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Advances in understanding of mycorrhizal-like associations in bryophytes

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Abstract

Mutually beneficial associations between plants and soil fungi, mycorrhizas, are one of the most important terrestrial symbioses. These partnerships are thought to have propelled plant terrestrialisation some 500 million years ago and today they play major roles in ecosystem functioning. It has long been known that bryophytes harbour, in their living tissues, fungal symbionts, recently identified as belonging to the three mycorrhizal fungal lineages Glomeromycotina, Ascomycota and Basidiomycota. Latest advances in understanding of fungal associations in bryophytes have been largely driven by the discovery, nearly a decade ago, that early divergent liverwort clades, including the most basal Haplomitriopsida, and some hornworts, engage with a wider repertoire of fungal symbionts than previously thought, including endogonaceous members of the ancient sub-phylum Mucoromycotina. Subsequent global molecular and cytological studies have revealed that Mucoromycotina symbionts, alongside Glomeromycotina, are widespread in both complex and simple thalloid liverworts and throughout hornworts, with physiological studies confirming that, in liverworts at least, these associations are mycorrhizal-like, and highlighting important functional differences between Mucoromycotina and Glomeromycotina symbioses. Whether a more prominent role of Mucoromycotina symbionts in plant nitrogen nutrition, as identified in liverworts, extends to other plant lineages, including the flowering plants, is a major topic for future research.

The latest finding that ascomycete symbionts of leafy liverworts are not restricted to one fungus, *Rhizoscyphus ericae*, but include species in the genus *Meliniomyces*, as shown here in *Mylia anomala*, together with the recent demonstration that *R. ericae* forms nutritional mutualisms with the rhizoids of *Cephalozia bicuspidata*, fill other major gaps in our growing knowledge of fungal associations across land plants.

Key words: Endogonales, fungal associations, hornworts, liverworts, *Meliniomyces*, *Mylia anomala*

Introduction

It has been known for a very long time that many bryophytes, with the exclusion of mosses, harbour in their living tissues filamentous fungi, which produce a variety of structures comparable to those formed by mycorrhizal fungi in the roots of modern vascular plants (Read *et al.* 2000, Ligrone *et al.* 2007, Pressel *et al.* 2010) and indeed in the prostrate and aerial axes of ancient fossil plants (Remy *et al.* 1994, Taylor *et al.* 1995). From their discovery by Gottsche in 1843, for over a 120 years these were simply described and illustrated by line drawings from a wide range of liverworts (see review in Schuster 1966) but with their real nature remaining purely conjectural. Electron microscope studies then revealed diverse kinds of fungi in both liverworts and hornworts with the demonstration of healthy hyphae in heathy host cells indicating that the associations were likely mutualistic (see Ligrone 1988, Read *et al.* 2000, Ligrone *et al.* 2007 and literature therein). In contrast, possible mutualistic associations have yet to be detected in mosses. Although numerous fungi have been described from mosses, we are not aware of a single micrograph showing healthy hyphae and host cytoplasm in the same cell. The most likely explanation is that, with diameters down to 2 µm, the ultimate ramifications of multicellular moss rhizoids match the nutrient-scavenging capabilities of fungal hyphae (Field *et al.* 2015b) and thus preclude the evolution of nutritional symbiosis between mosses and fungi.

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More recently, molecular studies have confirmed the identity of the fungi inhabiting the rhizoids and/or thalli of liverworts and hornworts as members of the three major clades of mycorrhizal fungi Glomeromycotina, Ascomycota and Basidiomycota (Chambers *et al.* 1999, Ligrone *et al.* 2007, Upson *et al.* 2007, Pressel *et al.* 2008a, b, Bidartondo & Duckett 2010, Newsham & Bridge 2010, Preußing *et al.* 2010). Based exclusively on cytological evidence, but with no confirmation from physiological studies, bryophyte-fungal associations were assumed to represent, like their counterparts in vascular plants, mutually beneficial symbioses with bidirectional exchange of resources between partners, i.e. to be mycorrhizal-like given the absence of true roots in bryophytes. This situation has now changed radically since our last review on fungal associations in bryophytes a decade ago (Pressel *et al.* 2010). Another major recent advance in understanding of bryophyte fungal associations has been the discovery, hinted at in Pressel *et al.* (2010), that the fungal symbionts of the earliest diverging liverwort clade, Haplomitriopsida (Forrest *et al.* 2006), are not arbuscular mycorrhizal fungi in the Glomeromycotina, as previously assumed (Wang & Qui 2006, Ligrone *et al.* 2007), but members of another major fungal clade, the Endogonales (Mucoromycotina), which has so far been little studied (Bidartondo *et al.* 2011, Bonfante & Venice 2020). This crucial discovery was a catalyst for much increased interest in bryophyte-fungal associations during the last decade, with ongoing research aimed at elucidating the diversity and functional significance of these partnerships globally.

Studies of bryophyte-fungal symbioses have also provided powerful insights into the origin and evolution of mycorrhizal associations in land plants. Both fossil (Remy *et al.* 1994, Taylor *et al.* 1995, Krings *et al.* 2007, Strullu-Derrien *et al.* 2014) and molecular evidence, the latter showing that the genes and biochemical pathways required for mycorrhization are conserved across all land plant lineages (Wang *et al.* 2010, Delaux *et al.* 2013, Oldroyd 2013) and that components of this symbiotic 'toolkit' are present within ancestral lineages of charophytic algae (Delaux *et al.* 2015), indicate that these associations are ancient and likely played a key role in the establishment and subsequent diversification of the land flora (Pirozynski & Malloch 1975, Krings *et al.* 2012). Here we review advances in understanding of bryophyte fungal associations over the last decade and present novel observations on the distinctive fungal association in the leafy liverwort *Mylia anomala* (Hook.) Gray.

Diversity of fungal associations in bryophytes

Liverworts

The history of liverwort symbioses with fungi is one of losses, reacquisitions, jumps, and swaps, including symbioses that are common to both liverworts and vascular plants (Pressel *et al.* 2010, Rimington *et al.* 2018, 2020 and references therein). Until recently, symbioses with arbuscular mycorrhizal forming fungi in the Glomeromycotina (AMF), like those in pteridophytes and angiosperms, were considered to be ancestral in liverworts, based on their apparent occurrence in Haplomitriopsida at the base of the liverwort tree and definitively in both the Marchantiidae and the Pelliidae in the complex and simple thalloid lineages respectively (Ligrone *et al.* 2007, Crandall-Stotler *et al.* 2009a, b). These symbioses were in turn lost in later clades of the Jungermanniopsida (Crandall-Stotler *et al.* 2009a, b). Thereafter fungal symbionts were either basidiomycetes in the derived thalloid family Aneuraceae in the Metzgeriidae (Bidartondo & Duckett 2010), but probably ascomycetes in the swollen rhizoids in the sister family Mizutaniaceae (Pressel *et al.* 2011), or, in the Jungermanniales, a mixture of either basidiomycetes (Arnelliaceae, Lophocoleaceae, Geocalycaeae, Scapaniaceae) or ascomycetes (Calypogeiaceae, Cephaloziaceae, Cephaloziellaceae, Lepidoziaceae, *Eremonotus* Lindb. & Kaal. ex Pearson in the Jungermanniaceae and *Geocalyx* Nees in the Geocalycaeae) (Pressel *et al.* 2008a, b, Bidartondo & Duckett 2010), with recent liverwort phylogenies indicating that the latter were first acquired in the rhizoids of the Schistochilaceae (Pressel *et al.* 2008a, b). In marked contrast Porellales, containing the most speciose liverwort family, Lejeuneaceae, are fungus-free, probably reflecting their high degree of epiphytism.

Fungal associations in Marchantiopsida and Pelliidae

The demonstration by Bidartondo *et al.* (2011) that *Treubia* K.I. Goebel and *Haplomitrium* Nees are consistently colonised by members of the Endogonales (Endogonaceae and Densosporaceae) in the subphylum Mucoromycotina and do not harbour Glomeromycotina AMF, has led to a major shift in understanding the diversity of fungal associations in liverworts and other plants. Since this discovery, made possible by technical advancements in the molecular detection of fungal symbionts, and through extensive global sampling, it has become apparent that associations with Mucoromycotina fungi are widespread in both complex and simple thalloids (Rimington *et al.* 2018, 2019, 2020). However, in these groups symbioses are not exclusive, with most liverwort species found to date to harbour Mucoromycotina fungi also able to host Glomeromycotina, sometimes with both fungi co-colonising the same host

