) the 12 sampling sites on the Thatcher Peninsula. The dashed box in (**A**) shows the location of the peninsula.

2.3. Taxonomic placement of fungal symbionts

Twenty-three specimens were selected for DNA sequencing, consisting of 1–4 samples of each liverwort species other than *C. humilis*, *Leptoscyphus antarcticus* and *P. spegazziniana* (Supplementary Table 2). Colonies of visibly healthy plants were carefully sorted to single species and stems were cleaned of any debris in water under a binocular microscope at ≥75× magnification. Rather than sampling entire stems of *Pachyschistochila splachnophylla*, which measured up to 30 mm in length, a sterile blade was used to excise ventral tissues of the species of 3–5 mm length with attached rhizoids. The stems were surface sterilised in 30 washes of sterile water (10 ml) in sterile tubes (15 ml capacity) on a vortexer set to maximum speed (50 rev. sec^{-1}). Water was drained from stems on sterile plastic mesh (1 mm) between washes, each of which lasted for 2 min. Surface sterilisation using hypochlorite or other sterilants was avoided because the stems of some of the liverwort species studied are only a few cells in thickness. The stems, which remained largely intact during the washing process, were then blotted dry on sterile filter paper and air-dried for 60 min under a sterile hood. The dried stems (10–20 mg) were then rehydrated in 200 μl of extraction solution from an Extract'n'Amp kit (Sigma–Aldrich, Gillingham, UK) and were homogenised for 30 s with a TissueRuptor (Qiagen, Hilden, Germany). DNA was extracted from the tissues following the manufacturer's instructions and fungal internal transcribed spacer (ITS) region ribosomal DNA was subsequently amplified in 20 μl reaction volumes containing a 4 μl aliquot of extraction solution and 0.4 μM final concentrations of the primers ITS1F (5′-CTTGGTCATTTAGAGGAGTAA-3′; Gardes and Bruns, 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990), generating amplicons of *c*. 641 bp length (Manter and Vivanco, 2007). Aliquots of extraction solutions were diluted by up to 20-fold with a 50:50 mixture of extraction and dilution solutions from the Extract'n'Amp kit to achieve efficient polymerase chain reaction (PCR) amplification. Sterile distilled water was used as a negative control. PCR thermocycling was carried out using an initial denaturation of 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and elongation at 72 °C for 2 min, with a final elongation step at 72 °C for 7 min. Gel visualization showed no amplicons in the negative controls.

The PCR products were cleaned using sodium acetate and ethanol precipitation (Sambrook and Russell, 2001) and were cloned using a TOPO® TA Cloning® Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Between 12 and 78 clones were generated from each specimen, with ≥39 clones being generated from three liverwort species that were frequently colonised by hyphal coils. Cloned DNAs were amplified using a commercially available reagent mix (MyTaq DNA polymerase, Meridian Bioscience, Memphis, TN, USA) with the primers M13F-20 (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-AACAGCTATGACCATGAT-3′) and were bidirectionally sequenced using the same primers at a commercial facility. A total of 406 high quality sequences were obtained, with 11–72 sequences being generated per liverwort species. Vector and primer sequences were trimmed, base calling was verified and consensus sequences generated using Geneious Prime 2019.2.3 (Biomatters, Aukland, New Zealand). These consensus sequences (GenBank accession codes OP088691–OP088709) were aligned using MUSCLE v5 (Edgar, 2021) with default settings and RNA gene boundaries were identified through comparison with similar annotated accessions in GenBank (Clark et al., 2016). The ITS2 region was then extracted and used to identify putative species groups at a 3 % ITS cut-off following Blaalid et al. (2013) using the BLASTCLUST-algorithm in the package blast-legacy v2.2.26 (available at https://ftp.ncbi.nlm.nih.gov/blast/executables/) with settings for 97 % identity over 90 % of sequence length. Representative ITS2 sequences from each species group were then subjected to searches using the UNITE database (https://unite.ut.ee/; Abarenkov et al., 2010).

