Conservative ecological and evolutionary patterns in liverwort–fungal symbioses

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Liverworts, the most ancient group of land plants, form a range of intimate associations with fungi that may be analogous to the mycorrhizas of vascular plants. Most thalloid liverworts contain arbuscular mycorrhizal glomeromycete fungi similar to most vascular plants. In contrast, a range of leafy liverwort genera and one simple thalloid liverwort family (the Aneuraceae) have switched to basidiomycete fungi. These liverwort switches away from glomeromycete fungi may be expected to parallel switches undergone by vascular plants that target diverse lineages of basidiomycete fungi to form ectomycorrhizas. To test this hypothesis, we used a cultivation-independent approach to examine the basidiomycete fungi associated with liverworts in varied worldwide locations by generating fungal DNA sequence data from over 200 field collections of over 30 species. Here we show that eight leafy liverwort genera predominantly and consistently associate with members of the *Sebacina vermifera* species complex and that Aneuraceae thalloid liverworts associate nearly exclusively with *Tulasnella* species. Furthermore, within sites where multiple liverwort species co-occur, they almost never share the same fungi. Our analyses reveal a strikingly conservative ecological and evolutionary pattern of liverwort symbioses with basidiomycete fungi that is unlike that of vascular plant mycorrhizas.

Keywords: ecology; evolution; fungi; mycorrhizas; plants; symbiosis

1. INTRODUCTION

Within the bryophytes (liverworts, mosses and hornworts), liverworts (Marchantiophyta) are now widely accepted as the most ancient clade of land plants (Forrest *et al.* 2006; Crandall-Stotler *et al.* 2008). Liverworts comprise a diverse and ecologically important group of approximately 8000 species that can form abundant biomass on moist soil and rocks, or as epiphytes. These plants share a dynamic history of intimate symbioses with three fungal phyla (Glomero-, Asco- and Basidiomycota), including fungi that form mutualistic arbuscular, ecto- and ericoid mycorrhizas with other plants (Goffinet & Shaw 2008; Smith & Read 2008). In contrast to liverworts, fungal symbioses are unknown in mosses, and in hornworts, the sister clade to vascular plants, glomeromycetes are a secondary acquisition restricted to a few genera (Renzaglia *et al.* 2008).

Among liverworts, basidiomycete and ascomycete fungi are secondary symbiotic acquisitions of recent origin following the loss of ancestral glomeromycetes. Complex thalloid liverworts (Marchantiopsida), the basal liverwort lineage and most simple thalloid liverworts (Metzgeriopsida) associate with glomeromycete fungi in a way similar to most vascular plants (Ligrone *et al.* 2007). The topology of liverwort phylogenies strongly supports symbioses with glomeromycete fungi as a basal trait of liverworts that long predates arbuscular mycorrhizas in other plants (Davis 2004; Kottke & Nebel 2005;

He-Nygrén *et al.* 2006). Underlining their extreme antiquity are remarkable structural similarities between the fungal associations in *Haplomitrium* and *Treubia* (the two genera at the very base of the extant land plant tree) and those in the Devonian fossil *Nothia* (Krings *et al.* 2007). All of these plants harbour inter- and extracellular glomeromycetes (Carafa *et al.* 2003; Duckett *et al.* 2006b). However, one derived family in the Metzgeriopsida (the Aneuraceae) and some genera of leafy liverworts (Jungermanniopsida) harbour intra-cellular basidiomycete fungi (Read *et al.* 2000; Kottke *et al.* 2003; Duckett *et al.* 2006a), while ascomycete fungi are prevalent in rhizoids of several other genera of leafy liverworts (Read *et al.* 2000). Overall, this scenario closely resembles the evolution of mycorrhizas in higher plants, where ancient ancestral arbuscular mycorrhizas with glomeromycete fungi gave way to ectomycorrhizal symbioses with many basidiomycete and some ascomycete fungi. Therefore, it appears likely that some extant higher plant mycorrhizas have arisen from ancestral liverwort–fungal symbioses and *vice versa*.