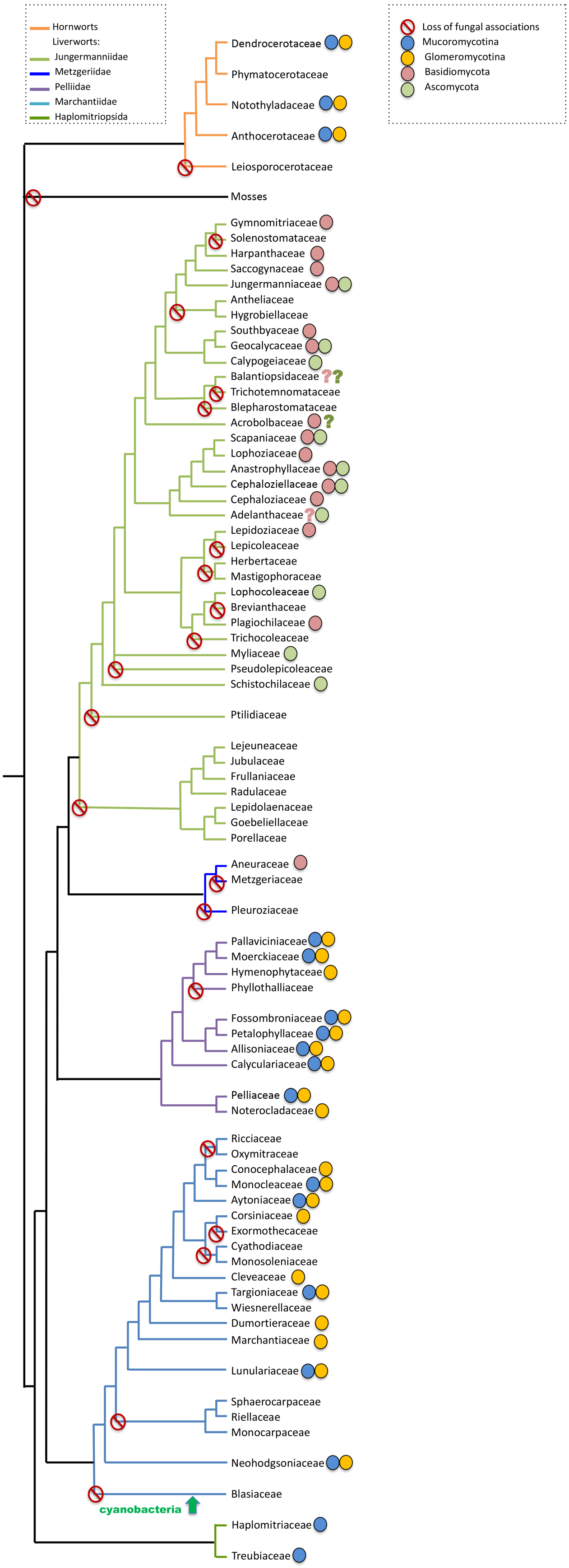

FIGURE 1. Phylogram showing the diversity and distribution of fungal assocations in bryophytes – adapted from Rimington *et al.* (2020).

in dual symbiosis (Field *et al.* 2016, Rimington *et al.* 2020). This situation parallels that in hornworts (Desirò *et al.* 2013) and pteridophytes (Rimington *et al.* 2015, Pressel *et al.* 2016), with the only currently known exception being the lycophyte *Lycopodiella inundata* (L.) Holub which, after detailed sampling of several populations, appears to form partnerships, like *Treubia* and *Haplomitrium,* only with Mucoromycotina (Rimington *et al.* 2015, Hoysted *et al.* 2019, 2020, Kowal *et al.* 2020).

The taxonomic distribution of Mucoromycotina symbioses in liverworts (Fig. 1) indicates that they represent the ancestral type and were subsequently lost in Marchantiopsida during the divergence of the Blasiales (the only early divergent lineage that is fungus-free but forms symbioses with cyanobacteria; Adams & Duggan 2008), and the Sphaerocarpales in the Marchantiidae, only to be regained elsewhere in the Marchantiidae, in the Neohodgsoniales, Lunulariales and several families in the Marchantiales, i.e. Marchantiaceae, Monocleaceae, Aytoniaceae and Targioniaceae. Absence of fungi in some marchantialean families (Monoseleniaceae, Cyathodiaceae, Exormothecaceae, Monocarpaceae, Oxymitraceae, Ricciaceae and probably Corsiniaceae and Wiesnerellaceae) is almost certainly a derived state. In Pelliidae, Mucoromycotina symbiosis has been maintained in *Pellia* Raddi*, Calycularia* Mitt.*, Allisonia* Herzog*, Fossombronia* Raddi and *Moerckia* Gottsche, lost in *Noteroclada* Taylor ex Hook. & Wilson*, Petalophyllum* Nees & Gottsche ex Lehm.*, Phyllothallia* E.A. Hodgs. and *Hymenophyton* Dumort. whilst other genera like *Makinoa* Miyake*, Sewardiella* Kashyap*, Sandeothallus* R.M. Schust.*, Greeneothallus* Hässel and *Xenothallus* R.M. Schust. require further study. It appears therefore that symbiosis with Mucoromycotina, like those with the more recently diverged Dikarya (Ascomycota and Basidiomycota) was lost and regained several times (Fig. 1). In contrast, symbiosis with Glomeromycotina was acquired once, after the divergence of the Haplomitriopsida, followed by several losses in the Marchantiopsida, from Sphaerocarpales and six or possibly eight families of the Marchantiales, but only one subordinal loss in the Pelliidae, from the Phyllothalliineae. While the Haplomitriopsida stand out at the base of the liverwort tree as the only lineage that apparently form exclusive symbioses with Mucoromycotina fungi, several families in the Marchantiopsida, including Conocephalaceae, Corsiniaceae, Cleveaceae, Dumortieraceae, and Marchantiaceae only harbour Glomeromycotina symbionts (Fig. 1). Absences of fungal symbionts linked to habitat preference are notable in saxicolous taxa, including members of the Cyathodiaceae, submerged aquatics such as *Monosolenium* Griff., taxa that grow on nutrient-rich substrates, including *Marchantia polymorpha* subsp. *polymorpha* (Nees) Gottsche *et al.* and *M. polymorpha* subsp. *ruderalis* Bischl. & Boissel.-Dub. and in otherwise mycorrhizal-like species from very wet habitats, i.e. *Conocephalum conicum* (L.) Dumort. (Pressel *et al.* 2010, Rimington *et al.* 2018). Absences are also notable in ephemeral taxa, viz. *Exormotheca* Mitt.*, Oxymitra* Bisch. ex Lindenb. and *Riccia* L. Recent genetic analyses of the model species *M. polymorpha* subsp. *polymorpha* have revealed that in this species genes required for successful arbuscular mycorrhizal colonization are missing (Bowman *et al.* 2017) while these are present in closely related mycorrhizal-like species such as *M. polymorpha* subsp. *montivagans* Bischl. & Boissel.-Dub. (Bowman *et al.* 2015). Thus, the inability to form mycorrhizal-like associations, through secondary loss of symbiosis, may represent an adaptation to specific habitats, in the case of *Marchantia* as a weedy colonizer of highly disturbed, nutrient-rich habitats (Bowman *et al.* 2017).

Glomeromycotina (syn. Glomeromycota) and Mucoromycotina are sub-phyla within Mucoromycota (Spatafora *et al.* 2016). While uncertainties remain on the timing of the origin of Mucoromycota (Berbee *et al.* 2017) and the order of divergence of Mucoromycotina and Glomeromycotina, with contrasting topologies placing either subphylum as more closely related to the Dikarya (Schüßler *et al.* 2001, James *et al.* 2006, Bidartondo *et al.* 2011, Chang *et al.* 2015, Spatafora *et al.* 2016, Uehling *et al.* 2017), recent phylogenomic analyses indicate that both lineages evolved prior to or concomitant with the initial colonisation of land by plants (Schüßler & Walker 2011, Chang *et al.* 2019). This evidence, together with the demonstration that the genes required for mycorrhization are conserved across all land plant lineages, including Haplomitriopsida liverworts (Wang *et al.* 2010, Delaux *et al.* 2013) strongly indicate that both symbioses are ancient and that symbiotic Mucoromycotina and Glomeromycotina likely co-evolved with liverworts since the dawn of plant terrestrialisation (Field & Pressel, 2018, Rimington *et al.* 2020).

Given the likely ancient origin of Glomeromycotina symbioses it was most surprising that, until recently, molecular identifications of glomeromycete symbionts in liverworts showed these to be invariably members of the Glomeraceae, the most recently diverged family (Russell & Bulman 2005, Ligrone *et al.* 2007, Bidartondo *et al.* 2011). This evidence, albeit based on only a handful of species, raised the possibility that the AM symbiosis in liverworts may in fact be derived via host-shifting from angiosperms (Selosse *et al.* 2005). However, more recent and sample-intensive molecular analyses have shown that liverworts frequently associate with ancient lineages of Glomeromycotina, more so than flowering plants, with every order detected in the Marchantiopsida and with diverse fungi often co-colonising the same host (Rimington *et al.* 2018), e.g. *Marchantia* was found to be colonised by members of the Claroideoglomeraceae, Diversisporaceae and Archaeosporaceae, alongside Glomeraceae. These findings confirm the ancient origin of the

Glomeromycotina-liverwort symbiosis, indicate that liverworts are a global reservoir of Glomeromycotina diversity likely acting as an overlooked source of arbuscular mycorrhizal inoculum for flowering plants, and dispel the notion that liverworts exhibit specificity towards members of the Glomeraceae (Rimington *et al.* 2018).

The cytology of colonisation by Glomeromycotina in liverworts, as described in detail before (Ligrone *et al.* 2007, Pressel *et al.* 2010, Rimington *et al.* 2018) is also indicative of a single and ancient origin of the symbiosis. Glomeromycotina fungi are known to produce a range of structures inside their liverwort host cells that are strikingly similar to those formed by the same fungi in vascular plants. While considerable variation exists in the size of these structures, e.g. vesicles and in their position, with diverse patterns of colonisation observed across genera (Ligrone *et al.* 2007, Rimington *et al.* 2018) (Fig. 2A–D), arbusculated coils and/or arbuscules, which are not always produced by Glomeromycotina in flowering plants (Smith & Smith 1997, Read *et al.* 2000), are a constant feature in liverworts.