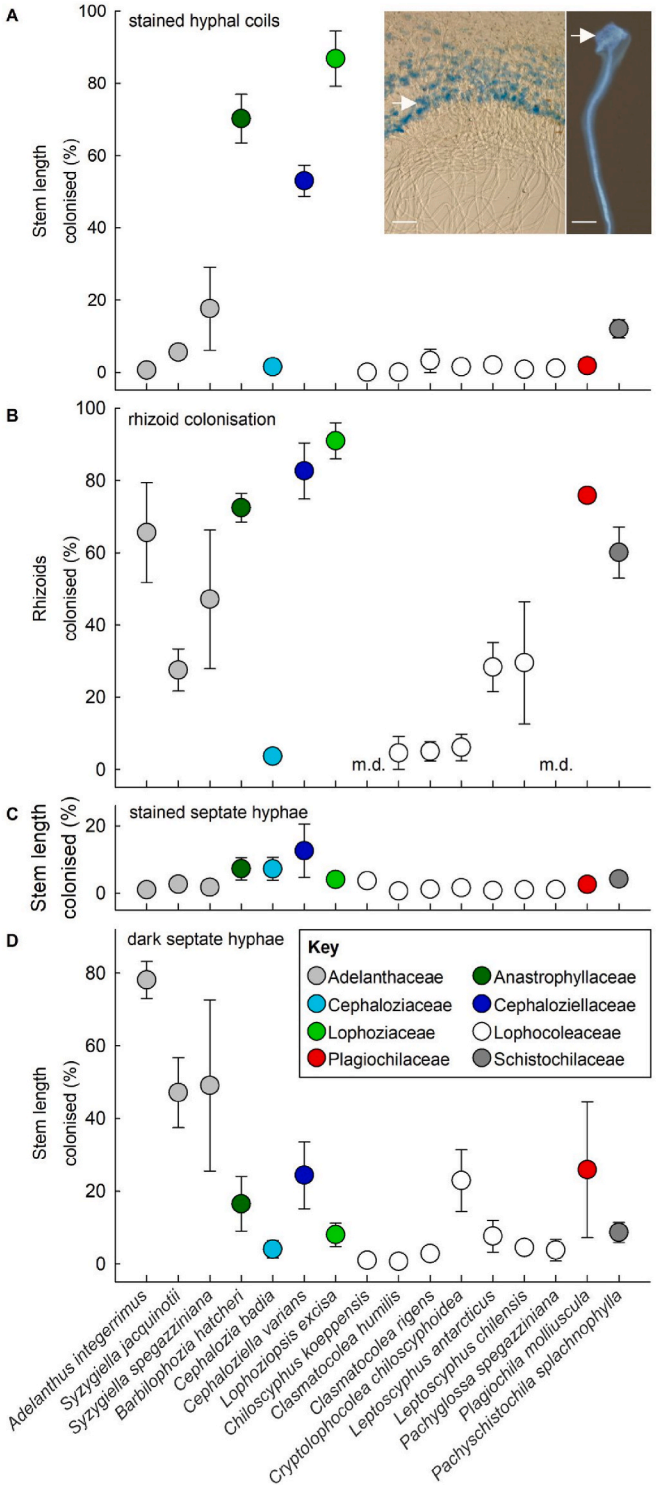

Fig. 2. Lengths of stem and percentages of rhizoids colonised by fungal structures in 16 leafy liverwort species. Different liverwort families are denoted by distinct colours (see key). See Table 1 for details of replication. Error bars are SEM. *Abbreviation*: m.d., missing data. The left hand inset in (**A**) shows a stem of *Barbilophozia hatcheri* viewed under light microscopy. Note the epidermal cells colonised by aniline blue-stained hyphal coils (arrow) and the numerous rhizoid cells on the ventral surface of the stem. The right hand inset in (**A**) shows an excised rhizoid of *B. hatcheri* viewed under epifluorescence microscopy with a stained fluorescing hyphal coil formed in its base (arrow). The scale bars in the left and right hand images are 100 μm and 20 μm in length, respectively. (For interpretation of the references to colour in the Figure, the reader is referred to the Web version of this article.)

2.4. Associations between fungal taxa and structures formed in liverworts

Principal components analysis (PCA) was used for exploratory analyses of the associations between fungal taxa and the structures formed in liverwort tissues. The absence of rhizoids from the *C. badia* and *C. koeppensis* specimens from which fungal DNA was amplified precluded the inclusion of these liverwort species in the PCA. *Penicillium citreonigrum* could also not be included in the PCA owing to its low abundance and presence in only one leafy liverwort species. Following PCA, one-way analysis of variance (ANOVA) was used to test for differences between the frequencies of fungal structures in leafy liverwort specimens from which the DNA of fungal taxa had been amplified or not. Probability values from the ANOVA tests were corrected for multiple comparisons using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). PCA and one-way ANOVA were conducted in MINITAB (version 19.2020.1).

2.5. Phylogeny of Serendipita

A full-amplicon-length (ITS1–5.8S–ITS2) sequence of *Serendipita* from the Thatcher Peninsula (GenBank accession code OQ002266) was used for a megaBLAST search, retaining the top 250 hits, of which 121 were from leafy liverwort-associated members of the genus. These 121 sequences were aligned using MUSCLE with the ITS1–5.8S–ITS2 *Serendipita* sequences generated for the present study (GenBank accession codes OQ002173–OQ002309), of which 137 were unique. Alignment positions for phylogenetic analysis were selected using GBlocks v0.91b (Castresana, 2000) with default settings and allowing up to half gap positions. The resulting 421 bp GBlocks-derived DNA matrix was interrogated for unique sequences, all 193 of which were extracted and used to infer a phylogeny with IQ-TREE v2.2.0 (Minh et al., 2020) incorporating ModelFinder (Kalyaanamoorthy et al., 2017) with a tree search including 10,000 ultrafast bootstrap replicates (Hoang et al., 2018). The geographical and host plant origins of samples were then mapped onto the phylogeny to explore the partitioning of these characteristics.

3. Results

3.1. Microscopy

Stained hyphal coils were observed in the tissues of 12 of the 16 leafy liverwort species. Septate hyphae apparently entered liverwort tissues *via* the tips of rhizoid cells, from where they grew along the shafts of the cells and formed hyphal coils in their bases and in adjacent stem medullary cells (Fig. 2A, insets). Stained coils were frequent (>40 % SLC) in the tissues of *Barbilophozia hatcheri* (Anastrophyllaceae), *Cephaloziella varians* (Cephaloziellaceae) and *Lophoziopsis excisa* (Lophoziaceae) (Fig. 2A). In contrast, coils attained frequencies of 6–18 % SLC in the tissues of *Syzygiella jacquinotii* and *S. spegazziniana* (both Adelanthaceae) and *Pachyschistochila splachnophylla* (Schistochilaceae), and only 1–3% SLC in *Cephalozia badia* (Cephaloziaceae), *Clasmatocolea rigens*, *Cryptolophocolea chiloscyphoidea*, *Leptoscyphus chilensis, Pachyglossa spegazziniana* (all Lophocoleaceae) and *Plagiochila molliuscula* (Plagiochilaceae). They were not recorded in the tissues of *Adelanthus integerrimus* (Adelanthaceae), *Chiloscyphus koeppensis, Clasmatocolea humilis* or *Leptoscyphus antarcticus* (Lophocoleaceae) (Fig. 2A).

