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Fungal symbioses in bryophytes: New insights in the Twenty First Century

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Abstract

Fungal symbioses are one of the key attributes of land plants. The twenty first century has witnessed the increasing use of molecular data complemented by cytological studies in understanding the nature of bryophyte-fungal associations and unravelling the early evolution of fungal symbioses at the foot of the land plant tree. Isolation and resynthesis experiments have shed considerable light on host ranges and very recently have produced an incisive insight into functional relationships. Fungi with distinctive cytology embracing short-lived intracellular fungal lumps, intercellular hyphae and thick-walled spores in *Treubia* and *Haplomitrium* are currently being identified as belonging to a more ancient group of fungi than the glomeromycetes, previously assumed to be the most primitive fungi forming symbioses with land plants. Glomeromycetes, like those in lower tracheophytes, are widespread in complex and simple thalloid liverworts. Limited molecular identification of these as belonging to the derived clade *Glomus* Group A has led to the suggestion of host swapping from tracheophytes. Ascomycetes, all identified to date as *Rhizoscyphus ericae* and having an extremely wide host range extending into the Ericales, occur in the rhizoids of a range of leafy liverwort families that are sister to the Schistochilaceae, where the fungus induces rhizoid branching and septation. Dating of the Schistochilaceae suggests that these liverwort rhizoid associations predated ericoid mycorrhizas. A more restricted distribution of basidiomycetes, all identified as members of the genus *Sebacina*, is interpreted as a secondary acquisition in leafy liverworts following the loss of ascomycetes. Unlike the latter and ectomycorrhizal basidiomycetes in tracheophytes, these are host specific. Whereas there is no evidence of host digestion for either the ascomycetes or basidiomycetes in leafy liverworts, basidiomycetes in the Aneuraceae display regular colonization and digestion cycles. Considerable molecular diversity in the aneuracean fungi, mainly in the genus *Tulasnella* with occasional sebacinoids, mirrors the same in the host liverworts. Nesting of the only mycoheterotrophic liverwort *Cryptothallus* within *Aneura* is congruent with the phylogeny of the endophytes. Glomeromycete fungi have been identified in *Phaeoceros* and *Anthoceros* whereas *Dendroceros, Megaceros* and *Nothoceros* lack endophytes but the distribution of fungi across hornworts has yet to be explored. In contrast to liverworts and hornworts, there is no evidence of mycorrhiza-like associations in mosses. Claims that the fossil *Prototaxites* was a fossil fungus-associated liverwort are discounted.

Key words: ascomycetes, basidiomycetes, co-evolution, fossils, glomeromycetes, mycorrhizas, phylogeny, *Rhizoscyphus ericae, Sebacina, Tulasnella*

Introduction

The recent discovery of mycorrhizal genes, probably homologous across all lineages of land plants (Wang *et al.* 2010, but also see the critique by Bonfante & Selosse 2010), provides a striking reaffirmation of the notion first proposed by Pirozynski & Malloch (1975) and variously elaborated subsequently with a liverwort-centred-focus (Selosse & LeTacon 1998; Nebel *et al.* 2004; Kottke & Nebel 2005) that fungal symbioses rank

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alongside stomata, cuticles, matrotrophy and conducting tissues as one of the key innovations of land plants. Since bryophytes are now firmly fixed at the base of the land plant tree of life, studies on their fungal symbioses probably represent the most likely source for new insights into the evolution of this phenomenon.

Our aim here is to review the state of knowledge of fungal symbioses in bryophytes with particular focus on new discoveries in the present century since the last overview by Read *et al.* (2000). As summarized in Schuster (1966) and Read *et al*. (2000), light microscope studies dating from the late 19th and first half of the 20th centuries documented the constant fungal associations in a variety of liverworts. The most striking are swollen hyphae-filled rhizoid tips in the Lepidoziaceae and allied families, a mosaic of infected and uninfected cells in stems of the Lophoziaceae, hyphal coils in specific regions of the thalli of many complex and simple thalloid genera and both inter- and extracellular fungal infection zones in *Treubia* K.I.Goebel. Today, a combination of electron microscope, molecular and experimental studies has not only confirmed all of these earlier findings but also has led to the identification of the endophytes and to the exploration of their host ranges. In addition, we are now beginning to map the distribution of fungi onto current liverwort phylogenies and provide estimates of their likely ages and patterns of evolution. However, there are still little or no data on the possible benefits of the associations except in the parasite *Cryptothallus* Malmb. (now renamed *Aneura mirabilis* (Malmb.) Wickett & Goffinet (Wickett & Goffinet 2008)). Here microcosm experiments have shown that the *Cryptothallus* fungus can form typical ectomycorrhizas with *Betula* L. and that the liverwort obtains its carbon indirectly from *Betula* via digestion of the common basidiomycetous endophyte (Bidartondo *et al.* 2003).

The extent of fungal associations in hornworts is less well known but a growing body of cytological evidence points to an independent origin from those in liverworts. In striking contrast we must underline that, despite a huge number of reports of fungal fruiting bodies growing on mosses (Dobbeler 1997), many of which are species-specific, a symbiotic relationship has yet to be demonstrated. Apart from specialized rostellopsid (chytrid) parasites that colonize the rhizoid apical cells in a handful of mosses (Martinez-Abaigar *et al.* 2005) all other fungal structures reported on mosses are confined to dead or dying host cells; we know not a single electron micrograph illustrating a healthy hypha in a healthy moss cell.

The first major advance beyond light microscope observations in furthering understanding of fungus-bryophyte associations came from electron microscope studies (Pocock & Duckett 1985; Ligrone 1988; Ligrone & Lopes 1989; Duckett *et al.* 1991; Ligrone *et al.* 1993; Ligrone & Duckett 1994). These studies led to the identification of the fungi as either glomeromycetes, ascomycetes or basidiomycetes and to the affirmation of the mutualistic nature of the interactions between the two partners. Subsequent experimental isolation and resynthesis studies began to identify different host ranges between groups (Duckett & Read 1995), whilst molecular studies produced much more precise identification of the fungi. The results to date have often been completely unexpected and the same is most likely in the future.

Haplomitrium and *Treubia*

Until the recent and detailed electron microscope studies by Carafa *et al.* (2003) and Duckett *et al.* (2006a) our knowledge of the endophytes in these two genera was limited to early light microscope investigations (Goebel 1891; Grun 1914; Lilienfeld 1911). These suggested that fungal associations in *Haplomitrium* Dedecek and *Treubia* might be different from those found elsewhere in land plants and so this proved to be.