In contrast to the well characterised cytology of the liverwort-AM symbiosis, a diagnostic range of structures and interfaces produced by Mucoromycotina symbionts colonising liverworts remains somewhat elusive. As described in detail before (Carafa *et al.* 2003, Duckett *et al.* 2006, Field *et al.* 2015a), the cytology of colonisation by Mucoromycotina symbionts in *Treubia* and *Haplomitrium* is highly distinctive, consisting of both inter and intracellular phases of colonisation characterised by ventral intracellular tightly wound coils of fine hyphae with small vesicles/lumps and pseudo-parenchyma-like hyphal structures filling the mucilage-containing dorsal intercellular spaces. The same cytology and colonisation patterns have also been described in the gametophytes and sporophytic protocorms of *Lycopodium* spp. (Duckett & Ligrone 1992, Schmidt & Oberwinkler 1993) and *Lycopodiella inundata* (Hoysted *et al.* 2019, 2020), which we now know to be also colonised by Mucoromycotina fungi (Rimington *et al.* 2015, Pressel *et al.* 2016, Hoysted *et al.* 2019). However, in other liverwort taxa where Mucoromycotina symbionts have been reported (Bidartondo *et al.* 2011, Field *et al.* 2016, Hoysted *et al.* 2019, Rimington *et al.* 2019, 2020), all of which lack intercellular spaces in their thalli and may form simultaneous symbioses with AMF, it has proved more difficult to identify which structures are produced by which fungal group (Field *et al.* 2016). The recent reclassification of fine root endophyte fungi (FRE – *Glomus tenue* (Greenall) I.R. Hall or *Planticonsortium tenue* (Greenall) C. Walker & D. Redecker; Walker *et al.* 2018) from the Glomeromycotina into the Mucoromycotina (Orchard *et al.* 2017a, Walker *et al.* 2018) has shed some light on what may represent Mucoromycotina-specific structures in liverworts and other plant lineages (Hoysted *et al.* 2019, 2020). Fine root endophytes, widespread globally and across vascular plants, have traditionally been distinguished from the 'coarse' glomeromycete endophytes on the basis of their gross morphology characterised by much finer hyphae (<2 µm in diameter), small intercalary and terminal vesicles and fine arbuscules (Orchard *et al.* 2017a, b). Similar structures have been variously but not consistently observed in liverworts engaged in dual associations with both Glomeromycotina and Mucoromycotina symbionts. In *Neohodgsonia mirabilis* (Perss.) Perss. both 'coarse' and 'finer' arbuscules have been described (Field *et al.* 2016, 2019) (Fig. 2E, F), and in *Fossombronia*, which harbours the greatest diversity of both Glomeromycotina and Mucoromycotina symbionts (Rimington *et al.* 2018, 2019), fine hyphae with intercalary and terminal small vesicles (Fig. 2H) are often present alongside typical Glomeromycotina arbuscules, coils and larger vesicles (Hoysted *et al.* 2019) (Fig. 2G). Resynthesis experiments using axenic cultures of a range of liverwort taxa other than Haplomitriopsida (Field *et al.* 2015a) and Mucoromycotina isolates combined with detailed cytological and ultrastructural analyses are now needed to characterise precisely Mucoromycotina specific structures and interfaces in liverworts and, potentially, across the land plant phylogeny.

The latest molecular analyses have shown that the same Mucoromycotina fungi colonising liverworts are also present in the roots of adjacent vascular plants (Hoysted *et al.* 2019), indicating that Mucoromycotina symbionts may be as widespread as AMF across the land plant phylogeny. In the last decade physiological studies in liverworts have not only demonstrated unambiguously that both Glomeromycotina (Humphreys *et al.* 2010, Field *et al.* 2012) and Mucoromycotina associations represent nutritional mutualisms (Field *et al.* 2015a, 2016) but have also identified important differences in the nutritional benefits afforded to liverwort hosts by the two fungal groups. It appears that Mucoromycotina symbionts have a more prominent role in nitrogen nutrition, being able to access and transfer N from both inorganic (Field *et al.* 2016) and organic sources (Field *et al.* 2019). A major research question now is whether the different functional significance of the two symbioses observed in liverworts, also extends to the rest of the land plants, with recent experiments in the lycophyte *Lycopodiella inundata* indicating that this may well be the case (Hoysted *et al.* 2019, 2020).

Ascomycota and Basidiomycota associations in the Jungermanniopsida

While research on the diversity and distribution of fungal symbioses in Marchantiopsida and Pelliideae has increased exponentially in the last decade, largely spurred on by the discovery of Mucoromycotina symbionts alongside AMF in both groups, parallel studies on basidiomycetous and ascomycetous associations in the Jungermanniopsida have lagged

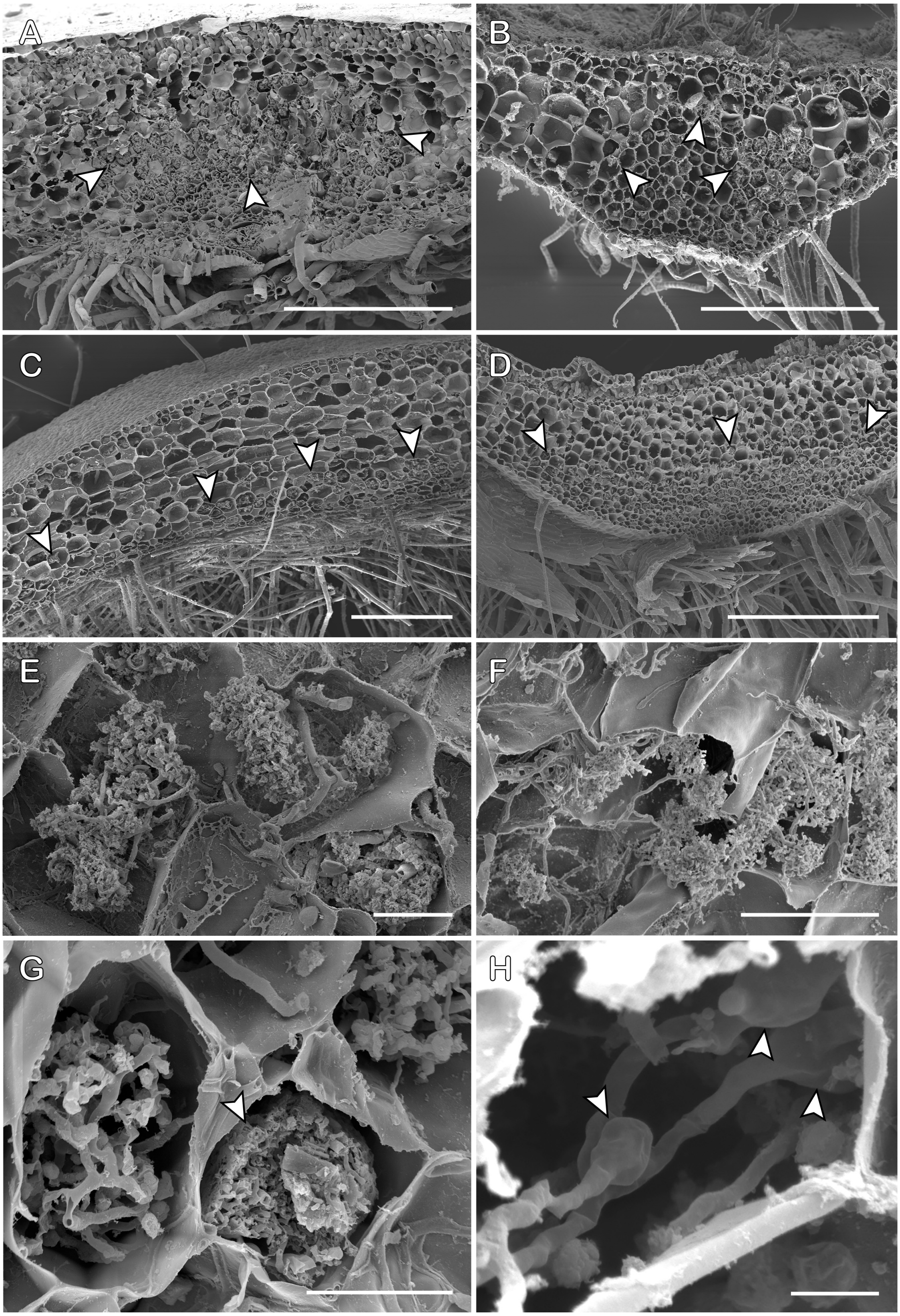

FIGURE 2. Fungal colonisation in thalloid liverworts. Scanning electron micrographs. A. Extensive fungal colonisation in the thallus central midrib in *Marchantia pappeana* Lehm. (arrowed), in the region overarching the midrib in *Dumortiera hirsuta* (Sw.) Nees (B, arrowed), and in the ventral cell layers in *Monoclea forsteri* Hook. (C, arrowed), and *Lunularia cruciata* (L.) Dumort. ex Lindb. (D, arrowed). E, F. Fungal structures in *Neohodgsonia mirabilis* (Perss.) Perss. include both 'coarse' (E) and much finer arbscules (F). G, H. Fungal colonisation in *Fossombronia foveolata* Lindb. G. Arbuscule and tighly wound coil of fine hyphae (arrowed). H. Fine hyphae with small swellings (arrowed). Scale bars: (A–D) 500 µm; (F) 50 µm; (E, G) 20 µm; (H) 5 µm.