Frequent colonisation of rhizoids (>40 % of cells colonised) was recorded in *A. integerrimus, S. spegazziniana*, *B. hatcheri*, *C. varians*, *L. excisa*, *P. molliuscula* and *P. splachnophylla* (Fig. 2B). In contrast, rhizoid colonisation was infrequent (4–30 % of cells colonised) in *S. jacquinotii*, *C. humilis*, *C. rigens*, *C. chiloscyphoidea*, *L. antarcticus* and

Table 2
The taxa of fungi recorded in leafy liverworts sampled from the Thatcher Peninsula. Distinct taxa are classified based on a 3 % cut-off for ITS2 sequence divergence.

Fungal taxon	GenBank accession code	Closest named match (percentage identity, accession code, source and geographical region)	Closest UNITE species hypothesis	Lineage
Basidiomycota				
(1) *Serendipita*	OP088691	99 %, KF636402, *Barbilophozia hatcheri*, Antarctica	SH1577252.08FU	Agaricomycotina; Agaricomycetes; Agaricomycetes *i.s.*; Sebacinales; Serendipitaceae
(2) Cantharellales	OP088692	95 %, KX403898, soil, Arctic	m.d.	Agaricomycotina; Agaricomycetes
(3) Cantharellales	OP088693	95 %, KX403898, soil, Arctic	m.d.	Agaricomycotina; Agaricomycetes
(4) Cantharellales	OP088694	95 %, KX403898, soil, Arctic	m.d.	Agaricomycotina; Agaricomycetes
(5) Hymenochaetales	OP088695	86 %, MH019916, *Nothofagus pumilio*, South America	SH1555449.08FU	Agaricomycotina; Agaricomycetes
(6) Hymenochaetales	OP088696	84 %, MF461619, moss, Australia	SH1555453.08FU	Agaricomycotina; Agaricomycetes
(7) *Exidia* sp.	OP088697	95 %, MK028399, sporocarp, Europe	SH1561164.08FU	Agaricomycotina; Agaricomycetes; Auriculariomycetidae; Auriculariales; Exidiaceae
(8) *Vishniacozyma carnescens*	OP088698	100 %, MN922485, wheat, Europe	SH1528208.08FU	Agaricomycotina; Tremellomycetes; Tremellomycetes i.s.; Tremellales; Bulleribasidiaceae
(9) Piskurozymaceae	OP088699	89 %, MT470199, *Cladonia pleurota*, Asia	SH3591906.08FU	Agaricomycotina; Tremellomycetes; Filobasidiales
Ascomycota				
(10) *Hyaloscypha hepaticicola*	OP088700	100 %, MN603749, soil, High Arctic	SH1523755.08FU	Pezizomycotina; Leotiomycetes; Helotiales; Hyaloscyphaceae
(11) *Gyoerffyella*	OP088701	99 %, MH128176, *Colobanthus quitensis* root, Antarctica	SH1509516.08FU	Pezizomycotina; Leotiomycetes; Helotiales; Discinellaceae
(12) *Gyoerffyella*	OP088702	100 %, UDB0779372, soil, Europe	SH1509534.08FU	Pezizomycotina; Leotiomycetes; Helotiales; Discinellaceae
(13) Helotiales	OP088703	85 %, AY699670, *Rhododendron lochiae* root, Australia	SH1522954.08FU	Pezizomycotina; Leotiomycetes
(14) Helotiales	OP088704	86 %, AY699670, *Rhododendron lochiae* root, Australia	SH1522954.08FU	Pezizomycotina; Leotiomycetes
(15) Helotiales	OP088705	98 %, MW215286, soil, Europe	SH1514525.08FU	Pezizomycotina; Leotiomycetes; Leotiomycetidae
(16) Helotiales	OP088706	89 %, KF297086, soil, High Arctic	SH1647639.08FU	Pezizomycotina; Leotiomycetes
(17) Helotiales	OP088707	87 %, HQ212323, soil, Arctic	SH1543029.08FU	Pezizomycotina; Leotiomycetes
(18) *Penicillium citreonigrum*	OP088708	100 %, MN794455, seawater, Africa	SH1529989.08FU	Pezizomycotina; Eurotiomycetes, Eurotiomycetidae, Eurotiales; Aspergillaceae
(19) Hyaloscyphaceae	OP088709	93 %, LC131018, *Phyllodoce aleutica* root, Asia	SH1522944.08FU	Pezizomycotina; Leotiomycetes; Helotiales

Abbreviations: m.d., missing data; *i.s.*, *incertae sedis*.

L. chilensis (Fig. 2B). Stained septate hyphae were infrequent (≤13 % SLC) in the tissues of all species (Fig. 2C). Similarly, DS hyphae were infrequent on the surfaces of all leafy liverwort species except for *A. integerrimus*, *S. jacquinotii* and *S. spegazziniana* (47–78 % SLC; Fig. 2D). Unstained DS coils were also recorded in the tissues of *S. jacquinotii*, *S. spegazziniana*, *C. humilis*, *L. antarcticus* and *L. chilensis*, but at low frequencies (<3 % SLC, data not shown).

3.2. Taxonomic placement of fungal symbionts

The DNA of Basidiomycota and Ascomycota was amplified from leafy liverworts (Table 2). The basidiomycetes were all members of the Agaricomycotina (Table 2). Based on a 3 % cut-off for ITS2 sequence divergence, a single species of *Serendipita*, which accounted for 39 % of the sequences generated in the study, dominated the basidiomycetes. It was the sole fungus recorded in *B. hatcheri* and *L. excisa*, but was also present with other fungi in the tissues of *A. integerrimus*, *C. badia*, *C. varians, C. chiloscyphoidea* and *L. chilensis* (Fig. 3). The best match to the ITS2 region of the fungus was at 99 % similarity to a mycobiont of *B. hatcheri* on Signy Island in maritime Antarctica (Table 2). The next best 29 matches, each also at 99 % similarity, were to symbionts of *B. hatcheri* on King George Island and Léonie Island in maritime Antarctica and on South Georgia, and to associates of the moss *Chorisodontium aciphyllum* on King George Island. Eight other members of the basidiomycota were recorded in leafy liverwort tissues (Table 2). Three members of the Cantharellales matching at 95 % similarity to fungi in Arctic soil were recorded in *A. integerrimus*, *C. badia*, *C. rigens*, *L. chilensis* and *P. molliuscula* (Table 2, Fig. 3). Two basidiomycetes with low (84–86 %) similarities to members of the Hymenochaetales inhabiting South American *Nothofagus* seedlings and Australasian mosses were also