Several recent findings may be relevant to these potentially intertwined mycorrhizal histories. So far, we know that: (i) an arbuscular mycorrhizal glomeromycete fungus of the vascular plant *Plantago* can colonize the simple thalloid liverwort *Pellia* (Read *et al.* 2000); (ii) the arbuscular mycorrhizal fungus *Glomus mosseae* can colonize the complex thalloid liverwort *Conocephalum* (Ligrone *et al.* 2007); (iii) *Glomus* colonization produces mycorrhiza-like effects in another complex thalloid genus, *Lunularia* (Fonseca & Berbara 2008); (iv) the non-photosynthetic liverwort *Cryptothallus mirabilis* (now *Aneura mirabilis*) acquires carbon via a shared *Tulasnella*

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basidiomycete that forms ectomycorrhizas with *Betula* trees (Bidartondo *et al.* 2003); (v) basidiomycete tulasnelloid fungi are not shared between the liverworts *Aneura* and *Riccardia* and some epiphytic orchids (Kottke *et al.*, 2008); (vi) basidiomycete sebacinoid fungi associated with three specimens of leafy liverworts (Kottke *et al.* 2003); and (vii) basidiomycete–liverwort symbioses may be more specific than ascomycete–liverwort symbioses (Chambers *et al.* 1999; Duckett *et al.* 2006a). These suggestive but fragmentary data are inadequate for accurately inferring and comparing the seminal early events in the evolution of land plant–fungal symbioses including mycorrhizas (Nebel *et al.* 2004; Kottke & Nebel 2005).

Globally, it is estimated that over 160 genera of diverse basidiomycete fungi form ectomycorrhizas with some 7000 species of seed plants (Smith & Read 2008; Rinaldi *et al.* 2009). The most distinct ectomycorrhizal symbiotic patterns are that (i) multiple distantly related basidiomycetes simultaneously colonize individual plants and (ii) there has been repeated independent evolution of ectomycorrhization both within plants and within basidiomycetes (Molina *et al.* 1992; Hibbett *et al.* 2000; Bruns & Shefferson 2004; Smith & Read 2008). Here we use molecular ecology techniques guided by electron microscopy information to identify fungi in by far the widest sample of basidiomycete-associated liverworts studied to date—embracing over 80 per cent of species likely to harbour these fungal symbionts in the European flora—to test whether their patterns of ecological and evolutionary association agree with those followed by higher plants engaged in ectomycorrhizal symbioses with basidiomycete fungi.

2. MATERIAL AND METHODS

At each site, we collected colonies (less than 5 cm diameter) of each species of liverwort potentially associated with basidiomycete fungi. In addition to liverworts for which basidiomycetes had been identified in cytological studies, we also included others where either the same was likely from their systematic position or where fungi of unknown affinities, but that extend into stem cells beyond the rhizoids (Paton 1999), had been reported. As controls, a range of liverworts with rhizoidal ascomycete fungi and some liverworts known to lack fungal symbionts were included in our analyses. Furthermore, we analysed five to ten ectomycorrhizas from *Betula* and *Salix* closely associated with liverwort samples at Beinn Dearg and Loch Affric (Scotland), and Susten Pass and Nufenen (Switzerland). Each field collection was subsampled within a week after field collection by removing five to ten thalli or stems. These were thoroughly cleaned of adhering soil and plant debris and they were rinsed several times in distilled water. Specimens were prepared for scanning electron microscopy via critical point drying (Duckett & Ligrone 2008a) and a representative selection (*Barbilophozia floerkii*, *Barbilophozia barbata*, *Lophozia excisa*, *Lophozia incisa*, *Lophozia opacifolia*, *Lophozia ventricosa*, *Tritomaria exsectiformis*, *Nardia scalaris*) were examined for the presence of dolipore septa by transmission electron microscopy (Duckett & Ligrone 2008a). Nomenclature follows Hill *et al.* (2008) for British taxa, otherwise authorities are cited. We retain *Cryptothallus* Malm. following common usage. Vouchers are either in the herbarium of J.G.D. or that of the Royal Botanic Gardens, Edinburgh. For molecular analyses, a total of 8–12 healthy colonized apical 2–3 mm lengths were placed in