behind. Indeed, our current understanding of the diversity and distribution of these associations in liverworts has not changed significantly since we last reviewed the topic in Pressel *et al.* (2010) (Fig. 1). It is well established that the Aneuraceae, which with the Metzgeriaceae and Pleuroziaceae form the most derived clade within the thalloid liverworts (Davis 2004, Forrest & Crandall-Stotler 2004, Forrest *et al.* 2006, He-Nygren *et al.* 2006) are the only simple thalloids to harbour basidiomycetes, mainly members of the genus *Tulasnella* J. Schröt. (Bidartondo *et al.* 2003, Kottke *et al.* 2003, Kottke & Nebel 2005, Bidartondo & Duckett 2010, Preußing *et al.* 2010, Krause *et al.* 2011), with only sporadic reports of *Serendipita* P. Roberts (*Sebacina* Tul. & C. Tul.) symbionts in this family (Duckett & Ligrone 2008, Bidartondo & Duckett 2010, Preußing *et al.* 2010). In contrast, sebacinoid symbionts are widespread in leafy liverworts, having been reported in members of diverse families including *Barbilophozia* Loeske, *Diplophyllum* (Dumort.) Dumort., *Lophozia* (Dumort.) Dumort., *Scapania* (Dumort.) Dumort. and *Tritomaria* Schiffn. ex Loeske in the Scapaniaceae, *Nardia* Gray in the Jungermanniaceae, *Saccogyna* Dumort. in the Geocalycaceae and *Southbya* Spruce, and possibly *Gongylanthus* Nees, in the Arnelliaceae (Read *et al.* 2000, Kottke *et al.* 2003, Kottke & Nebel 2005, Nebel *et al.* 2004, Duckett *et al.* 2006b, Bidartondo & Duckett 2010, Newsham & Bridge 2010). However, the most widespread symbiosis by far in leafy liverworts (Kowal *et al.* 2018, Rimington *et al.* 2020) involves ascomycetes symbionts, first acquired in the rhizoids of the Schistochilaceae (Pressel *et al.* 2008a, b) with several families in Jungermanniideae, including Schistochilaceae, Lepidoziaceae, Calypogeiaceae, Cephaloziaceae and Cephaloziellaceae shown to be consistently colonized by *Rhizoscyphus* (*Pezoloma, Hymenoscyphus* or *Pezizella) ericae* (D.J. Read) W.Y. Zhuang & Korf (Upson *et al.* 2007), the same fungus known to form ericoid mycorrhizas with the roots of ericaceous plants (Read *et al.* 2004, Smith & Read 2008). Because the symbiotic status of many Jungermanniideae families remains to be investigated, it is not yet possible to obtain a comprehensive picture of the distribution of Basidiomycota and Ascomycota associations in this subclass and thus determine whether these symbioses had a single or multiple origins (Pressel *et al.* 2010, Rimington *et al.* 2020). Current understanding of the diversity and distribution of Basidiomycota in liverwort phylogenies, and demonstrations that basidiomycete symbionts are highly host specific, indicate that Basidiomycota associations likely had multiple origins (Duckett *et al.* 2006b, Bidartondo & Duckett 2010). On the other hand, DNA sequencing data identifying but one fungus, *Rhizoscyphus ericae* (Upson *et al.* 2007), in the rhizoids of all ascomycete-containing lineages, together with cross-inoculation experiments (Duckett & Read 1995, Chambers *et al.* 1999, Read *et al.* 2000, Upson *et al.* 2007) point to a possible monophyletic origin of the liverwort-Ascomycota symbiosis. However, cytological differences between the rhizoidal colonisation of different liverwort groups (Pressel *et al.* 2010) suggest that multiple origins are equally likely. A decade ago we proposed that identifying the fungus colonising the swollen rhizoid apices of *Mylia anomala*, which stand out by being considerably larger than those in the ascomycete-containing genera but resemble those of the related southern hemisphere Balantiopsidaceae (de Roo *et al.* 2007, Duckett & Ligrone, 2008a), should be a priority for investigation (Pressel *et al.* 2010). Here we report for the first time the results of a molecular and cytological study aimed at resolving the identity of the rhizoidal fungus in *M. anomala* and test the hypothesis that *Mylia* also harbours *Rhizoscyphus ericae* via its likely schistochilaceous ancestry (de Roo *et al.* 2007, Pressel *et al.* 2010).

The 'odd' case of *Mylia anomala* swollen rhizoids

From the distribution of fungi overall in the Jungermanniales, the swollen rhizoids of *Mylia anomala* immediately stand out as highly enigmatic; without cytological and molecular data it is very difficult to even guess at the nature of their mycobiont. Growing over *Sphagnum* L., together with a mixture of ascomycete and basidiomycete-associated liverworts, members of both of these fungal groups should be readily available as possible colonists of its rhizoids.

Mylia anomala is the only *Mylia* species with swollen rhizoids; the other three species (Paton, 1999) all appear to lack fungi (pers. obs., Duckett *et al.* 1991). Traditionally located in the Jungermanniaceae (e.g., Paton 1999), Grolle (1963) placed *Mylia* in an independent family, Myliaceae, whereas Schuster (1969) erected the subfamily Mylioideae only for the familial level to be reinstated by Engel & Braggins (2005). The last two authors, on the basis of cell wall characters, but ignoring the unique rhizoids, suggested that *M. anomala* was best retained in the Jungermanniaceae but as a new genus, *Leiomylia,* that they placed in its own subfamily within Jungermanniaceae, the Leiomylioideae. In earlier molecular analyses, but based only on *M. taylorii* (Hook.) Gray, *Mylia* is an isolated lineage (Hentschel *et al.* 2006, 2007). However, de Roo *et al.* (2007) demonstrated that *Leiomylia* J.J. Engel & Braggins/*Mylia anomala* and *M. taylorii* have a well-supported sister relationship, thus calling into doubt the generic status of *Leiomylia*. The two species form a long branch in both Bayesian and parsimony phylogenetic analyses. They are resolved as either sister to the majority of Jungermanniales (parsimony) including all the taxa with either ascomycete or basidiomycete symbionts or, more likely, within the Jungermanniineae (Bayesian analysis) as sister to a clade that is mostly fungus-free, except for basidiomycetes in a few scattered genera (*Gongylanthus, Southbya, Nardia*) and a few ascomycetes in others

(*Anastrophyllum, Calypogeia* Raddi*, Eremonotus, Geocalyx, Roivainenia* Perss.) (Bidartondo & Duckett 2010). With *Pachyschistochila* (R.M. Schust.) R.M. Schust. & J.J. Engel as the immediate sister to *Mylia* in both topologies the most likely hypothesis for the origin of its symbiosis is that it retained *Rhizoscyphus* from a schistochilaceous ancestor. This appears all the more likely since cross-colonisation experiments revealed that the *Pachyschistochila* fungus forms typical rhizoidal associations across a range of liverworts (Pressel *et al.* 2008b). More recent phylogenetic analyses have confirmed *L. anomala* and *M. taylorii* as sister taxa in a strongly supported clade and the return of *Leiomylia* to the Myliaceae together with the removal of Myliaceae from the Jungermanniineae, and validation of a newly established monogeneric suborder Myliineae to house it (Shaw *et al.* 2015).

In order to determine the identity of the fungus colonising the rhizoids of *Mylia anomala* and to compare the cytology of the association with that of other liverworts we prepared samples of *M. anomala*, collected from three widely separate sites in Southern England, Scotland and Ireland for scanning and transmission electron microscopy. Further, we sequenced the fungal nuclear ribosomal internal transcribed spacer region, aligned the DNA sequences obtained together with the most similar sequences retrieved from GenBank via BLAST, and then we used the alignment to generate a distance tree using neighbour-joining as described previously (Bidartondo & Duckett 2010). Representative newly obtained DNA sequences are accessioned in GenBank (MZ062410-MZ062415).

It is immediately apparent from scanning electron micrographs (Figs. 3, 4) that the swollen-tipped rhizoids in *Mylia* are much larger than those that harbour *Rhizoscyphus*. Those in the other families have rhizoid shafts 10 µm or less in diameter with swollen tips between 25 and 35 µm (Fig. 3A) compared to the 20–30 µm shafts and 50–80 µm terminal swellings in *Mylia* (Fig. 3B, C). The swollen-tipped rhizoids in *Cephalozia* (Dumort.) Dumort. are thin-walled (Fig. 4C) whereas those in *Mylia* are thick with hyphae often running along their multiple stratifications (Fig. 3D, Fig. 4A). Numerous Golgi bodies in the host cytoplasm presumably contribute to the prominent interfacial matrix (Fig. 3D–G) around the hyphae where amyloid deposits are frequent (Fig. 3G). Simple septa and Woronin bodies confirm the ascomycetous nature of the symbiont (Fig. 4G). Two other striking features of the host rhizoids are the compound oil bodies typical of *Mylia anomala* (Fig. 3E), compared with the simple oil droplets in *Cephalozia* (Paton,1999) and starch grains in the plastids (Fig. 3E–G). The latter are notably rare in the infected cells in other liverworts (Fig. 4D) and hornworts.