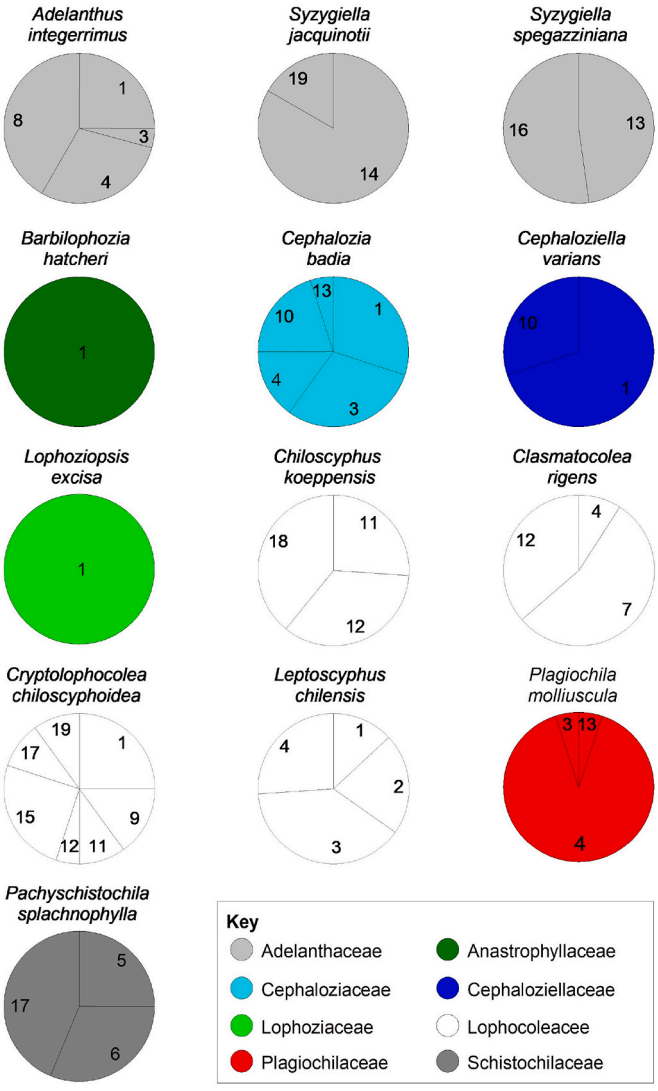

Fig. 3. Pie charts showing the taxa of fungi recorded in leafy liverworts. The numbers in each segment correspond to the fungal taxa shown in the first column of Table 2. Different liverwort families are denoted by distinct colours (see key). (For interpretation of the references to colour in the Figure, the reader is referred to the Web version of this article.)

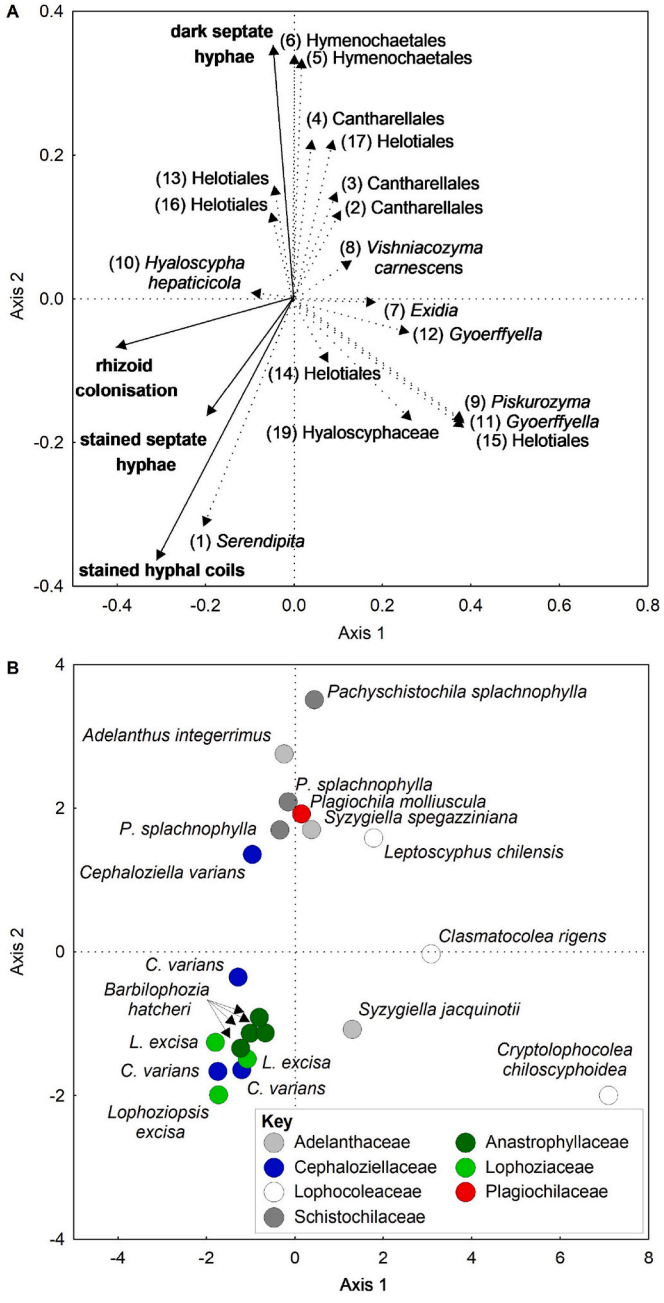

Fig. 4. Principal component analysis (**A**) loading and (**B**) score plots for associations between fungal structures and the identities of fungi colonising leafy liverworts. Solid and dotted arrows in (**A**) denote vectors for fungal structures (in bold) and fungal taxa, respectively. See Table 2 for details of the fungal taxa. Different colours in (**B**) represent distinct liverwort families (see key). Components 1 and 2 explained 18 % and 14 % of variance, respectively. (For interpretation of the references to colour in the Figure, the reader is referred to the Web version of this article.)