Electron microscopy confirmed the presence of both extra and intracellullar hyphae in *Treubia* (Fig. 1). The former forms pseudoparenchyma-like structures within the mucilage-filled intercellular spaces in the thalli (Fig. 1D, E). In the older thalli these hyphae produce spores with highly distinctive multilamellate walls (Fig. 1F). The intracellular fungal zone lies in the lower part of the thalli and comprises coils of fine hyphae some of which terminate in short-lived fungal lumps (Fig. 1B, C). Exactly the same kind of intracellular cytology is found in the epidermal and sometimes the subepidermal cells in the subterranean mucilageinvested 'roots' of *Haplomitrium*. Spores with multilamellate walls like those in *Treubia* can also be seen in this mucilage. Unlike all other bryophyte-fungus associations where hyphal entry is via the rhizoids, in *Treubia* the rhizoids remain uninfected and entry is via mucilage-filled spaces between the epidermal cells. In *Haplomitrium*, which lacks rhizoids, hyphae penetrate directly through the epidermal cell walls.

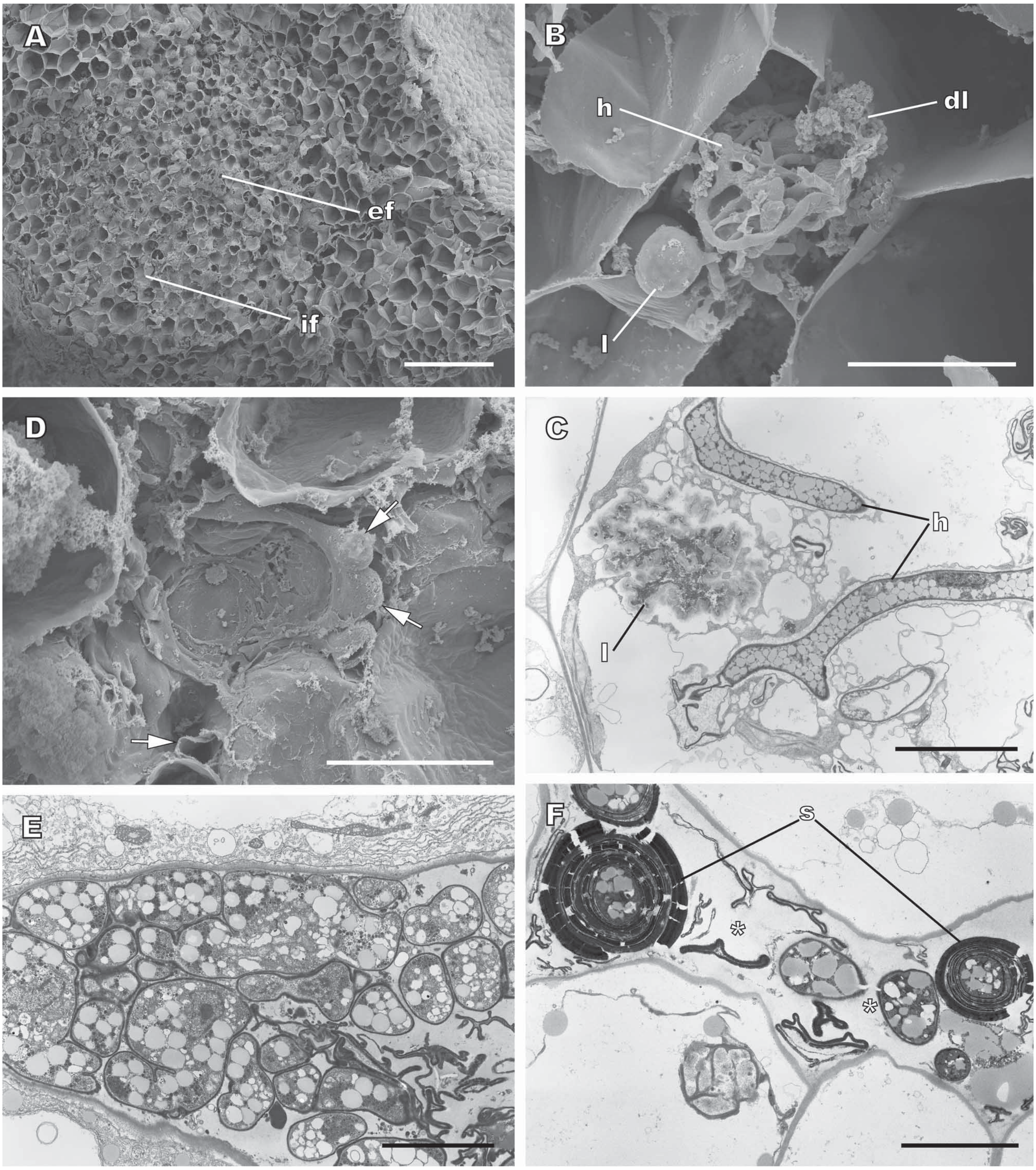


FIGURE 1. The highly differentiated endophyte in *Treubia pygmaea* R.M.Schust.. **A, B, D.** Scanning electron micrographs. **C, E, F.** Transmission electron micrographs. **A.** Profile of thallus showing the intercellular fungal zone (ef) and the intracellular zone below it (if). **B, C.** Details of the intracellular fungus showing hyphal coils (h), fungal lumps (l) and a degenerating lump (dl). **D, E.** Intracellular fungus showing pseudoparenchymatous hyphae (arrowed). **F.** Fungal spores (s) with multilayered walls in the intercellular mucilage (*). Scale bars = (A) 200 µm; (B, D) 20 µm; (C, E, F) 5 µm.

The presence of an intercellular phase for the infections in *Treubia* draws parallels with the same in *Lycopodium* L. (Duckett & Ligrone 1992) and even more strikingly with the Devonian plant *Nothia aphylla* Lyon ex Høeg. (Krings *et al.* 2007a, b). Given this distinctive ultrastructure and the fact that *Treubia* and *Haplomitrium* are now considered to have had a very long evolutionary history in the land plant tree of life, it is extremely disappointing that the only published molecular study to date (Ligrone *et al.* 2007) revealed the presence of a *Glomus* in *Haplomitrium chilensis* R.M.Schust. closely allied to the *Glomus* Tul. & C.Tul. Group A endophytes found in *Conocephalum* Hill*, Fossombronia* Hazsl. and *Pellia* Raddi. However, a much more extensive sequencing programme of the *Treubia* and *Haplomitrium* endophytes now in progress (Bidartondo & Duckett 2010, unpublished data) is revealing that these two genera, plus several primitive taxa in the thalloid liverworts, lack *Glomus* but contain zygomycetous endophytes, a group of fungi recently resolved as ancestral to the glomeromycetes (James *et al.* 2006). Thus, we now have the first glimpses of what may well turn out to be a hitherto unsuspected spectrum of novel fungal symbioses very early in the ancestry of land plants.