Molecular analyses show that the fungi colonising *Mylia* rhizoids comprise several species of *Meliniomyces* Hambl. & Sigler (Hambleton & Sigler 2005) alongside *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew and *Rhizoscyphus ericae* (Fig. 5), all currently placed in the *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan aggregate. Fungi in the genus *Meliniomyces*, including *M. variabilis* Hambl. & Sigler and *M. bicolor* Hambl. & Sigler are known to form ericoid mycorrhizas with several species in the Ericaceae (Grelet *et al.* 2009) and some, e.g. *M. bicolor*, can be ectomycorrhizal with temperate forest trees (Villarreal-Ruiz *et al.* 2004). *Meliniomyces* and *Cadophora finlandica* have often also been reported to grow as endophytes in the roots of non-host plants (Tedersoo *et al.* 2009, Perotto *et al.* 2018).

The present demonstration that the association in *Mylia anomala* is different from others previously described in liverworts, all of which include only *Rhizoscyphus ericae*, points to possible independent evolution and underlines the current subordinal status of the Myliineae. We have failed to find fungi in a range of genera in the allied families Trichotemnomataceae (*Trichotemnoma* R.M. Schust.), Balantiopsidaceae (*Balantiopsis* Mitt.*, Isotachis* Mitt.), Acrobolbaceae (*Acrobolbus* Nees, *Goebelobryum* Grolle, *Lethocolea* Mitt., *Marsupidium* Mitt., *Tylimanthus* Mitt.), Blepharidophyllaceae (*Blepharidophyllum* Ångstr., *Clandarium* (Grolle) R.M. Schust.) whilst the next nearest family the Arnelliaceae harbours basidiomycetes. Having also drawn a blank from cytological observations on other leafy families whose fungus status was previously unknown, it is now reasonable to conclude that *Mylia anomala* plugged the biggest gap in knowledge.

It would now be interesting to determine if the *Mylia* association with members other than *Rhizhoscyphus ericae* of the *Hymenoscyphus ericae* aggregate are also mycorrhizal-like in terms of function. Recently it has been shown that *R. ericae* forms nutritional mutualisms with leafy liverworts. Isotope tracer experiments have demonstrated that *R. ericae* colonising the rhizoids of *Cephalozia bicuspidata* (L.) Dumort. transfers nitrogen and phosphorus to its host in exchange for photosynthesis-fixed carbon (Kowal *et al.* 2018). Whether associations with other ascomycete fungi, such as those inhabiting *Mylia* rhizoids, are also mycorrhizal-like now need experimental investigation. Another major functional knowledge gap as far as liverworts are concerned, are the basidiomycete symbioses in leafy Scapaniaceae and Arnelliaceae (Bidartondo & Duckett 2010, Kowal *et al.* 2018) and thalloid Aneuraceae except in the achlorophyllous epi-parasite *Aneura* (*Cryptothallus*) *mirabilis* (Malmb.) Wickett & Goffinet which obtains its carbon via an interlinked *Tulasnella* fungus that simultaneously forms ectomycorrhizas with trees (Bidartondo *et al.* 2003).

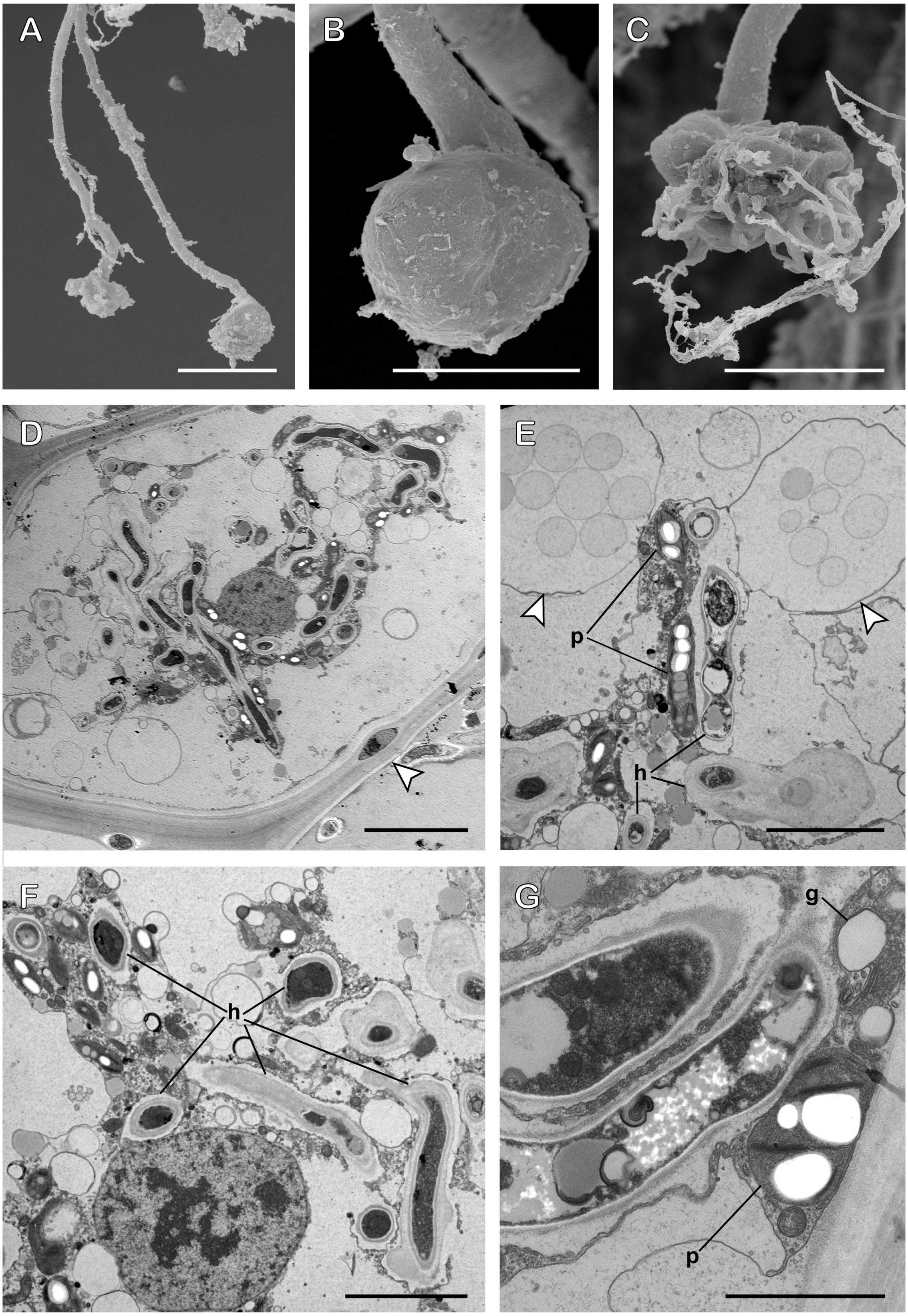

FIGURE 3. The distinct fungal assocation in *Mylia anomala* (Hook.) Gray vs. that in *Cephalozia lunulifolia* (Dumort.) Dumort.. A–C. Scanning electron micrographs. D–G. Transmission electron micrographs. A. Swollen rhizoid tip in *Cephalozia lunulifolia*. B-G. *Mylia anomala*. B, D. Large, swollen rhizoid tips. E. Thick-walled, swollen rhizoid tip packed with hyphae and showing hyphae in the wall multiple stratifciations (arrowed). E. Plastids (p) with prominent starch grains adjacent to fungal hyphae (h) and compound oil bodies (arrowed). F. Dense host cytoplasm surrounding fungal hyphae with prominent interfacial matrix (h). G. Golgi body (g) and plastid (p) adjacent to an hypha with amyloid deposits. Scale bars = (A–C) 50 µm; (D) 10 µm; (C, F) 5 µm; (G) 1 µm.