recorded in *P. splachnophylla* (Table 2, Fig. 3). A species of *Exidia*, which matched at 95 % identity to a sporocarp of the genus from Switzerland, was also found in *C. rigens*. Two basidiomycetous yeasts in the Tremellomycetes were also recorded. *Vishniacozyma carnescens*, matching at 100 % to an endophyte of *Triticum durum* from Italy, was found in *A. integerrimus*, and a member of the Piskurozymaceae, with low (89 %) similarity to an Asian lichen symbiont, was recorded in *C. chiloscyphoidea* (Table 2, Fig. 3).

Ten species of ascomycete were found in the leafy liverworts. All were members of the Pezizomycotina, and all but one were assigned to the Helotiales (Table 2). *Hyaloscypha hepaticicola*, which matched at 100 % similarity to a soil fungus on Svalbard, was recorded in *C. badia* and *C. varians* (Table 2, Fig. 3). Two *Gyoerffyella* species, matching at 99–100 % to fungi inhabiting Swedish and Antarctic soil, were found in *C. koeppensis*, *C. rigens* and *C. chiloscyphoidea* (Table 2, Fig. 3). Five members of the Helotiales that could not be placed with any certainty below order level were also recorded in *S. jacquinotii*, *S. spegazziniana*, *C. badia*, *C. chiloscyphoidea*, *P. molliuscula* and *P. splachnophylla* (Table 2, Fig. 3). These fungi had low (85–98 %) similarities to inhabitants of Australasian *Rhododendron* roots and Arctic and European soils (Table 2). *Penicillium citreonigrum*, matching at 100 % similarity to a fungus in Mediterranean seawater, was also found in *C. koeppensis*, and a member of the Hyaloscyphaceae, matching at 93 % to an Asian *Phyllodoce aleutica* root endophyte, was recorded in *S. jacquinotii* and *C. chiloscyphoidea* (Table 2, Fig. 3).

3.3. Associations between fungal taxa and structures formed in leafy liverworts

The PCA of associations between the abundances of fungal taxa and the structures formed in liverwort tissues indicated a positive association between the occurrence of the *Serendipita* species and the formation of stained hyphal coils (Fig. 4A), notably in *B. hatcheri* and *L. excisa*, but also in *C. varians* (Fig. 4B). ANOVA showed that stained hyphal coils