300 µl of lysis buffer solution (2% acetyl trimethylammonium bromide, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris, 1% polyvinylpyrrolidone 40 000 MW) and stored at –20°C or –80°C. From each of these collections, two lengths were sampled arbitrarily and, for larger specimens, the meristems and/or leaves were manually removed under a dissecting microscope. Each of these two liverwort samples, or a single ectomycorrhiza, was then used for a separate genomic DNA extraction using methods described elsewhere (Gardes & Bruns 1993), modified to include a silica emulsion and purification step using GeneClean (QBioGene) instead of precipitation. We amplified (PicoMaxx, Stratagene or JumpStart, Sigma), cloned (if we could not sequence directly; TOPO TA, Invitrogen) and sequenced bidirectionally (BigDye v. 3.1 on ABI3730 Genetic Analyzer, Applied Biosystems) the following: (i) fungal nuclear ribosomal internal transcribed spacer region (ITS1F/ITS4; White *et al.* 1990; Gardes & Bruns 1993), (ii) tulasnelloid nuclear ribosomal transcribed spacer region (ITS1/ITS4Tul; White *et al.* 1990; Taylor 1997), (iii) fungal mitochondrial ribosomal large subunit region (Mlin3/cML7.5; White *et al.* 1990), and/or (iv) fungal nuclear ribosomal large subunit 5' region (ITS1F/TW14). The TW14 oligonucleotide sequence is gctatcctgagggaacttc. Genomic region 1 was targeted at every sample, regions 2 and 3 at every Aneuraceae sample and region 4 at a broad sample of *Sebacina*-harbouring liverwort samples that maximized diversity according to results from analysis of genomic region 1. The tulasnelloid nuclear ribosomal internal transcribed spacer region and the sebacinoid nuclear ribosomal large subunit 5' region were visually aligned together with closely similar DNA sequences retrieved from GenBank and used to generate distance trees using neighbour-joining with branch support assessed by bootstrap in PAUP* v. 4.0b10 (Swofford 2009). Representative DNA sequences are deposited in GenBank (GQ907046–149).

3. RESULTS

(a) *Patterns of symbiosis*

A total of 379 samples from 211 liverwort collections were analysed for the target taxa, and from these at least one, and usually at least two, different rDNA regions were sequenced. We used a relatively conservative near-identity (99%) cut-off to define putative fungal lineages (Nilsson *et al.* 2008). Phylogenetic species concepts from multi-locus gene genealogical analyses are not available for any of the fungal lineages detected, and the fungi detected are still poorly represented in GenBank. Thus, our fungal nrDNA sequence-based delimitation and species recognition, though state-of-the-art, are tentative.

The internal transcribed spacer (630–712 bp), mitochondrial large subunit (334 bp) and nuclear large subunit (653 bp) regions' data reveal remarkably simple patterns of symbiotic association: members of the Aneuraceae liverwort species examined associate with several *Tulasnella* spp. (table 1, figure 1) and the rest of the basidiomycete-associated liverworts form symbioses with diverse members of the putatively non-ectomycorrhizal *S. vermifera* species complex (i.e. Sebacinales clade B, *sensu* Weiss *et al.* 2004; table 1, figure 2). Therefore, even though both *Tulasnella* and *Sebacina* include some ectomycorrhizal fungal lineages, we only detected overlap between ectomycorrhizas and *Tulasnella* from *Cryptothallus* and a few collections of *Aneura pinguis*. Only in the case of Loch

Table 1. The families and species of basidiomycete-associated liverworts sampled, their origin, their fungi, the number of field collections obtained and the number of samples analysed.