Complex & simple thalloid liverworts (Marchantiopsida & Jungermanniopsida, Metzgeriidae)

Extensive cytological studies (reviewed by Ligrone *et al.* 2007) have revealed that glomeromycete fungi forming associations that mirror those in Paris-type vesicular arbuscular mycorrhizas in vascular plants are extremely widespread in both these groups (Fig. 2). In the Marchantiopsida, fungi are absent from both the basal (Blasiales and Sphaerocarpales) and several derived clades including the Monosoleniaceae, Cyathodiaceae, Exormothecaceae, Monocarpaceae, Oxymitraceae and Ricciaceae. *Marchantia* L. is an interesting genus in that the fungi are absent from taxa growing on nutrient-rich substrates e.g. *M. polymorpha* subsp. *polymorpha* (Nees) Gottsche *et al.* and subsp. *ruderalis* Bischl. & Boisselier.

The exclusively intracellular endophytes enter their hosts through the rhizoids (only the smooth rhizoids in the Marchantiopsida (Fig. 2D)) and colonize particular taxon-specific regions of the thalli where they form trunk hyphae with numerous short-lived, regularly-dichotomizing arbuscular side branches (Fig. 2A, C). Unlike the lumps in *Haplomitrium* and *Treubia* which normally occupy less than 10% of the volume of colonised cells, single smooth-surfaced vesicles almost completely fill some of the thallus cells (Fig. 2B). The hyphae are surrounded by a narrow interfacial matrix which in *Pellia* (Fig. 2F) has a regular tubular substructure. Ubiquitous in these endophytes, as they are in *Haplomitrium* and *Treubia,* are Gram-positive bacteria (Fig. 2E). These bacteria, recently identified as members of the Mollicutes, appear to be ancestrally associated with glomeromycotean fungi (Naumann *et al.* 2010).

The much more fragmentary molecular data published to date show the presence of *Acaulospora* Gerd. & Trappe in *Monoclea* Hook, and fungi within or allied to *Glomus* Group A in *Marchantia, Conocephalum, Fossombronia* and *Pellia* (Russell & Bulman 2005; Ligrone *et al.* 2007). Experimental resynthesis studies (Read *et al*. 2000; Fonseca & Berbara 2008) confirm that *Glomus* can form typical associations in *Pellia* and *Lunularia* Adans.. Fonseca & Berbara (2008) go on to show that *Glomus* hyphae, extending from the liverworts, capture and translocate phosphorus into *Lunularia*, but do not demonstrate any major advantage in terms of increased vigour accruing to the host. However, most recently Beerling *et al.* (2010) have elegantly demonstrated that colonization of *Marchantia paleacea* Bertol. with *Glomus* promotes photosynthetic carbon uptake, growth and asexual reproduction. A simulated CO_2-rich atmosphere, similar to that of the Palaeozoic when land plants originated, amplifies the net benefits of the association. This study thus provides essential missing evidence supporting fungal symbioses as drivers of plant terrestrialization in early Palaeozoic land ecosystems.

The taxonomic distribution of the endophytes in the Marchantiopsida and Metzgeriidae indicates that glomeromycete associations are a plesiomorphy in these liverworts. Since the glomeromycete associations in liverworts involve cellular interactions like those in arbuscular mycorrhizas (Paszkowski 2006), Wang & Qiu (2006) have hypothesized homology in terms of their biological interactions. Indeed, cytologically the associations in gametophytes of lycopods and basal ferns (Duckett & Ligrone 1992, 2005; Schmid & Oberwinkler 1993, 1994, 1995) are virtually identical to those in the liverworts. The near basal position of the Marchantiopsida and Metzgeriidae in embryophyte phylogeny and the widespread occurrence of glomeromycetes within them fit the notion that the co-evolution of liverworts with these fungi predated the appearance of arbuscular mycorrhizas in tracheophytes (Kottke & Nebel 2005; Wang & Qiu 2006). However,