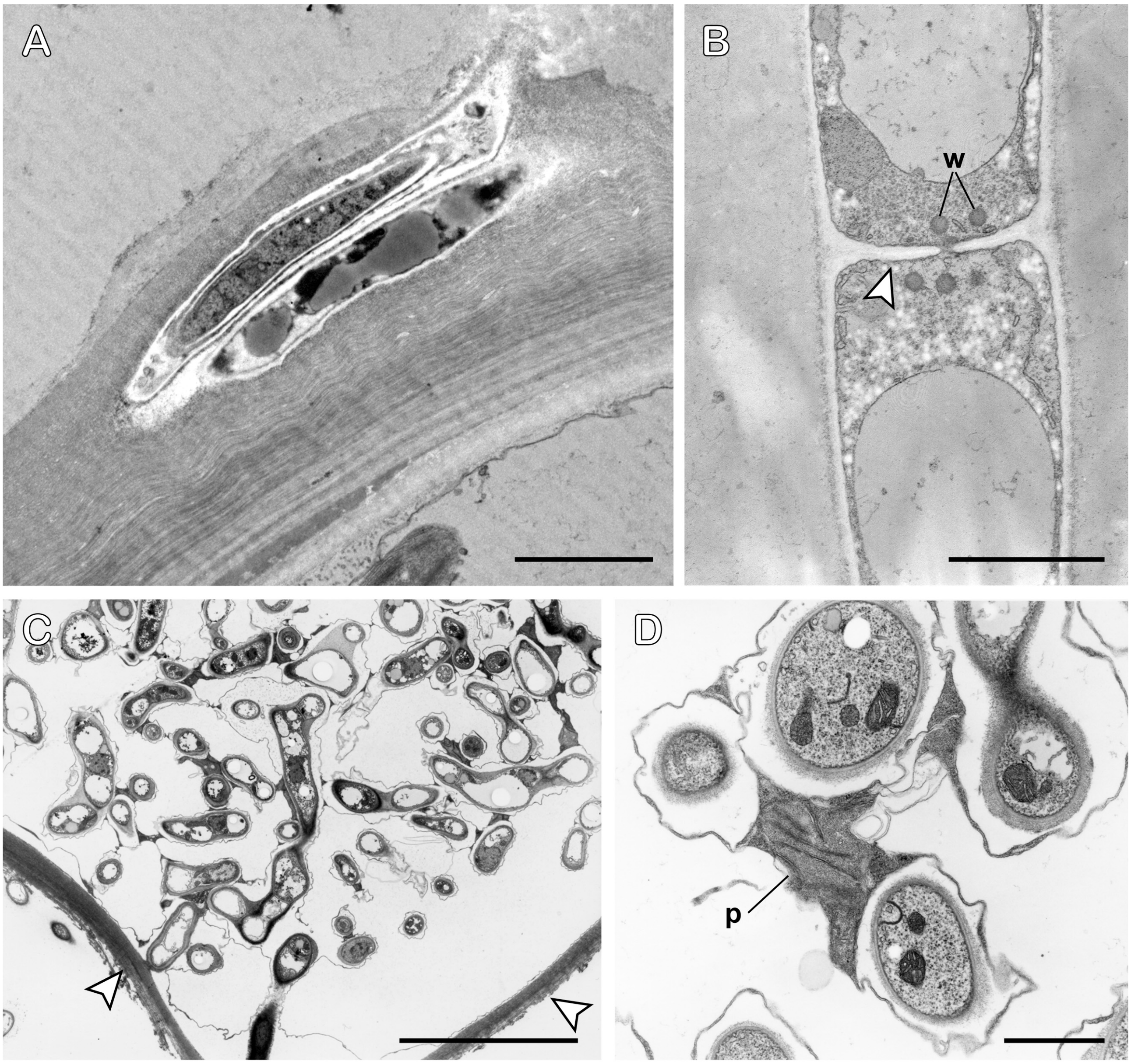

FiGURE 4. The distinct fungal association in *Mylia anomala* vs. that in *Cephalozia connivens* (Dicks.) Lindb. Transmission electron micrographs. A, B. *Mylia anomala*. A. Detail of fungal hyphae in the multistratose wall of a swollen rhizoid tip. B. Simple septum (arrowed) with Woronin bodies (w). C, D. *Cephalozia connivens* C. Thin-walled (arrowed) rhizoid tip packed with fungus. D. Starch-free plastid (p) adjacent to fungal hyphae. Scale bars = (C) 10 µm; (A) 2 µm; (B, D) 1 µm.

Fungal associations in hornworts

Until recently, investigations of fungal associations in hornworts have been limited to two investigations. The study by Ligrone (1988) showed that the ultrastructure of the fungus colonizing the thallus of *Phaeoceros laevis* (L.) Prosk. was very similar to that of AM fungi in vascular plants and thalloid liverworts. A decade later, the demonstration by Schüßler (2000) that *Anthoceros punctatus* L. was able to form an arbuscular mycorrhizal-like symbiosis with two isolates of *Glomus claroideum* Schenck & Smith, corroborated the notion that hornworts are symbiotic with members of the Glomeromycotina. The subsequent molecular detection of *Endogone*-like Mucoromycotina fungi in two species of *Phaeomegaceros* R.J. Duff, J.C. Villarreal, Cargill & Renzaglia alongside that of Glomeromycotina in *Phaeoceros carolinianus* (Michx.) Prosk., *P. laevis* (L.) Prosk., *Anthoceros laminifer* Steph. and *A. husnotii* Steph. (Bidartondo *et al.* 2011) indicated that hornworts, *on a par* with thalloid liverworts, might be able to engage with a wider repertoire of symbiotic fungi than previously thought, as subsequently confirmed by the first global molecular and cytological study of fungal associations in this group (Desirò *et al.* 2013). Desirò *et al.* (2013) showed that both Glomeromycotina and Mucoromycotina symbioses are widespread across hornworts, with nine of the eleven recognized hornwort genera

(Villarreal & Renner 2012) found to harbour diverse glomeromycete and mucoromycete symbionts, often together in the same plant host. Fungal associations are notably absent in *Leiosporoceros dussii* (Steph.) Hässel, the only species in *Leiosporoceros* Hässel sister genus to all other hornworts, with secondary losses of symbioses associated, as in liverworts (Pressel *et al.* 2010), with change of habitat from terrestrial to epiphytic, in *Dendroceros* Nees, or in species within symbiotic genera growing in permanently wet habitats such as *Megaceros flagellaris* (Mitt.) Steph. and the New Zealand endemic *Nothoceros giganteus* (Lehm. & Lindenb.) J. Haseg. ex J.C. Villarreal, Hässel & N. Salazar. The symbiotic status of *Phymatoceros* Stotler, W.T. Doyle & Crand.-Stotl., reported to harbour fungal endophytes (Stahl 1949) remains to be confirmed by cytological and molecular analyses.

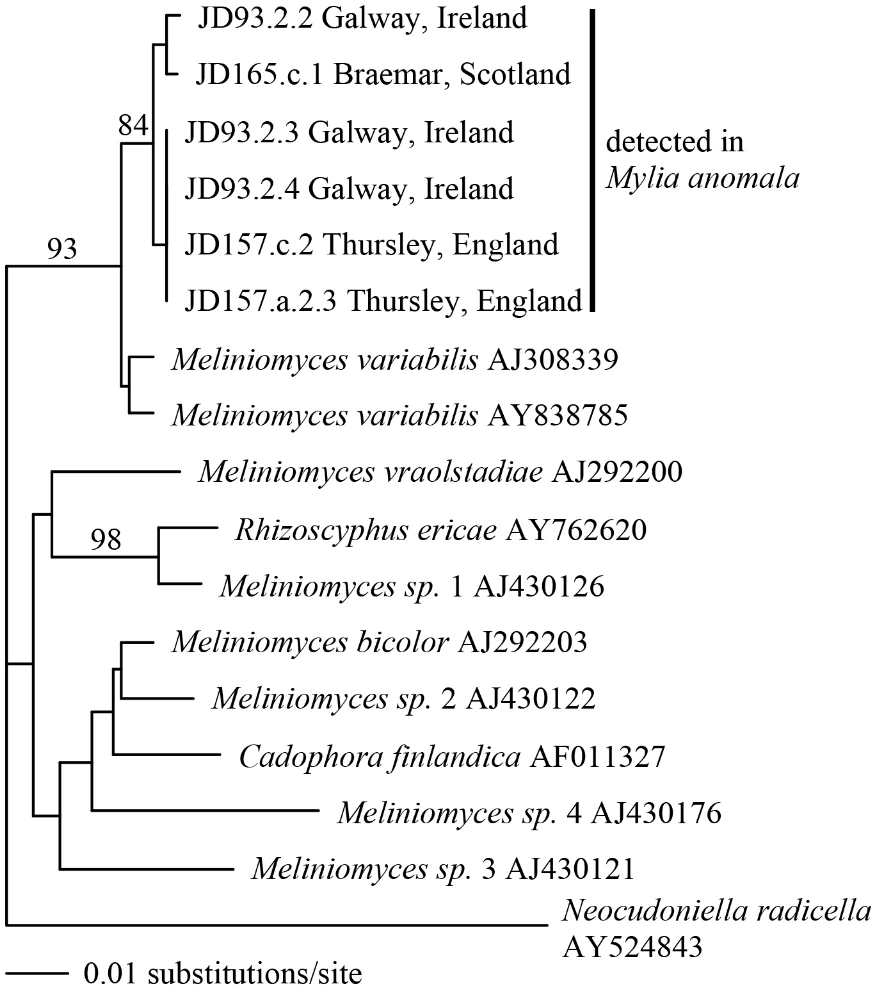

FIGURE 5. Phylogenetic placement of fungal associates of *Mylia anomala* within the "*Hymenoscyphus ericae* aggregate" (sensu Hambleton & Sigler 2005). This fungal nuclear internal transcribed spacer phylogram is based on neighbour joining with bootstrap (values > 70% are shown) of ten representative fungal isolate DNA sequences retrieved from GenBank and six sequences from thalli representing three sites. *Neocudoniella radicella* L.M. Kohn, Summerb. & Malloch was used as the outgroup.

Similarly to thalloid liverworts, hornworts form symbioses with nearly all clades of Glomeromycotina (Desirò *et al.* 2013), and are more often colonized by early divergent lineages than flowering plants (Rimington *et al.* 2018). While this finding indicates that both symbioses are likely ancient, the taxonomic distribution of Glomeromycotina and Mucoromycotina fungi in hornworts does not allow to infer conclusively the symbiotic history of these fungi and whether either lineage or both were present in the ancestor of hornworts (Desirò *et al.* 2013, Rimington *et al.* 2018). The absence of symbiosis in the earliest *Leiosporoceros* and the presence of both fungi in the Anthocerotaceae (Fig. 1), which include *Anthoceros* and *Folioceros*, and is the sister group to the remaining taxa (Söderström *et al.* 2016), point to three possible scenarios: Glomeromycotina as ancestral and followed by at least five losses of AM fungi together with three gains of Mucoromycotina; ancestral Mucoromycotina with five subsequent losses of symbiosis (Desirò *et al.* 2013); or both fungal lineages having formed the ancestral hornwort-fungus symbiosis (Rimington *et al.* 2018).