families	plants	countries	fungi	collections	samples
Aneuraceae	<i>Aneura maxima</i>	Indonesia, UK, USA	<i>Tulasnella</i>	5	5
Aneuraceae	<i>Aneura pinguis</i>	Chile, Ireland, Switzerland, UK, USA	<i>Tulasnella</i> , <i>Sebacina</i>	37	56
Aneuraceae	<i>Aneura</i> spp.	Chile, China, Malaysia, Peru, UK, USA	<i>Tulasnella</i>	15	23
Aneuraceae	<i>Aneura mirabilis</i>	UK	<i>Tulasnella</i>	5	6
Aneuraceae	<i>Lobatiriccardia lobata</i>	New Zealand	<i>Tulasnella</i>	1	1
Aneuraceae	<i>Lobatiriccardia</i> spp.	China, Venezuela	<i>Tulasnella</i>	2	2
Scapaniaceae	<i>Barbilophozia attenuata</i>	UK	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Barbilophozia barbata</i>	Switzerland, UK	<i>Sebacina</i>	6	12
Scapaniaceae	<i>Barbilophozia floerckii</i>	Switzerland, UK	<i>Sebacina</i>	6	12
Scapaniaceae	<i>Barbilophozia hatcheri</i>	Chile, UK	<i>Sebacina</i>	5	10
Scapaniaceae	<i>Barbilophozia lycopodioides</i>	Switzerland	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Barbilophozia quadriloba</i>	UK	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Diplophyllum albicans</i>	UK, Ireland	<i>Sebacina</i>	15	30
Scapaniaceae	<i>Diplophyllum apiculatum</i>	USA	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Diplophyllum obtusifolium</i>	UK	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Diplophyllum</i> sp.	Malaysia	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Lophozia bicrenata</i>	UK	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Lophozia crispata</i>	Chile	<i>Sebacina</i>	3	6
Scapaniaceae	<i>Lophozia excisa</i>	Switzerland, UK	<i>Sebacina</i>	5	10
Scapaniaceae	<i>Lophozia incisa</i>	Ireland, UK	<i>Sebacina</i>	6	12
Scapaniaceae	<i>Lophozia longidens</i>	UK	<i>Sebacina</i>	2	4
Scapaniaceae	<i>Lophozia opacifolia</i>	Switzerland, UK	<i>Sebacina</i>	4	8
Scapaniaceae	<i>Lophozia sudetica</i>	Switzerland, UK	<i>Sebacina</i>	4	8
Scapaniaceae	<i>Lophozia ventricosa</i>	Germany, Ireland, Switzerland, UK	<i>Sebacina</i>	18	36
Scapaniaceae	<i>Lophozia wenzelii</i>	Switzerland	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Scapania calcicola</i>	UK	<i>Sebacina</i>	3	6
Scapaniaceae	<i>Scapania cuspiduligera</i>	UK	<i>Sebacina</i>	2	4
Scapaniaceae	<i>Scapania irrigua</i>	Switzerland, UK	<i>Sebacina</i>	3	6
Scapaniaceae	<i>Scapania umbrosa</i>	UK	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Tritomaria exsectiformis</i>	UK	<i>Sebacina</i>	2	4
Scapaniaceae	<i>Tritomaria polita</i>	Switzerland	<i>Sebacina</i>	1	2
Scapaniaceae	<i>Tritomaria quinquidentata</i>	Ireland, Switzerland, UK	<i>Sebacina</i>	11	22
Jungermanniaceae	<i>Nardia geoscyphus</i>	UK	<i>Sebacina</i>	1	2
Jungermanniaceae	<i>Nardia scalaris</i>	Switzerland, UK	<i>Sebacina</i>	4	8
Geocalycaceae	<i>Saccogyna viticulosa</i>	UK	<i>Sebacina</i>	10	20
Arnellaceae	<i>Southbya nigrella</i>	France	<i>Sebacina</i>	2	4
Arnellaceae	<i>Southbya tophacea</i>	Italy, France, UK	<i>Sebacina</i>	3	6

Affric did we retrieve identical DNA sequences from ectomycorrhizas and liverworts; these were from *Betula pendula* and *C. mirabilis*, respectively, sharing a *Tulasnella* (electronic supplementary material, table S1).