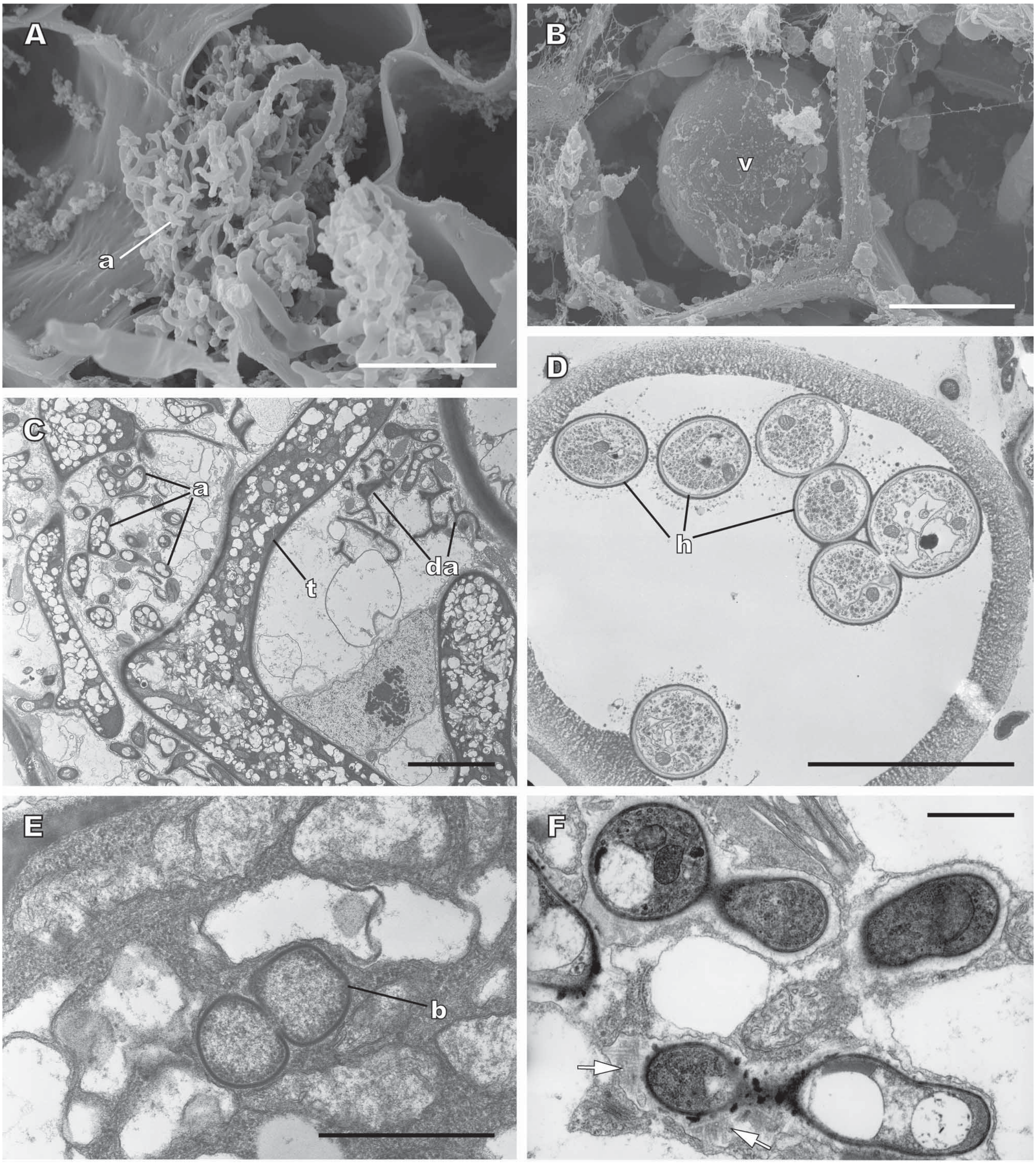


FIGURE 2. Typical features of glomeromycote associations in thalloid liverworts. **A, B.** Scanning electron micrographs. **C-F.** Transmission electron micrographs. **A.** *Preissia quadrata* (Scop.) Nees; dichotomously branching arbuscules (a). **B.** *Pellia epiphylla* (L.) Corda; fungal vesicle (v) almost completely filling a host cell. **C-E.** *Marchantia foliacea* Mitt. **C.** trunk hyphae (t), arbuscules (a) and degenerating arbuscules (da). **D.** Numerous hyphae (h) in a smooth rhizoid. **E.** Gram-positive dividing bacteria-like organism (b) in a fungal cell. **F.** *Pellia epiphylla*; arbuscules. Note the healthy host cytoplasm and the highly structured interfacial matrix (arrowed). Scale bars = (A, B) 20 µm; (C, D) 5 µm; (E, F) 1 µm.

the fact that members of *Glomus* Group A, a derived clade within the genus that also forms mycorrhizas in tracheophytes, are widespread in these liverwort taxa that are widely separate both geographically and phylogenetically led Selosse (2005) to an alternative hypothesis: host shifting from tracheophytes to liverworts. Ligrone *et al.* (2007) noted that these two notions are not necessarily mutually exclusive since, in a tracheophyte-dominated world, advanced glomeromycetes might well replace more primitive endophytes. Examples include *Acaulospora* in *Monoclea* (Ligrone *et al.* 2007) and particularly the recent discovery of zygomycetous fungi in basal genera like *Neohodgsonia* Perss. and *Allisonia* Herzog (Bidartondo & Duckett 2010 unpublished data). It should also be noted that all the thalloid liverwort fungus sequences published to date are from derived (*Fossombronia, Pellia*) (Forrest *et al*. 2006), weedy (*Conocephalum, Lunularia*, *Marchantia*) or ephemeral taxa (*Fossombronia*).

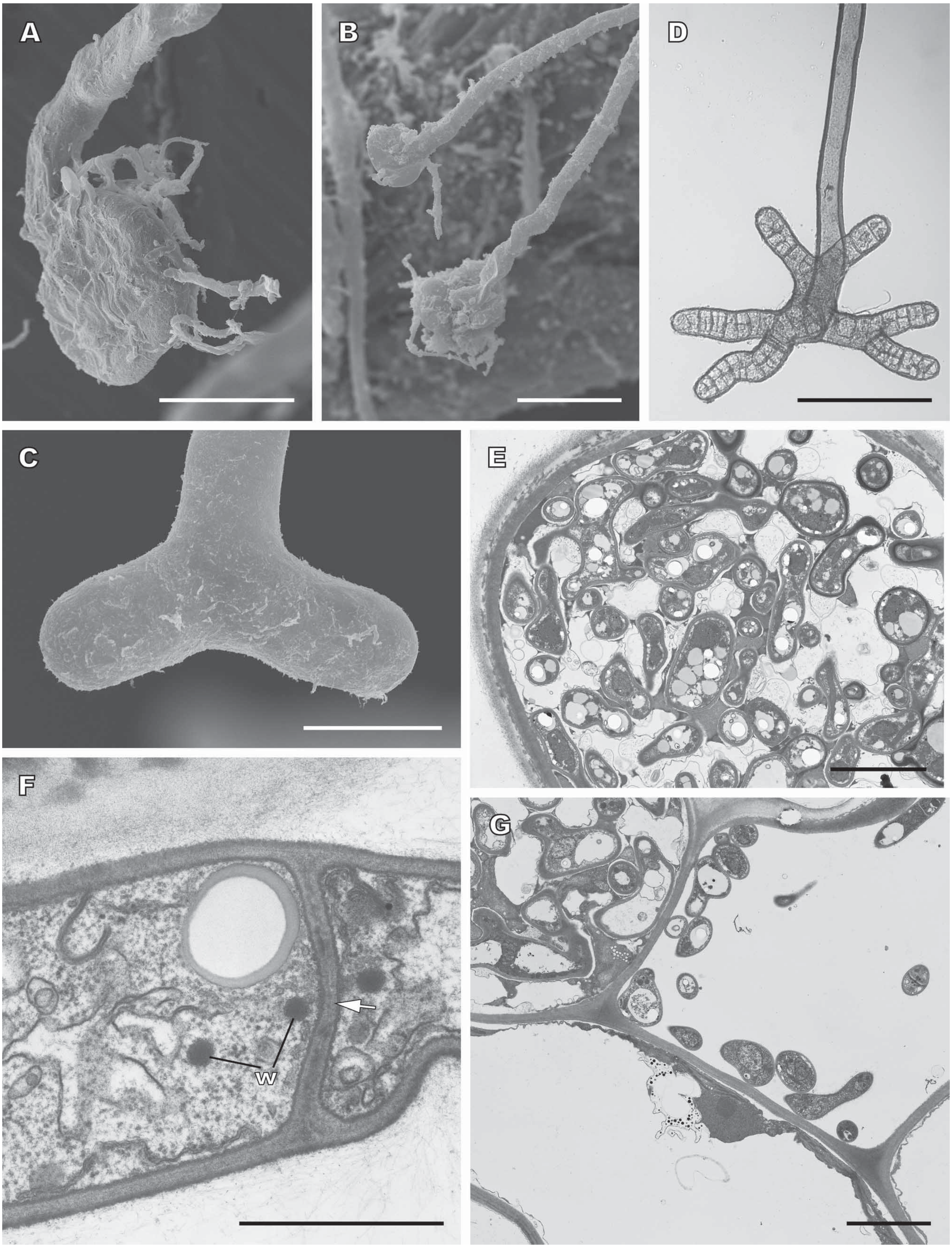
These considerations point to a pressing need for systematic molecular screening of the fungi across thalloid liverworts plus further ultrastructual studies to see whether or not the cytology seemingly diagnostic of a zygomycetous endophyte in *Treubia* and *Haplomitrium* also occurs in the thalloid liverworts. Only in the light of these, together with functional studies, we will gain a proper understanding of the early evolution of liverwort symbioses and the relationships between these and tracheophyte mycorrhizas.