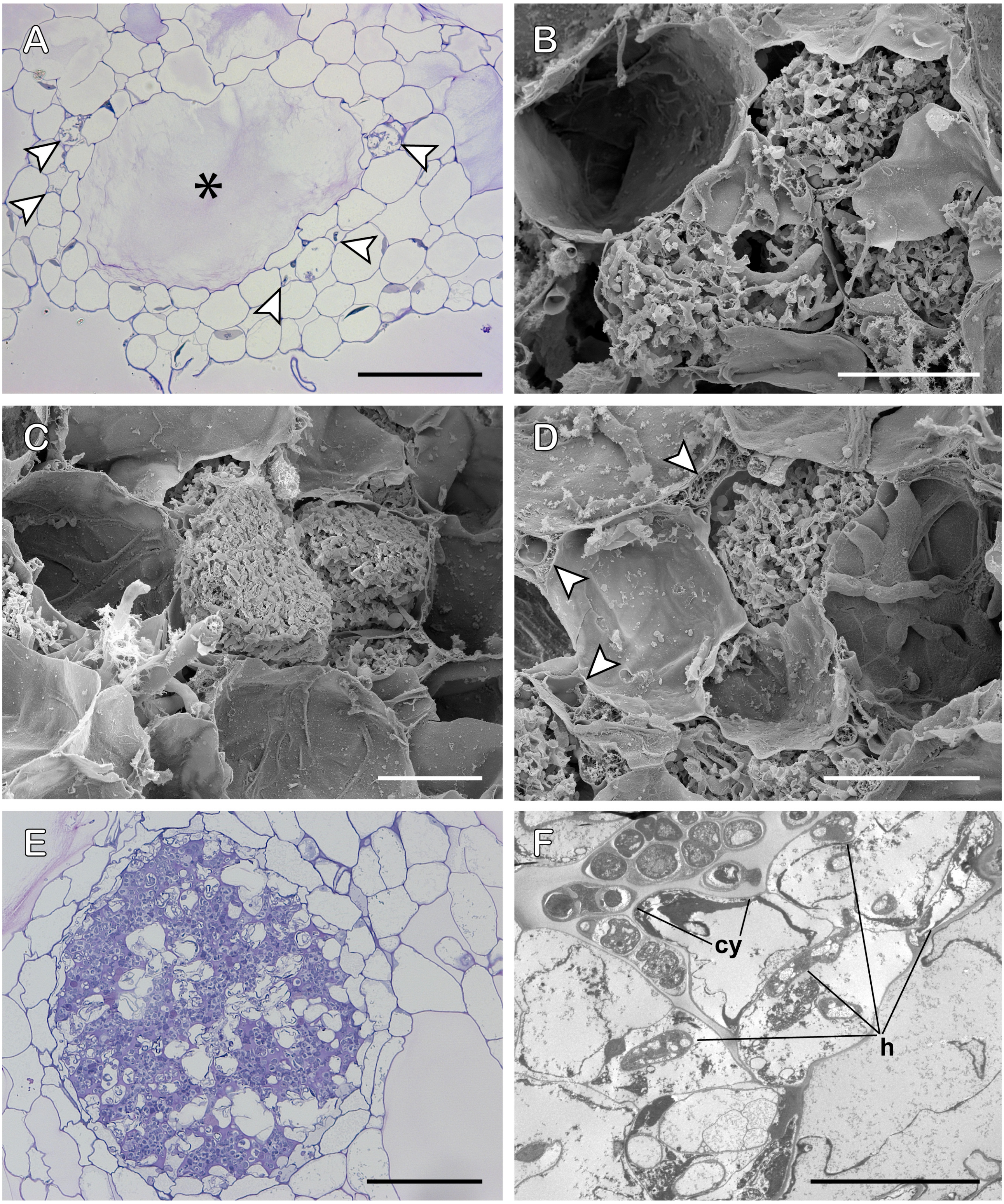

FIGURE 6. Fungal colonisation in hornworts. A, E. Light micrographs. B-D. Scanning electron micrographs. F. Transmission electron micrographs. A. Intracellular and intercellular fungal hyphae scattered in the thallus of *Anthoceros cristatus* Steph., the large mucilage cavities (*) are fungus-free. B–D. Fungal structures in the thallus of *Phaeoceros himalayensis* (Kashyap) Prosk. ex Bapna & G.G. Vyas include intracellular arbuscules (B), tightly wound coils (C) and intercellular hyphae (D, arrowed). E, F. *Anthoceros punctatus* L. cyanobacterial colonies (cy) with closely associated hyphae (h). Scale bars = (A, E) 100 µm; (B–D, F) 20 µm.

Compounding these uncertainties is the currently unresolved position of hornworts in land plant phylogeny. Several recent phylogenomic studies have challenged the general consensus of liverworts as sister to all other embryophytes and hornworts sister to the vascular plants (Qiu *et al.* 2006) and found strong support for the monophyly of liverworts and mosses (Setaphyta) (e.g. Wickett *et al.* 2014, Puttick *et al.* 2018, Sousa *et al.* 2018, 2020). However, whether hornworts are the sister group to the Setaphyta in monophyletic bryophytes (Harris *et al.* 2020) or, in paraphyletic bryophytes, either the earliest divergent embryophyte lineage or the one most closely related to vascular plants (Puttick *et al.* 2018, Sousa *et al.* 2020) remains to be established.

The cytology and distribution of fungal colonization in hornworts (Desirò *et al.* 2013) show both parallels and differences with those in thalloid liverworts (Fig. 6). Fungal structures attributable to both Glomeromycotina, i.e. arbuscules with trunk hyphae and large vesicles, and Mucoromycotina symbionts (Fig. 6A–D), e.g. intercellular semi-parenchymatous hyphal structures and thick, multilayered hyphae in the mucilage-filled intercellular spaces, as typical of Mucoromycotina associations in *Treubia* and *Haplomitrium* (Carafa *et al.* 2003, Duckett *et al.* 2006, Field *et al.* 2015a), have been described in the thalli of diverse hornwort species (Desirò *et al.* 2013). However, while in liverworts fungi usually occupy specific regions of the thalli (Fig. 2A–D) (Ligrone *et al.* 2007, Pressel *et al.* 2010, Field *et al.* 2016, Rimington *et al.* 2018) or in the case of *Haplomitrium* are restricted to the epidermal layers of its rhizoidless underground axes (Carafa *et al.* 2003, Field *et al.* 2015a), such distinct zonation is not present in hornworts, where fungi are more randomly distributed in the thallus except for the large mucilage cavities, when these are present (Fig. 6A). In contrast to the ubiquitous role of rhizoids as conduits for fungal entry in thalloid liverworts (Ligrone *et al.* 2007, Pressel *et al.* 2010), hornwort rhizoids are invariably devoid of fungi (Ligrone 1988, Desirò *et al.* 2013, pers. observations), a situation with a single liverwort counterpart in *Treubia*. In *Treubia* fungal entry is via mucilage-filled spaces between the epidermal cells (Duckett *et al.* 2006a, Pressel *et al.* 2010); whether in hornworts fungal entry is via mucilage clefts (Ligrone 1988) remains to be confirmed.

The functional significance of fungal associations in hornworts also remains to be established. In contrast to liverworts, where different taxa are either obligate symbiotic or lack fungi altogether, the occurrence of fungal associations in hornworts appears variable, with the same species variously found to harbour either or both Glomeromycotina and Mucoromycotina symbionts or to be fungus-free (Desirò *et al.* 2013). In liverworts, the ability to form associations with both fungal groups is thought to confer nutritional advantages to the host plant. Dual symbioses in *Neohodgsonia mirabilis* and *Allisonia cockaynei* (Steph.) R.M. Schust. have been shown to afford greater gains of phosphorus and nitrogen to their liverwort hosts than symbioses involving a single fungal group, i.e. Mucoromycotina in *Treubia lacunosa* (Colenso) Prosk. and *Haplomitrium gibbsiae* (Steph.) R.M. Schust. (Field *et al.* 2015a) and Glomeromycotina in *Marchantia* (*Preissia*) *quadrata* Scop. and *Marchantia paleacea* Bertol. (Field *et al.* 2012), but at a greater carbon cost (Field *et al.* 2016). Recent physiological analyses using microcosm and isotope tracer experiments have indicated that Mucoromycotina symbionts are functionally distinct yet complementary to Glomeromycotina, because of their greater ability to access and transfer nitrogen to their liverwort hosts from both inorganic and organic sources (Field *et al.* 2019). Together these findings suggest that the ability to engage with both Mucoromycotina and Glomeromycotina fungal partners may offer additional plasticity in terms of nutrient uptake to liverworts (Field *et al.* 2016) by increasing the availability and range of nutrient pools. The apparent greater ability of Mucoromycotina over Glomeromycotina symbionts to supply liverwort hosts with organically derived nitrogen is likely to provide a competitive advantage in disturbed, primary successional habitats where nitrogen may be limiting and the distribution of organic nutrients patchy (Field *et al.* 2019).