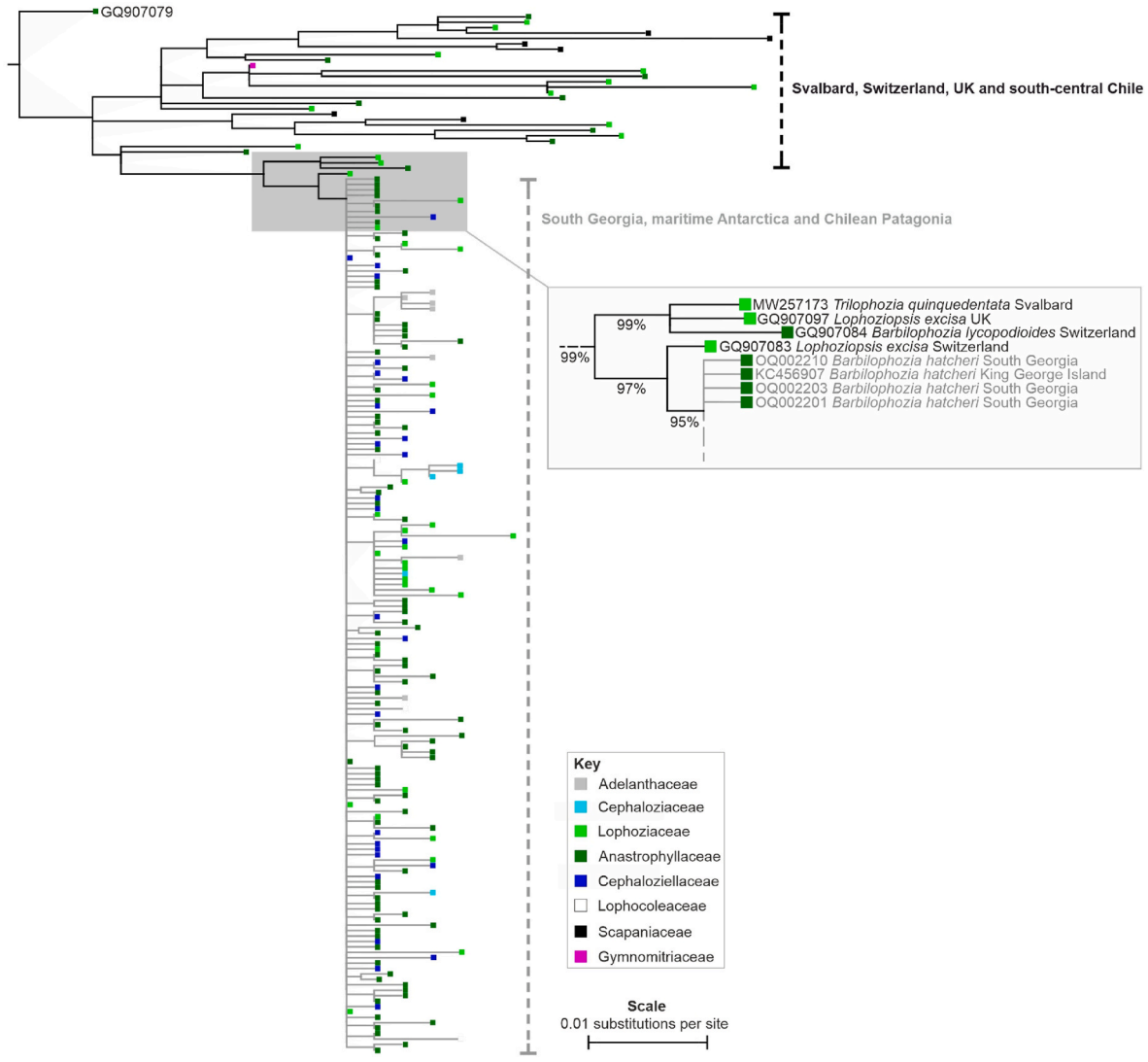

Fig. 5. Maximum likelihood phylogeny of *Serendipita* symbionts of leafy liverworts. Accessions in the upper clade, marked by the vertical black dashed line, are from Svalbard, Europe and south-central Chile, and those in the lower clade, marked by the vertical grey dashed line, are from South Georgia, three maritime Antarctic islands and Chilean Patagonia. Near-zero internal branches (<0.0023 expected substitutions per site) have been collapsed. The phylogeny was rooted with a *Serendipita* sequence from Swiss *Barbilophozia barbata* (GenBank accession code GQ907079). The coloured boxes at branch ends denote the family to which each leafy liverwort species belongs (see key). The grey box shows ultrafast bootstrap support (%) for basal branches within the main clade that contain the accessions generated for this study and associated BLAST hits. See Supplementary Fig. 2 for details of the accession codes and liverwort species for each sequence, which, for clarity, have not been included here for the majority of sequences. A TNe + R2 substitution model was used, which had a log-likelihood of −3294.8242 (s.e. 159.7512) and equal state frequencies and two FreeRate categories (site proportion and rates: 0.857, 0.5363 and 0.143, 3.778). (For interpretation of the references to colour in the Figure, the reader is referred to the Web version of this article.)

were six times more frequent in liverwort specimens from which the DNA of *Serendipita* had been amplified than in those from which the fungus was apparently absent (54.3 % *v*. 8.8 % SLC, respectively, $F_{1,21} = 10.60$, $P = 0.008$). Although the PCA also suggested positive associations between the occurrence of the *Serendipita* species and rhizoid colonisation and the formation of stained septate hyphae (Fig. 4A), ANOVA indicated that neither of these fungal structures differed in frequency between liverwort specimens colonised by *Serendipita* and those apparently lacking the fungus (both $F_{1,21} \leq 1.64$, $P \geq 0.26$).

The PCA indicated that the occurrence of the five basidiomycetes in the Cantharellales and Hymenochaetales was negatively associated with the formation of stained hyphal coils (Fig. 4A). ANOVA accordingly showed that hyphal coils were an order of magnitude lower in frequency in the tissues of liverwort specimens from which the DNA of these five basidiomycetes had been amplified, compared with those from which their DNA was not amplified (5.7 % *v*. 59.6 %, respectively, $F_{1,21} = 20.53$, $P = 0.001$). Similarly, the frequency of rhizoid colonisation was approximately halved in liverwort specimens from which the DNA of these five basidiomycetes had been amplified, compared with those apparently lacking these fungi (44.4 % v. 81.7 %, respectively, $F_{1,19} = 12.63$, $P = 0.006$). The PCA also suggested that, along with five members of the Cantharellales and Hymenochaetales, the frequencies of three members of the Helotiales were positively associated with the presence of DS hyphae (Fig. 4A), notably in *A. integerrimus*, *S. spegazziniana*, *C. varians*, *L. chilensis*, *P. molliuscula* and *P. splachnophylla* (Fig. 4B). However, ANOVA showed no difference in the frequency of DS hyphae in the tissues of liverwort specimens from which the DNA of these eight taxa had been amplified, compared with those apparently not colonised by these fungi ($F_{1,21} = 3.41$, $P = 0.119$).

3.4. Phylogeny of Serendipita

Phylogenetic analyses, based on full-length ITS1–5.8S–ITS2 sequences and incorporating leafy liverwort-associated *Serendipita* accessions from Antarctica, South America, Europe and the High Arctic, indicated that the *Serendipita* species on the Thatcher Peninsula formed a clade with members of the genus associated with *B. hatcheri* and *L. excisa* sampled previously from three maritime Antarctic islands and South Georgia (Supplementary Fig. 2, Fig. 5). A single *Serendipita* accession, from *Lophozia crispata* sampled from the Brunswick Peninsula in southern Chile (GQ907138), also fell within this clade, which, after collapsing near-zero internal branches (<0.0023 expected substitutions per site), consisted of a large polytomy of multiple, largely poorly supported sub-groups and orphan lineages (Supplementary Fig. 2, Fig. 5). There was 95 % bootstrap support for this clade of southern hemisphere accessions, which was sister to a single *Serendipita* accession from Swiss *Lophoziopsis excisa* (GQ907083) and a clade of *Serendipita* from High Arctic *Trilophozia quinquedentata* (MW257173), *L. excisa* from the United Kingdom (GQ907097) and Swiss *Barbilophozia lycopodioides* (GQ907084) (Fig. 5). BLASTCLUST analysis of the ITS2 region extracted from these sequences revealed divergence levels of <3 %, consistent with a single species under the 3 % cut-off criterion. This putative single-species group was separated from the remainder of the phylogeny with 99 % support (Fig. 5). With the exception of a single sequence of *Serendipita* from *Lophozia crispata* sampled from Chiloé Province in south-central Chile (GQ907137), the remainder of the phylogeny consisted of fungal accessions obtained from northern hemisphere members of the Anastrophyllaceae, Lophoziaceae, Scapaniaceae and Gymnomitriaceae (Supplementary Fig. 2, Fig. 5). It formed the backbone of the phylogeny within which the southern hemisphere *Serendipita* were nested (Supplementary Fig. 2, Fig. 5) and comprised multiple accessions with >3 % differences over ITS2, indicative of an additional 27 distinct species lineages under the 3 % criterion (Fig. 5). The phylogeny showed no clear grouping of *Serendipita* lineages within specific liverwort families (Fig. 5) or species (Supplementary Fig. 2).