Ascomycetes in the Jungermanniopsida

Cytological and cross-infection studies clearly demonstrated that the swollen rhizoids found in a range of leafy liverwort families including the Lepidoziaceae, Cephaloziaceae, Calypogeiaceae and Cephaloziellaceae are packed with one and the same ascomycete fungus (simple septa and Woronin bodies) (Fig. 3E, F) that is almost certainly *Rhizoscyphus ericae* (D.J.Read) W.Y.Zhang & Korf., the mycorrhiza-forming mycobiont in ericoid mycorrhizas (Duckett *et al.* 1991; Duckett & Read 1995). The identity of the fungus has subsequently been confirmed from extensive sequencing studies on Antarctic *Cephaloziella* (Spruce) Schiffn. populations (Chambers *et al.* 1999; Upson *et al.* 2007).

A subsequent major advance in understanding liverwort ascomycete associations came from the chance discovery that the branched septate rhizoids in the Schistochilaceae (Fig. 3C, D) are packed with fungi. This led to cytological, cross-infection and molecular studies (Pressel *et al.* 2008a, b). The endophyte was identified as *Rhizoscyphus*, like that in Antarctic *Cephaloziella* (Upson *et al.* 2007) while the presence of healthy hyphae and host cytoplasm in the rhizoids indicated a balanced relationship. Cross-infection experiments revealed that fungal isolates not only from the host *Pachyschistochila* R.M.Schust. & J.J.Engel species but also from a range of other liverworts with swollen rhizoids induce the rhizoid septation and branching characteristic of the Schistochilaceae. The presence of this fungus also induces swelling of the rhizoids in *Cephalozia* (Dumort.) Dumort. while those in the Lepidoziaceae often swell prior to infection (Duckett *et al.* 1991). It is also of interest that experimental studies on the ascomycete ‘lichen’ *Mniaecia jungermanniae* (Nees ex Fr.) Boud., originally considered as the possible perfect stage of the liverwort rhizoid fungus, in fact showed it to have no effect on rhizoid development in *Cephalozia* but rather to be a parasite that induces giant perichaetia and apogamous sporophytes (Pressel & Duckett 2006).

FIGURE 3. Rhizoidal ascomycete associations**. A-C.** Scanning electron micrographs. **D.** Light micrograph. **E-G.** Transmission electron micrographs. **A.** Swollen rhizoid tip in *Mylia anomala*. **B.** Swollen rhizoid tip in *Cephalozia lunulifolia* (Dumort.) Dumort.. **C, D.** Branched and septate tips of infected rhizoids in *Pachyschistochila splachnophylla* J.J.Engel & R.M.Schust.. Note the much smaller rhizoids in *Cephalozia* compared with the other two genera. **E.** Rhizoid tip of *Calypogeia muelleriana* (Schiffn.) Müll. Frib. packed with hyphae. **F.** Detail of simple septum (arrowed) with Woronin bodies (w). **G.** Rhizoid base in *Cephalozia connivens* (Dicks) Lindb.. Note the failure of the hyphae to penetrate the host walls. Scale bars = (A-D) 50 µm; (E, G) 5 µm; (F) 1 µm.


A
B
D
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W
G

Each of the swollen rhizoids has several fungal entry sites (Fig. 3B). Within the tips the hyphae form extensive coils which outlive the host cytoplasm and show no signs of digestion (Fig. 3E). Ingress into other host cells at the bases of the rhizoids is marked by overgrowths of host wall in *Calypogeia* Raddi (Němec 1904) whilst in other genera like *Cephalozia* and *Cephaloziella* there is no obvious morphological barrier preventing the spread of the endophytes into the stems (Fig. 3G). In the rhizoids of most taxa a thin transparent interfacial matrix separates the fungus from the host cytoplasm whereas in *Pachyschistochila* the hyphae are enveloped by irregular thickenings of the host cell wall (Pressel *et al.* 2008a, b).

The discovery of ascomycete associations in the Schistochilaceae has major implications for the origins and evolution of all liverwort and land plant symbioses when examined in the context of current liverwort phylogenies. In these, whatever some of the finer configurations of the trees, the Schistochilaceae are always resolved as sister to all the other fungus-containing leafy liverwort lineages in a clade designated the Jungermanniales (Heinrichs *et al.* 2005) or Leafy II (Davis 2004). Most clades within the Jungermanniales include taxa with rhizoidal ascomycetes whereas all the other leafy liverworts form a clade called either Leafy I or the Porellales and are fungus-free. The far more restricted distribution of taxa with basidiomycetes in the Jungermanniales suggests that these were predated by those with ascomycetes. Taking account of the southern hemisphere/Gondwanaland-centred distribution of most of the ascomycete-containing liverworts together with the dating of the divergence of the fungus-free Porellales in the Permian and the origins of the Schistochilaceae in the Triassic (Heinrichs *et al.* 2007) Pressel *et al.* (2008a, b) argued that the origins of the ascomycete associations in liverworts perhaps date back to in excess of 250 MYA. Thus, they long predate their counterpart in the Ericales estimated to have arisen 106-114 MYA (Wikström *et al.* 2001). Descriptions of ascomycetes from the Lower Devonian (Taylor *et al.* 1999) are in line with an ancient origin for their liverwort associations. However, it should also be noted that a recent fungus phylogeny (James *et al*. 2006) places the Leotiomycetes, the clade containing *Rhizoscyphus,* as highly derived and therefore of relatively recent origins. The same could also be argued from the highly specialized nature of the ascomycete association in the Schistochilaceae and the restriction of ascomycetes to the rhizoids in all the other taxa. For the future it will be of considerable interest to find out if the swollen rhizoid apices of *Mylia anomala* (Hook.) Gray (Fig. 3A)*,* which are considerably larger than those in the ascomycete-containing genera, inherited *Rhizoscyphus* via its likely schistochilaceous ancestry as indicated by the latest molecular phylogeny of the Lophoziaceae (de Roo *et al.* 2007)

Basidiomycete associations

a) Aneuraceae

The Aneuraceae are the only thalloid liverworts found to date to contain basidiomycetous endophytes clearly identified as such by the presence of dolipore septa (Fig. 4D, F) (Bidartondo *et al.* 2003; Duckett & Ligrone 2008a, b; Ligrone *et al.* 1993; Nebel *et al.* 2004; Preußing *et al*. 2010). The endophyte-host relationships recall those in orchids with the formation of hyphal coils in the colonised cells. These are then digested by the host to be followed by one or more reinfection cycles. As in the Marchantiophyta, the number of cell layers colonized by the fungus varies among taxa: from two to five in Chilean and New Zealand species of *Aneura* to approximately half the thallus in northern hemisphere *A. pinguis* (L.) Dumort. to virtually every cell in *Cryptothallus.*