As expected from the absence of fungi in microscopic examination of fresh specimens and literature reports, we did not detect fungi in the following Aneuraceae: *Aneura pellioidea*, *Riccardia chamaedryfolia*, *Riccia glauca* and *Pleurozia purpurea*—the basal genus in Metzgeriidae (Forrest *et al.* 2006). Similarly, we did not detect fungi in the following leafy liverworts: *Diplophyllum taxifolium*, *Douinia ovata* and *Scapania undulata*. Also as expected, we detected only ascomycetes in *Cephalozia connivens*, *Calypogeia fissa*, *Calypogeia muelleriana* and *Lepidozia reptans*. However, we detected only ascomycetes in the putatively basidiomycete-containing *Eremonotus myriocarpus*, *Anastrophylum helleranum*, *Geocalyx graveolens* and *Roivainenia jacquinotii* (electronic supplementary material, table S1). We failed to obtain fungal DNA sequences from *Acrobolbus wilsonii*, *Gongylanthus ericetorum*,

Harpanthus scutatus and *Pedinophyllum interruptum* (data not shown). It is important to note that in many cases relatively few samples were analysed and thus only wider field surveys will lead to robust generalizations regarding any predominant fungal symbionts or lack thereof.

To unravel fungal–liverwort symbiotic patterns within sites, within liverwort species and within liverwort individuals, we focused on the highly variable internal transcribed spacer DNA sequence data. Within seven sites where several (i.e. 5–12) *Sebacina*-associated liverwort species were sampled, we searched for identical fungal nrITS sequences shared between species. We only detected two cases in which samples from liverworts of different species share a fungus with identical DNA sequences: *L. ventricosa* and *N. scalaris* at Ben Wyvis (Scotland), and *L. opacifolia* and *N. scalaris* at St Gotthard (Switzerland). Conversely, within the 21 liverwort species that were sampled at more than one site, we searched for identical fungal nrITS sequences retrieved from two or more sites. We found five species

Figure 1. Phylogenetic placement of fungi associated with Aneuraceae liverworts within Tulasnellales based on neighbour-joining and bootstrap from an alignment of partial mitochondrial large subunit ribosomal DNA sequences generated for this study and retrieved from GenBank.

(*A. pinguis*, *Diplophyllum albicans*, *L. excisa*, *Scapania cuspiduligera* and *Tritomaria quinquentata*) that shared identical fungal DNA sequences between two different sites, all in the British Isles. For example, *S. cuspiduligera* produced the same fungal DNA sequence at Killin and Morrone (Scotland). These findings suggest long-distance clonal dispersal of liverworts and/or fungi; however, they were exceptional for all of these liverworts. That is, identical fungal DNA sequences shared between sites were not detected from more than two sites in any of the five liverwort species. Lastly, of the 70 collections from which we obtained nrITS sequence data from more than one clone (two to five), or from the two samples of each collection, only four generated different fungal DNA sequences.

(b) Morphology of symbiosis

The cytology of liverwort–basidiomycete associations has been described in detail elsewhere (Kottke *et al.* 2003; Duckett *et al.* 2006a; Duckett & Ligrone 2008a), thus, our observations are included solely to illustrate the commonalities in features of taxa not hitherto examined.

Throughout the Aneuraceae, the sequence of colonization of ventral thallus cells via the rhizoids by hyphae with multi-stratose walls, followed by their proliferation (figure 3b) and degeneration (figure 3c), often with multiple cycles in the same cells, appears to be the rule. These commonalities extend to the Swiss and Chilean collections with sebacinoid symbionts. Indeed, the only consistent difference was in the number of liverwort cell layers occupied by the fungal symbionts, ranging from almost the entire thallus in *A. mirabilis* to approximately eight ventral layers in European *A. pinguis*, two to five

Figure 2. Phylogenetic placement of fungi associated with liverworts within Sebacinales based on neighbour-joining and bootstrap from an alignment of partial nuclear large subunit ribosomal DNA sequences generated for this study and retrieved from GenBank. The clade designations A and B correspond to those used by Weiss *et al.* 2004.

layers immediately above the lower epidermis in Chilean collections (figure 3a), and absence in *A. pellioides* (figure 3d) and Californian *A. pinguis*.