4. Discussion

Previous studies that have isolated fungi, or PCR-amplified fungal DNA, from leafy liverworts sampled from South Georgia and Bird Island have shown the presence of *Serendipita* and *Hyaloscypha hepaticicola* in the tissues of *B. hatcheri* and *C. varians* (Upson et al., 2007; Newsham et al., 2014). Here, we used the latter approach to further confirm the presence of these, and closely related, fungi in these two leafy liverwort species, and extended current knowledge of mycothalli to a further 14 hepatics on South Georgia. The patterns of colonisation observed in mycothalli on the Thatcher Peninsula were identical to those previously reported for leafy liverworts in maritime Antarctica, South America, Asia, Europe and the High Arctic, with septate hyphae apparently entering rhizoids by their tips, growing along the shafts of the cells and forming hyphal coils in their bases and in adjacent stem epidermal cells (Kottke et al., 2003; Upson et al., 2007; Newsham and Bridge, 2010; Pressel et al., 2010; Newsham et al., 2014; Newsham and Goodall-Copestake, 2021). However, by comparison with a similar study on the Brøgger Peninsula on Svalbard in the High Arctic, where the hepatic flora is dominated by the Anastrophyllaceae and Lophoziaceae, and where mycothalli are consequently abundant (Newsham and Goodall-Copestake, 2021), the symbiosis was relatively infrequent on the Thatcher Peninsula. This is attributable to the abundance on South Georgia of members of the Lophocoleaceae (Bednarek-Ochyra et al., 2000), the seven members of which, in agreement with previous observations (Duckett and Read, 1995), were either not colonised, or were only sparsely colonised, by stained hyphal coils in the present study.

Although the amount of divergence in the ribosomal DNA ITS region used to delineate *Serendipita* species is presently undefined (Cao et al., 2021), the analyses here, using the frequently-applied 3 % cut-off for fungal sequence divergence (Blaalid et al., 2013), indicated that a single species of the genus is apparently present on the Thatcher Peninsula on South Georgia. In support of the view that the Serendipitaceae consists of globally distributed species (Weiß et al., 2016), the phylogenetic analysis here showed that the *Serendipita* species on the peninsula is conspecific with symbionts of hepatics in other geographical regions. It indicated that the same species is associated with *B. hatcheri* and *L. excisa* on three maritime Antarctic islands, with *Lophozia crispata* on the Brunswick Peninsula in Chilean Patagonia, and with *L. excisa*, *B. lycopodioides* and *T. quinquedentata* in the UK and Switzerland and on Svalbard in the High Arctic. The analysis also showed strikingly higher genetic diversity of *Serendipita* outside sub- and maritime Antarctica, with 27 distinct *Serendipita* species lineages being found in association with leafy liverworts in other geographical regions. Of these 27 species, one third alone are found on the Brøgger Peninsula on Svalbard, where nine distinct *Serendipita* species, again separated at the 3 % threshold, occur in 13 leafy liverworts, including *B. hatcheri* and *L. excisa* (Newsham and Goodall-Copestake, 2021).

That more *Serendipita* species symbiotic with leafy liverworts do not have global distributions incorporating Antarctic representatives may be owing to the biogeographical history of the genus. Our study showed that the *Serendipita* found on the Thatcher Peninsula is nested predominantly within accessions from Europe, suggesting an origin of these fungi in the northern hemisphere. Similarly, ectomycorrhiza-forming members of the Sebacinales, in which the Serendipitaceae is placed (Weiß et al., 2016), are inferred to have an origin in North American coniferous forests (Tedersoo et al., 2014). In further accordance with the observations here, of the 11 biogeographic regions studied by Tedersoo et al. (2014), the lowest phylogenetic diversity of ectomycorrhizal Sebacinales is found in southern South America, the closest of the regions to Antarctica. In addition, the polytomy backbone inferred for the multiple Antarctic lineages of liverwort-associated *Serendipita*, as well as their more derived placement, suggest a more recent evolutionary origin. Molecular clock-based age estimates indicate that geographically mixed clades of Sebacinales are similarly relatively young (Garnica et al., 2016) with recent dispersal of *Sebacina* in Holarctic regions

(Tedersoo et al., 2014). Accordingly, our finding of a single, apparently globally distributed *Serendipita* species containing all of the Antarctic representatives of the genus follows a similar pattern, and suggests that it is the first successful extant colonisation of Antarctica by *Serendipita*. Although other fungi are known to have bipolar distributions (e.g., Cox et al., 2016), we are unaware of similar examples in the literature of a cosmopolitan fungal taxon exhibiting such low diversity in Antarctica.