Whilst this kind of association is also found in *Verdoornia* R.M.Schust. (Duckett & Ligrone 2008a) and in nearly all collections of *Aneura* Dumort. examined to date with the exception of *A. pellioides* (Horik.) and *A. pinguis* from California (Bidartondo & Duckett 2010), the sister genus *Riccardia* Gray is almost fungus-free. In three *Riccardia* taxa, all New Zealand endemics, where an endophyte is present the associations appear distinctively different from those in *Aneura*. Two of these we describe here for the first time (Fig. 4A–D), but a third species *R. intercellula* A.E.Brown is known from but a single collection and has never been refound in nature (Brown & Braggins 1989). In *R. pennata* A.E.Brown*,* prominent basidiomycetous hyphal coils, which show no signs of digestion by the host, fill the ventral epidermal cells (Fig. 4C). Even more remarkable is *R. cochleata* (Hook.f. & Taylor) Kuntze. This species produces achlorophyllous radially symmetrical ‘rhizomes’

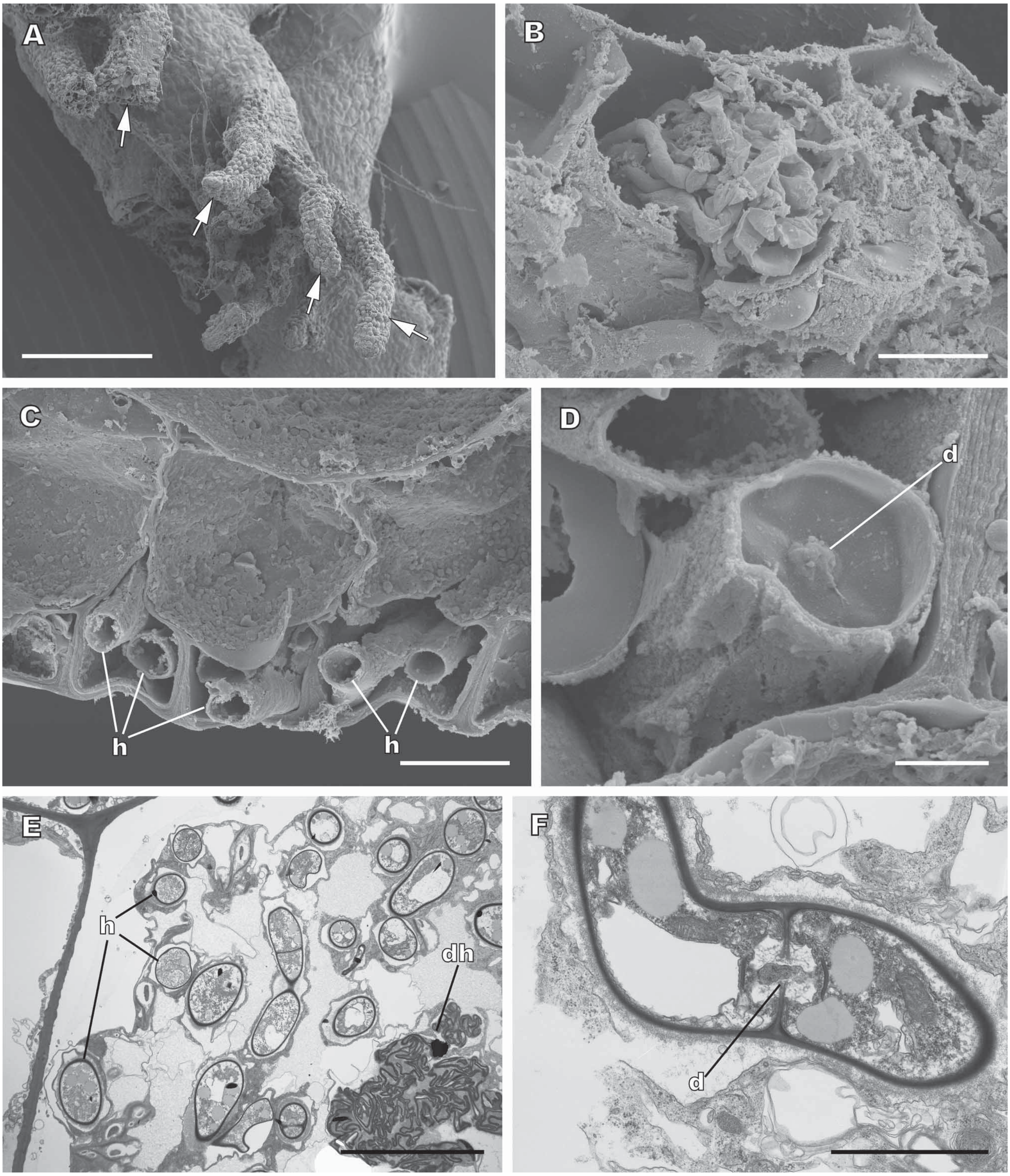

FIGURE 4. Basidiomycetes in the Aneuraceae. **A-D.** Scanning electron micrographs. **E, F.** Transmission electron micrographs. **A, B.** *Riccardia cochleata*. **A.** Rhizomes extending into the substratum from the lower surface of a thallus (arrowed). **B.** Hyphal coils in an epidermal cell of a rhizome. **C, D.** *Riccardia pennata*. **C.** Lower epidermal cells almost completely filled by hyphae (h) showing no sign of degeneration**. D.** Detail of septum with dolipore (d). **E, F.** *Verdoornia succulenta* R.M.Schust.. **E.** Infected cell with healthy (h) and degenerating (dh) hyphae. **F.** Detail of a typical dolipore septum (d). Scale bars = (A) 500 μm; (B, C, E) 10 μm; (D, F) 2 μm.

(Brown & Braggins 1989) recalling the 'roots' of *Haplomitrium* but lacking the mucilage investiture of the latter (Fig. 4A). While the fleshy surface-growing thalli are fungus-free, the first investigation of the anatomy of the rhizomes revealed that the epidermal cells are packed with hyphal coils (Fig. 4B) that undergo digestion cycles.

The fact that the Aneuraceae forms the crown group of an otherwise fungus-free lineage comprising *Pleurozia* Dumort., *Phyllothallia* E.A.Hodgs. and the Metzgeriaceae and the discovery from the initial molecular studies that the fungus from both *Aneura* and *Cryptothallus* belongs to the derived genus *Tulasnella* J.Schröt. (Bidartondo *et al.* 2003; Kottke *et al.* 2003) indicate that the aneuracean associations are of recent origin. Much more extensive sequencing investigations (Bidartondo & Duckett 2010; Preußing *et al*. 2010), embracing over one hundred collections of the Aneuraceae, revealed that whilst in the vast majority the fungus is *Tulasnella,* two gatherings (from Chile and Switzerland) contained *Sebacina* Tul., the basidiomycete genus characteristic of leafy liverworts (Kottke *et al.* 2003). The molecular diversity discovered within the *Tulasnella* symbionts (Bidartondo & Duckett 2010; Preußing *et al*. 2010), plus the rare occurrence of sebacinoids, in seemingly closely allied aneuracean taxa was at first sight most surprising. However recent molecular studies on the liverworts (Wackowiak *et al.* 2007; Baczkiewicz *et al.* 2008; Wickett & Goffinet 2008) have revealed that conservative thallus morphology within the Aneuraceae conceals remarkable genetic diversity. In fact, the diversity in the endophytes closely mirrors that in the hosts and includes noteworthy congruence between the fungal and liverwort phylogenies. Indeed the nesting of *Cryptothallus* within a single clade of *Aneura* (Wickett & Goffinet 2008), leading to the subsuming of the former into this genus is reinforced by the fungal data. Systematic considerations aside, with wholesale fungal digestion a standard feature of the aneuracean associations compared to those with basidiomycetes in leafy liverworts, it is perhaps not surprising that in this group we find the only achlorophyllose liverworts. Although on the one hand the fungus in the achlorophyllose rhizomes in *Riccardia cochleata* is most likely an independent acquisition, on the other the rhizomes perhaps provide an example as to how mycoheterotrophy may have evolved in the Aneuraceae.