In leafy liverworts, hyphal degeneration has not been reported (Duckett *et al.* 2006a,b) and it was not observed in the additional ones sampled here (figure 4a; electronic supplementary material, fig. S1a). There are two distinct patterns of colonization. First, in *Southbya tophacea*, the colonized cells occupy a strand in the centre of the stems, and there are no obvious interactions between fungi and liverwort cell walls. Nonetheless, fungal hyphae traversing the cortex are overgrown by liverwort cell wall material. In *Southbya nigrella* (electronic supplementary material, fig. S1a), and in the closely allied *G. ericetorum*, hyphal coils fill most of the ventral stem cells (Paton 1999). Second, in all other basidiomycete-associated leafy liverworts, there is a mosaic of colonized and uncolonized cells (electronic supplementary material, fig. S1b–d) with numerous pegs of cell walls overgrowing the hyphae along the interfaces between the two kinds of cells (figure 4b; electronic supplementary material, fig. S1e–g). Similar host wall modifications were not detected in liverworts where fungi occupy virtually all the cells in the ventral region of the stems: *Acrobolbus*, *Anastrophylum*, *Eremonotus*, *Geocalyx*, *Pedinophyllum* and *Roivainenia*.

Whatever the pattern of colonization inside stems, rhizoids invariably contain numerous hyphae and fungal

Figure 3. Scanning electron micrographs of basidiomycetes in Aneuraceae. (a–c) Sebacinoid-containing *Aneura* species from Navarino, Southern Chile. (a) General view showing up to five ventral thallus layers packed with hyphae (arrowed). (b) Cell packed with hyphal coils. (c) Cells with degenerating hyphal masses. (d) A fungus-free thallus of *Aneura pellioidea*. Scale bars for (a) and (d) are 200 µm and for (b) and (c) are 20 µm.

entry sites are normally at apices that are often branched (figure 4c). We observed fungal entry/exit sites through ventral epidermal cells (figure 4d) in *L. incisa* and *L. opacifolia*, but nowhere else.

4. DISCUSSION

Using the broadest taxonomic and geographical sampling to date, this study reveals that the evolutionary and ecological trajectories followed by liverworts and basidiomycete fungi are distinctly divergent from those followed by seed plants and basidiomycetes forming mycorrhizas. We can identify two major differences. (i) Liverworts appear to target members of only one of two cosmopolitan fungal genera: *Tulasnella* and *Sebacina*. (ii) With only rare exceptions, these are not the *Tulasnella* and *Sebacina* lineages that are known to form ectomycorrhizas with seed plants. Liverwort symbiotic conservatism differs fundamentally from the generalist pattern of mycorrhization in seed plants that evolved repeatedly to form ectomycorrhizas simultaneously with numerous distantly related basidiomycete genera. Nevertheless, we found evidence of high within-site diversity in the *Tulasnella* and *Sebacina* lineages that associate with liverworts. This diversity can result from: (i) simultaneous colonization of a single liverwort individual by genetically different congeneric fungal individuals; (ii) heterokaryosis or heterozygosity within a single fungal individual; or (iii) having sampled two different liverwort individuals, each colonized by a genetically different fungal individual in cases where two samples were analysed from a single collection.

Our findings are largely in line with previous much more taxonomically and geographically restricted studies (Kottke *et al.* 2003), with only two exceptions: the occurrence of sebacinoid fungi in a small number of *Aneura* collections, and the identification by both electron microscopy and molecular methods by Kottke *et al.* (2003) of a sebacinoid mycobiont in *Calypogeia*. As

Figure 4. Scanning electron micrographs of basidiomycete fungi in Jungermanniales. (a–d) *Lophozia incisa*. (a) Hyphal coils in ventral stem cells with no evidence of fungal degradation. (b) Liverwort wall ingrowths preventing hyphal colonization of an uncolonised stem cell. (c–e) Hyphal entry/exit sites. (c) Hyphae extending from the base of a severed rhizoid. (d) Direct hyphal entry sites (arrowed) through ventral stem cells. (e) *Saccogyna viticulosa* showing numerous hyphae associated with a branched rhizoid apex. Scale bars for (a), (c) and (e) are 10 µm and for (b) and (d) are 5 µm.

earlier microscopy and cross-colonization studies (Duckett *et al.* 1991; Duckett & Read 1995) and the present molecular analyses revealed only ascomycetes in this genus, the disparity in findings highlights a need for further investigation of variation in this genus. It is noteworthy that Calypogeiaceae lies between the basidiomycete-associated Arnelliaceae and Jungermanniaceae (Crandall-Stotler *et al.* 2008), and *Calypogeia*'s cell wall ingrowths preventing fungal hyphal colonization beyond the rhizoid bases, first illustrated by Némec (1904), are more typical of colonization by basidiomycetes than by ascomycetes.