An added 'symbiotic' dimension in hornworts is the ubiquitous presence of N_2-fixing cyanobacteria endophytes in this group (Renzaglia *et al.* 2009, Frangedakis *et al.* 2021), mostly in the genus *Nostoc* Vaucher ex Bornet & Flahault, which have been shown to transfer up to 80% of their fixed nitrogen to their hornwort hosts (Adams 2002, Adams & Duggan 2008) (Fig. 6E). Cyanobacteria endophytes in liverworts are restricted to *Blasia* L. and *Cavicularia* Steph. in the Blasiales (Adams 2002, Rikkinen & Virtanen 2008), the only early divergent liverwort lineage which consistently lack fungal symbionts (Pressel *et al.* 2010), and are absent from the pteridophytes except for the aquatic fern *Azolla* Lam., which also lack fungi (Whitton 1993). Some moss species associate with cyanobacteria but here the association is strictly epiphytic (Warshan *et al.* 2017) or within the dead hyalocysts in *Sphagnum* (Meeks 1989) and endosymbioses with fungi have never been reported in this group (Pressel *et al.* 2010, Field *et al.* 2015b). As such, hornworts stand out as the only spore-producing extant embryophyte lineage to associate with both fungal and cyanobacterial symbionts. Seed plant counterparts are found in the cycads (Lindblad & Bergman 2018) and possibly *Gunnera* L. (Bergman *et al.* 1992). It has been suggested that in cycads AM fungi may provide phosphorus in phosphorus-limited environments to the cyanobacteria (Fisher & Vovides 2004), a well-known phenomenon in the more recently evolved tripartite symbioses between legumes, rhizobia and arbuscular mycorrhizal fungi (Azcón *et al.* 1991, Xavier & Germida 2002, 2003, Jia *et al.* 2004). Mycorrhizas in *Gunnera* have only been reported once, in the endemic Hawaiian *Gunnera petaloidea* ssp. *kauaiensis* (A. Gray) Doyle (Koske *et al.* 1992), but here fungus and cyanobacterium are spatially separated, the latter being restricted to specialised stem glands.

Observations that in hornworts fungal hyphae often occur in close associations with cyanobacterial colonies (Fig. 6E, F) may be indicative of a functional relationship between microbes (Desirò *et al.* 2013). It has been argued that the more abundant the cyanobacteria the less likely are hornworts to harbour fungi, since fungi are lacking from *Leiosporoceros*, which has the most extensive cyanobacterial colonies (Villarreal & Renzaglia 2016) and in the only cyanobacteria-containing liverworts, the fungus-free Blasiales, *Nostoc* are more numerous than in hornworts (Desirò *et al.* 2013). However, it should be noted that cyanobacterial colonisation in *Leiosporoceros* is distinct from that in all other hornworts, where continuous formation of mucilage clefts supports multiple invasions of a single thallus (Villarreal & Renzaglia 2016) and, presumably, fungal entry (Desirò *et al.* 2013, Ligrone 1988). In *Leiosporoceros* bifurcating and interconnected strands of *Nostoc* are locked inside the thallus following a single colonisation event in the sporeling stage through mucilage clefts whose production ceases once cyanobacterial colonies are established (Villarreal & Renzaglia 2016). In the Blasiales, the whole symbiotic consortium (bryophyte and cyanobiont) presumably can disperse together via their *Nostoc*-containing stellate gemmae, possibly allowing for specific symbiotic consortia to be maintained (Costa *et al.* 2001). Probably the most striking cytological difference between the *Nostoc* colonies in *Blasia* and hornworts is that, in the former the surrounding cells and the filaments traversing the colonies have prominent wall ingrowths diagnostic of transfer cells (Duckett *et al.* 1977) whereas the corresponding cells in hornworts are thin-walled and highly vacuolate (Figure 6E, F). These features suggest very different functional relationships. Interesting to note here is that in *Blasia* and *Cavicularia* the slime papillae of the cavities housing *Nostoc* play a crucial role in the plant-cyanobacterium symbiosis by attracting the cyanobiont and by developing into the transfer cells mentioned before (Rikkinen 2017 and literature therein), whilst slime papillae are absent in hornworts. Whether slime papillae in other liverworts also play a role in their interactions with soil microbes (Rikkinen 2017) is currently unknown but certainly deserves further scrutiny.

Clearly, there is an urgent need to determine whether fungal associations in hornworts, like those in thalloid liverworts, are mycorrhizal-like and to investigate possible functional relationships between co-occurring fungal and cyanobacterial symbionts. Latest genomic analyses of the emerging model species *Anthoceros agrestis* Paton (Szövényi *et al.* 2015, Szövényi 2016, Frangedakis *et al.* 2020) have identified orthologues of all the key angiosperm genes required for the establishment and maintenance of the AMF symbiosis (Li *et al.* 2020). Given that other bryophyte model species with published genomes, the moss *Physcomitrium* (*Physcomitrella*) *patens* and the liverwort *Marchantia polymorpha* subsp. *polymorpha*, both lack fungal symbionts, physiological and molecular analyses of fungal associations in *Anthoceros* may hold the key to unravel the origin and evolutionary history of mycorrhizal associations in land plants.

Further, the presence of cyanobacteria in the prostrate mycorrhizal axes of the 400 million years old fossil plant *Aglaophyton major* (Krings *et al.* 2009) together with genomic evidence placing the evolution of endosymbiotic *Nostoc* clades at ca. 950 million years ago (Warshan *et al.* 2018), all indicate that the land plant-cyanobacterial symbiosis may be as ancient as that formed with fungi. It is possible therefore that associations involving both fungi and cyanobacteria, as seen today in hornworts, were common at the dawn of plant terrestrialisation. An alternative hypothesis is that earliest symbioses on land involved fungi and cyanobacteria and that, through these, fungi might have become pre-adapted for mycorrhization (Schüßler 2012). Today *Geosiphon pyriforme* (Kütz.) v. Wettstein is the only known example of a glomeromycete fungus in endobiotic association with a cyanobacterium (Schüßler 2002, Fisher & Vovides 2004) and, interestingly, its hyphal networks are often found associated with the rhizoids of *Anthoceros* and other bryophytes (Chapman & Margulis 1988).

Whether this association represents a modern oddity or a relic of an ancient association between fungi and cyanobacteria that preceded plant terrestrialisation and the evolution of plant-fungal symbiosis remains to be established. Fungi and cyanobacteria are thought to have inhabited the terrestrial environment as part of microbial mats long before the establishment of the land flora (Edwards *et al.* 2015, Lenton & Daines 2017) and Glomeromycotina have been shown to possess two additional prokaryotic (class I) ribonuclease III protein-coding genes arising by putative horizontal gene transfer from cyanobacteria, suggestive of an ancient symbiotic history between Glomeromycotina and cyanobacteria (Lee *et al.* 2018).

Conspectus

The last decade witnessed major advances in understanding of bryophyte fungal associations. Extensive DNA sequencing studies have revealed that these are formed with a wider repertoire of fungal symbionts than ever envisaged at the start of the 21st Century, including newly discovered partnerships in Haplomitriopsida, Marchantiopsida and Pelliidae and throughout hornworts with members of the ancient fungal sub-phylum Mucoromycotina (Bidartondo *et al.* 2011, Desirò *et al* 2013, Rimington *et al.* 2018, 2019, 2020). Parallel physiological studies of liverwort-fungus associations have not only confirmed that these are mycorrhizal-like but have also highlighted key differences between Glomeromycotina and Mucoromycotina symbionts both in their sensitivity to changes in atmospheric CO_2 concentrations and functionality, with Mucoromycotina having a more prominent role in liverwort nitrogen nutrition in line with their apparent ability to assimilate and transfer N from both inorganic and organic sources (Field *et al.* 2015a, 2016, 2019). These studies have provided important insights into the origin and evolution of mycorrhizal associations in land plants, indicating that members of both fungal lineages might have engaged with the earliest embryophytes and played a key role in plant terrestrialisation. Major remaining knowledge gaps are the phylogenetic position of hornworts in the plant tree of life, whether their fungal associations are also mycorrhizal-like and whether hornworts, their ubiquitous cyanobacterial partners and varied fungal endophytes form tripartite symbiotic consortia, possibly representative of the ancestral symbiotic condition of land plants. Expanding genomic resources, including the development of *Anthoceros agrestis* as the first hornwort model species (Szövényi *et al.* 2015) hold much promise for answering these major evolutionary questions in the next decade.

Recent findings that the same Mucoromycotina symbionts of bryophytes are ‘shared’ by vascular plants (Hoysted *et al.* 2019), including angiosperms open up a new exciting chapter in mycorrhizal research. Whether the functional significance of symbioses with Mucoromycotina demonstrated in liverworts extend to the flowering plants is currently a key area of research with potentially major implications for our understanding of ecosystem functioning, both natural and agroecosystems. The recent development of methodologies for the isolation of Mucoromycotina symbionts and resynthesis of the symbiosis *in vitro* now provides a powerful experimental system for dissecting the role of these fungi across the land plant phylogeny and for determining their diagnostic structures and interfaces when colonising diverse host plants.

Our finding that the swollen rhizoids in *Mylia anomala*, reported here for the first time, contain *Meliniomyces*, rather than exclusively *Rhizoscyphus ericae*, and are cytologically distinct from those in all other ascomycete-containing liverworts fills the biggest gap in our knowledge of fungal associations across liverworts.

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