b) Jungermanniales

Electron microscopy and molecular studies (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) have shown that eight or nine leafy liverwort genera; *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 almost certainly *Gongylanthus* Nees*,* in the Arnelliaceae, consistently and predominantly associate with members of the *Sebacina vermifera* Oberw. species complex, in striking contrast to *Tulasnella* as the predominant endophyte in the Aneuraceae. As in most liverwort-fungus associations, these endophytes enter the hosts via the rhizoids. However colonization by these basidiomycetes causes the rhizoids to become highly branched (Fig. 5A) rather than eliciting the terminal swellings typical of ascomycetous infections. Within the liverwort stems two kinds of association have been described. In the Arnelliaceae the endophytes colonize a cluster of cells in the centre of the stems (Fig. 5D) and there are no visible features in the host walls indicating why the fungus is so restricted. In the Scapaniaceae, Jungermanniaceae and Geocalycaceae, the central region of the stems contains a mosaic of infected and fungus-free cells. Growth of hyphae into the latter is prevented by overgrowths of host wall material (Fig. 5B). In both kinds of infection there is no evidence of fungus digestion by the host and healthy hyphae persist long after the death of the host cells in older stems (Fig. 5F). In fact, host cell death occurs very rapidly in the mosaic infections (Duckett *et al.* 2006b). One previously overlooked effect of fungal colonization in the Arnelliaceae is that oil bodies in infected cells rapidly break down (Fig. 5E) as hyphae proliferate suggesting that their contents may be a nutrient source for the endophytes.

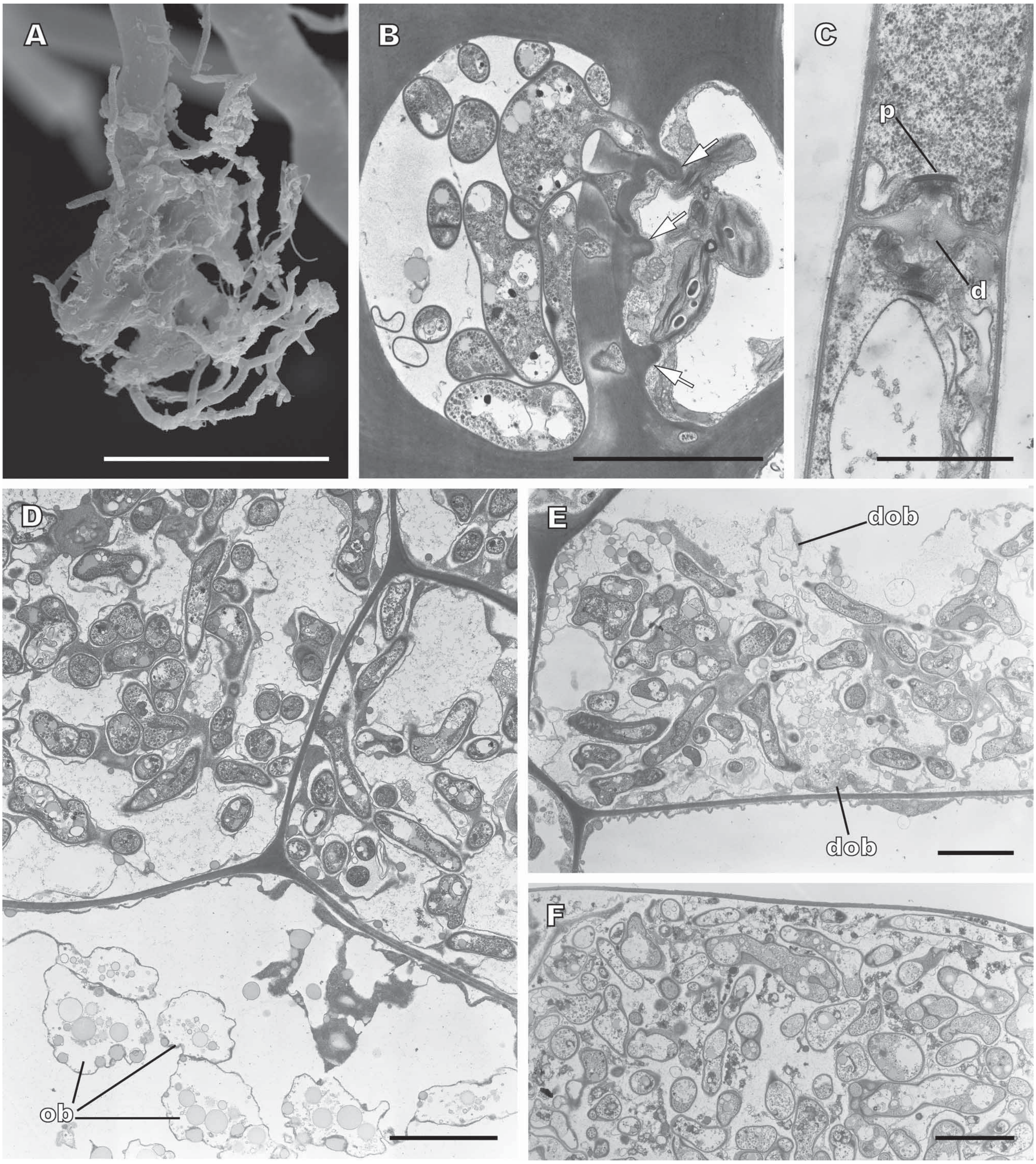

FIGURE 5. Basidiomycetes in Jungermanniales. **A.** Scanning electron micrograph. **B-F.** Transmission electron micrographs. **A.** *Saccogyna viticulosa* (L.) Dumort.; branched rhizoid tip associated with numerous hyphae. **B.** *Scapania cuspiduligera* (Nees) Müll. Frib.; infected and uninfected cells. Note the outgrowths of host wall (arrowed) preventing the ingress of hyphae into the uninfected cell. **C.** Dolipore septum (d) with imperforate parenthosome (p), typical of the genera *Sebacina* and *Tulasnella*. **D-F.** *Southbya nigrella* (De Not.) Henriq.. **D.** Infected cells packed with hyphae lying next to an uninfected cell containing numerous oil bodies (ob). **E.** Newly infected cell showing degenerating oil bodies (dob). **F.** Older infected cell. Note that the hyphae outlive the host. Scale bars = (A) 50 µm; (B, D-F) 5 µm; (C) 1m.