Although our dataset is predominantly from the northern hemisphere, it is likely to portray accurately basidiomycete symbioses of leafy liverworts worldwide. Overall, basidiomycete-associated leafy liverworts are far more phylogenetically restricted than those with ascomycete fungi and in every case they are in lineages with ascomycete-containing sister groups; thus, we can infer that basidiomycetes were a secondary acquisition following the loss of rhizoidal ascomycetes. That is, within the Jungermaniidae (leafy liverworts; *sensu* Hentschel *et al.* 2006), fungus associates are confined to the Jungermanniales; in liverwort phylogenies that include Jungermanniales (Davis 2004; Heinrichs *et al.* 2005, 2007; Forrest *et al.* 2006), the ascomycete-associated Schistochilaceae (Pressel *et al.* 2008) are sister to all other fungus-associated families, with continuous lineages to nearly all of these. The *Southbya* sebacinoid fungi fall into clades sister to those in the remaining leafy liverworts, suggesting that this may represent an ancestral state, a situation in agreement with its less pronounced modifications compared with those in other families. This is congruent with the placement of all the other basidiomycete-containing genera in

more derived clades than the Arnelliaceae (Davis 2004; Heinrichs *et al.* 2005, 2007; Forrest *et al.* 2006).

The present study confirms the basidiomycete identity of the vast majority of taxa where this was inferred to be likely from light microscopy studies and from their systematic position (Duckett *et al.* 2006a). Putative corrections to fungal identity are limited to two species, *E. myriocarpus* and *G. graveolens*, shown here to harbour ascomycetes rather than basidiomycetes. Meanwhile, DNA sequencing results from the fungi of *Harpanthus*, *Acrobolbus*, *Gongylanthus* and *Pedinophyllum* remain inconclusive, but all of these liverworts' cell walls lack the modifications characteristic of the majority of basidiomycete symbioses. The presence of fungi, in both cases sebacinoid, is described here for the first time in *Lophozia wenzelii* and *Tritomaria polita*, as it is typical of other members of these genera, whereas the occurrence of an ascomycete in *R. jacquinotii* is surprising because all allied liverworts associate with basidiomycetes. These symbioses merit further investigation.

We observed that liverworts symbiotic with a range of co-occurring sebacinoid fungi are mostly concentrated in a small number of specialized habitats. Whereas taxa with rhizoidal ascomycetes are widespread in waterlogged nutrient-poor habitats, particularly *Sphagnum* bogs (Duckett *et al.* 1991), those with basidiomycetes are most diverse on well-drained humus-rich soils, or on rocks, both acidic and base-rich, covered with the same, or on decorticated logs. Late snow patch communities are dominated by liverworts containing both basidiomycetes and ascomycetes, whereas the northern Atlantic liverwort mat communities are remarkably fungus-free, as are aquatic habitats. Two traits common to the liverworts growing in these habitats are thick stem cell walls and a general absence of rhizoids along the stems. Both ascomycetes and basidiomycetes are also conspicuously absent from liverworts that commonly grow on bare rocks and bark.

The widespread occurrence of basidiomycetes in *Scapania*, *Diplophyllum*, *Barbilophozia* and *Lophozia* agrees with their close evolutionary relationship (Schill *et al.* 2004; Yatsentyuk *et al.* 2004; He-Nygrén *et al.* 2006). The placement of basidiomycete-associated liverworts (*Lophozia sudetica*, *B. barbata*, *Barbilophozia lycopodioides*, *Barbilophozia hatcheri*) at the base of the Lophoziaaceae (de Roo *et al.* 2007) suggests that the presence of fungi is ancestral, that absence in genera like *Anastrophyllum* is derived and that colonization by ascomycetes in *R. jacquinotii* and *A. helleranum* is a recent acquisition. In *Diplophyllum*, containing some 20 species (Paton 1999) where fungi appear to be almost ubiquitous, including those from Malaysia (present study) and New Zealand (J. G. Duckett 2007, unpublished data), the absence of a fungal symbiont in *D. taxifolium* can be interpreted as a recent loss. Basidiomycetes are present only in a minority of species in *Diplophyllum*'s sister genus *Scapania*. In contrast to the predominance of basidiomycetes in the Lophoziaaceae, the Geocalyceae is heterogeneous with both asco- and basidiomycete symbionts. The presence of ascomycetes in *Eremonotus* agrees with the finding that this monospecific genus is not allied with the Scapaniaceae but with the Gymnomitriaceae (Hentschel *et al.* 2006), a generally fungus-independent family.