It is plausible that the low diversity of *Serendipita* in Antarctica, and the clustering of all Antarctic representatives of the genus within a single recent offshoot with an apparent northern hemisphere centre of origin, is a consequence of the limited aerial dispersal of the genus, compounded by the geographical isolation and glaciation of the continent. *Serendipita* species, in which sexual structures have been very rarely observed, do not form basidiomata, but instead produce vermiform basidiospores in clusters of gelatinous basidia (Oberwinkler et al., 2014; Cao et al., 2021). The basidiospores are large, with those produced by *S. vermifera*, the type species, measuring 30–60 μm in length (Oberwinkler et al., 2014). They are hence most probably not lofted into the atmosphere and dispersed on air currents to the same extent as the tiny dry conidia of some other fungal taxa, such as *Penicillium*, *Paecilomyces* and *Aspergillus*, cosmopolitan genera found in Antarctica with conidia rarely exceeding 6 μm × 4 μm in size (Cox et al., 2019). Alone, inefficient aerial dispersal would most probably have only slowed the colonisation of sub- and maritime Antarctica by *Serendipita*. However, Antarctica's geographical isolation, coupled with its extensive current and past glaciation (Barnes et al., 2016; Graham et al., 2017), would have presented significant barriers to the progression of the genus towards the most southerly edge of its distribution.

In the absence of pure cultures of leafy liverwort-associated *Serendipita* with which to perform resynthesis experiments, we used ordination to determine associations between the frequency of the basidiomycete and the fungal structures formed in hepatic tissues. Despite amplifying the DNA of *Serendipita* from *Adelanthus integerrimus* and *Leptoscyphus chilensis*, species in which coils were not observed, we found positive associations between the presence of the fungus and the occurrence of stained hyphal coils in liverwort tissues, with coils being six times more frequent in specimens from which the DNA of *Serendipita* had been amplified, compared with specimens from which the fungus was apparently absent. Electron microscopy has similarly shown heterobasidiomycetes with dolipore septa and imperforate parenthesomes – which are most likely members of *Serendipita* – to form intracellular coils in members of the Anastrophyllaceae and Lophoziaceae (Read et al., 2000; Kottke et al., 2003; Pressel et al., 2010). Given these observations and those of Newsham and Goodall-Copestake (2021), it is likely that *Serendipita* does indeed form the stained hyphal coils that are frequently observed in the tissues of leafy liverwort species in these families and the Scapaniaceae, Arnelliaceae, Jungermanniaceae and Geocalycaceae (Read et al., 2000; Pressel et al., 2010). As shown by the analyses here, it is much less likely that other basidiomycetes, such as members of the Cantharellales and Hymenochaetales, form hyphal coils in liverwort tissues.

It is evident from the observations reported here that *Serendipita* may have a wider host range than has previously been thought. The heterobasidiomycete was present on the Thatcher Peninsula not only in the Anastrophyllaceae and Lophoziaceae, but also in the Adelanthaceae, Lophocoleaceae, Cephaloziaceae and Cephaloziellaceae, families that are not normally regarded as hosts of the basidiomycete (Pressel et al., 2010). Members of the latter two families are usually regarded as being symbiotic with *H. hepaticicola* and other ascomycetes (Duckett and Read, 1995; Chambers et al., 1999; Upson et al., 2007; Pressel et al., 2010; Newsham and Goodall-Copestake, 2021), but here we found all five specimens of *C. badia* (Cephaloziaceae) and *C. varians* (Cephaloziellaceae) that were examined to be colonised by *Serendipita*. As previously observed for members of the Anastrophyllaceae and Lophoziaceae on Svalbard (Newsham and Goodall-Copestake, 2021), the DNA of both *Serendipita* and ascomycetes was amplified from the same specimens of these two hepatic species. As in dual-mycorrhizal plant species, in which arbuscular mycorrhizal and ectomycorrhizal fungi often co-occur in the same root apices (Teste et al., 2020), it is hence possible that phylogenetically distant fungal taxa may colonise individual stems of leafy liverworts.

Ascomycetes, and particularly members of the Helotiales, were frequently recorded in leafy liverwort tissues sampled from the Thatcher Peninsula. Previous studies have suggested that members of the Ascomycota form DS hyphae on leafy liverwort stems (Upson et al., 2007; Newsham and Goodall-Copestake, 2021), but the analyses here did not indicate a clear association between the occurrence of ascomycetes and the formation of these hyphae. Instead, although the PCA suggested a positive association between the frequency of DS hyphae on the stems of liverworts colonised by three members of the Helotiales and five basidiomycetes in the Cantharellales and Hymenochaetales, ANOVA indicated no difference in DS hyphal frequencies between the liverworts colonised by these fungi and those that were not. Further studies, utilising secondary DNA barcodes in addition to the ITS for enhanced taxonomic precision (Lücking et al., 2020), are hence needed to identify which fungi form these hyphae on the stems of liverworts. By PCR-amplifying fungal DNA from leafy liverworts, we showed the ascomycete *H. hepaticicola* to be present in the tissues of both *C. badia* and *C. varians*, confirming earlier studies that have isolated closely related fungi from the latter species in sub-, maritime and continental Antarctica (Chambers et al., 1999; Upson et al., 2007). Given that a previous study has shown positive effects of *H. hepaticicola* on the phosphorus uptake of *Cephalozia bicuspidata* in temperate regions (Kowal et al., 2018), it is plausible that this fungus might similarly benefit the nutrient acquisition of sub-Antarctic leafy liverworts.

5. Conclusions

Our study indicated that a single *Serendipita* taxon, equating to a single species at the 3 % ITS cut-off level, which apparently forms stained hyphal coils in the bases of rhizoid cells and in adjacent stem epidermal cells of leafy liverworts, frequently forms mycothalli with *B. hatcheri*, *L. excisa* and *C. varians* on the Thatcher Peninsula on sub-Antarctic South Georgia. A global phylogeny of leafy liverwort-associated *Serendipita* showed the same species to be frequent in maritime Antarctica and to be present in the UK, Switzerland, Chile and on Svalbard, and indicated that the genus *Serendipita* exhibits much lower species richness in sub- and maritime Antarctica than in other geographical regions.

Authors' contributions

KKN conceived the study, and, with GWF, conducted fieldwork. KKN, GWF and CJS carried out labwork and WPG-C conducted sequence editing and phylogenetic analyses. KKN and WPG-C wrote the article. All authors approved the submitted version.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2023.11.006.

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