In striking contrast to the vast host range of the rhizoidal ascomycetes, cross-infection experiments on the leafy liverwort basidiomycetes strongly suggested these to be highly host specific (Duckett *et al.* 2006b). This situation has now been confirmed by a molecular study (Bidartondo & Duckett 2010) revealing a high level of fungal specificity to the extent that where multiple liverworts co-occur they almost never share the same endophyte. This remarkable symbiotic conservatism differs fundamentally from the generalist pattern of mycorrhizas in seed plants that repeatedly evolved to form ectomycorrhizas simultaneously with a range of distantly related basidiomycete genera.

The phylogenetic position of these basidiomycete-associated leafy liverworts (Davis 2004; Forrest *et al.* 2006; Heinrichs *et al.* 2005, 2007; Hentschel *et al.* 2006) and the fact that they are far more restricted than those with ascomycetes suggest that the basidiomycetes were a secondary acquisition following the loss of ascomycetes. However, the position of the Sebacinales in fungal phylogenies suggests that they may be considerably more ancient than the *Tulasnella* associations in the Aneuraceae (Kottke *et al.* 2003; Kottke & Nebel 2005). The discovery that basidiomycetes are widespread in *Scapania, Diplophyllum, Barbilophozia* and *Lophozia* is congruent with their close evolutionary relationship (Schill *et al.* 2004; Yatsentyuk *et al.* 2004; He-Nygrén *et al.* 2006) whilst the placement of basidiomycete-associated liverworts (*Lophozia sudetica* (Nees & Huebener) Grolle*, Barbilophozia barbata* (Schmieder ex Schreb.) Loeske, *B. lycopodioides* (Wallr.) Loeske and *B. hatchery* (A.Evans) Loeske) sister to other members of the Lophoziaceae (de Roo *et al.* 2007) suggests ancestral presence of fungi in this family.

Hornworts

The occurrence of unequivocally symbiotic fungal relationships in hornworts, first demonstrated in an ultrastructual study on *Phaeoceros laevis* (L.) Prosk. (Ligrone 1988) was subsequently confirmed by the experimental establishment of an arbuscular mycorrhiza-like symbiosis between *Anthoceros punctatus* L. and *Glomus claroideum* Schenck & Smith (Schußler 2000). Cytologically these associations exactly match those with glomeromycetes in thalloid liverworts. Since these pioneering works we are not aware of any systematic investigation of fungi in hornworts. However, our current research reveals that endophytes are widespread in both the above genera but are absent in *Dendroceros* Nees*, Megaceros* Campbell and *Nothoceros* (R.M.Schust.) J.Haseg.. As in thalloid liverworts *Leiosporoceros dussii* (Steph.) Hässel.*,* the sister taxon to other hornworts, lacks an endophyte (Villarreal & Renzaglia 2006). The same also appears to be the case in *Notothylas* Sull. but we have yet to examine many taxa and fresh specimens in this speciose genus. Whether or not *Folioceros* Bharad. contains endophytes has yet to be determined. Exclusively intracellular fungi are found in *Phaeomegaceros* Steph. but in *Phaeoceros* (Ligrone and Lopes, 1988) and *Paraphymatoceros* Hässel. hyphae can also be seen in mucilage-filled intercellular spaces. Thus our knowledge of fungal symbioses in hornworts remains very limited and we eagerly await the results of future cytological and molecular studies.

Fossils

Whilst incontrovertible fossil evidence from the Rhynie chert (Hass *et al.* 1994; Remy *et al.* 1994; Berbee & Taylor 2007; Krings *et al*. 2007a, b) would at first sight firmly cement the palaeozoic origins of glomeromycete associations and thus their considerable antiquity, the close parallels between *Nothia* and *Treubia* suggest that an earlier episode of symbioses featuring the zygomycetous fungi may have predated glomeromycetes as the primeval land plant fungus symbiosis. Setting aside this speculative consideration based on sound morphological evidence, this overview would not be complete without reference to the truly remarkable suggestions by Graham *et al.* (2010a). These are based on a new look at the enigmatic Late Silurian to Late Devonian fossil *Protaxites* variously interpreted previously as a giant alga, fungus or lichen

(Selosse 2002). Building on their earlier study (Graham *et al.* 2004) Graham *et al*. (2010a) suggest that *Prototaxites* was formed from partially degraded wind-, gravity- or water-rolled mats of mixotrophic liverworts having fungal and cyanobacterial associates much like *Marchantia*. They go on to argue that *Prototaxites* was derived from degradation-resistant rhizoids of marchantioid liverworts intermixed with tubular microbial elements, that liverworts were important components of Devonian ecosystems and that fossils previously attributed to nematophytes actually represent the remains of thalloid liverworts.

We take serious issue with both the evidence Graham *et al.* (2010a) present and their conclusions. 1) Their figure (4D) purporting to show a septum in a smooth rhizoid in *Marchantia* like those in *Prototaxites* is simply a tubular overgrowth of host wall over a fungal hypha which is commonly seen in extant liverworts. 2) Their figures 4F-H more closely resemble moss rhizoid systems than liverwort rhizoids and the former are much more resistant to decay than those of liverworts. 3) The tubes in *Prototaxites* are extensively branched like moss protonemata (Pressel *et al*. 2008c) whereas liverwort rhizoids, except for terminal ramifications associated with adherence to solid substrates (Duckett *et al.* 1991) are not. 4) There is absolutely no evidence that any extant liverworts roll up in mats. Given their extraordinary claims it is unsurprising that the Graham *et al*. (2010a) article stimulated both a letter (Boyce & Hotton 2010) and a commentary (Taylor *et al.* 2010), which were followed by a rapid response from Graham *et al.* (2010b). Whereas our critique comes from a bryophyte perspective, the equally sceptical views of Boyce & Hotton (2010) and Taylor *et al.* (2010) focus on the methodology of Graham *et al.* (2010a) including taphonomy, stable isotope analyses and interpretation of *Prototaxites* from a palaeobotanical standpoint. Graham *et al*. (2010b) fail to produce any convincing counter evidence: for their claim of liverwort mats rolling down a Devonian slope to have any credibility whatsoever at the very least a convincing demonstration from extant plants is required. Stimulating though these debates might be as contributions to elucidating the real nature of *Prototaxites*, in our view they add absolutely nothing to furthering understanding of the origins of land plant symbioses.

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