The variety of sebacinoid fungi in the Jungermanniales is perhaps not as surprising, given the diverse liverwort families, genera and species involved, as the variety of tulasnelloid symbionts plus rare sebacinoids in just a handful of species in the three seemingly closely allied Aneuraceae genera, *Aneura*, *Lobatiriccardia* and *Verdoornia*. Recent studies (Wachowiak *et al.* 2006; Bączkiewicz *et al.* 2008; Wickett & Goffinet 2008; L. Forrest & D. Long 2009, unpublished data) can provide an explanation for this apparent disparity. *Aneura pinguis*, the only pan-European member of the genus, was regarded by some as a single variable species (Paton 1999), while others described a series of varieties (Damsholt 2002). Molecular studies have revealed that conservative thallus morphology in the Aneuraceae conceals much genetic diversity (e.g. Wickett & Goffinet 2008). L. Forrest & D. Long (personal communication 2009) are finding much molecular heterogeneity including the nesting of *Cryptothallus*, *Aneura maxima* and *Aneura sharpii* as single clades within *A. pinguis*. The diversity of *Tulasnella*, plus the scattered occurrence of *Sebacina* that we detect in *Aneura*, may mirror the molecular diversity of the liverwort itself. There is noteworthy congruence between liverwort and fungal phylogenies in the nesting of *Cryptothallus*, and its fungi within single clades. In contrast, the occurrence of a range of tulasnelloid symbionts in *A. maxima* suggests multiple origins. However, as with host morphology, there appear to be no obvious correlations between fungal symbionts, liverwort morphology or ecology. For example, nearly identical fungal DNA sequences were retrieved from *A. pinguis* collected in Welsh dune slacks and in a variety of English, Scottish and Welsh upland and lowland sites.

Undoubtedly, further sampling of both liverworts and fungi will provide a fuller understanding of what we have shown to be a conservative history of liverwort–basidiomycete symbioses. Our results indicate that priorities for investigation include: (i) the southern hemisphere leafy Balantiopsidaceae, with swollen rhizoids (Duckett & Ligrone 2008b) resembling those of the related ascomycete-associated *Mylia* (de Roo *et al.* 2007; S. Pressel, M. I. Bidartondo & J. G. Duckett 2009, unpublished data); (ii) *Verdoornia*, sister to *Lobatiriccardia* and *Aneura* (Wickett & Goffinet 2008); (iii) *Lobatiriccardia* (*Aneura lobata* and *A. novaeguineensis*; Duckett & Ligrone 2008a) where the fungi—as in the sebacinoid-associated Chilean *Aneura*—occupy up to five ventral layers of the thallus; (iv) *Aneura sharpii*, similar to *A. lobata* (Duckett & Ligrone 2008a) with fungi only in the ventral subepidermal layer of the thallus (Inoue & Miller 1985); (v) *Aneura pseudopinguis* (re-designated *A. pinguis*) from Southern Africa (Perold 2001) with a similar fungal symbiont distribution to northern hemisphere *A. pinguis* (J. G. Duckett 2007, unpublished data); (vi) *Riccardia* from New Zealand, including *Riccardia pennata*, where fungi are restricted to the ventral epidermis without evidence of hyphal degeneration, and *Riccardia intercellula*, where fungi are exclusively intercellular (Brown & Braggins 1989); (vii) *Cryptothallus hirsutus*, a second myco-heterotrophic Aneuraceae (Crum & Bruce 1996); and, crucially, in view of their abundance as global sources of symbionts to liverwort communities, (viii) understanding the range of abilities of sebacinoid and tulasnelloid fungi to form symbioses with plants in nature.

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