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Review

Pteridophyte fungal associations: current knowledge and future perspectives

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SHORT RUNNING TITLE: Fungal associations in pteridophytes

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Abstract

Current understanding of the nature and function of fungal associations in pteridophytes is surprisingly patchy given their key evolutionary position, current research foci on other early-branching plant clades, and major efforts at unravelling mycorrhizal evolution and the mechanisms underlying this key interaction between plants and fungi. Here we provide a critical review of current knowledge of fungal associations across pteridophytes and consider future directions making recommendations along the way.

From a comprehensive survey of the literature, a confused picture emerges: suggestions that members of the Lycopsida harbour Basidiomycota fungi contrast sharply with extensive cytological and recent molecular evidence pointing to exclusively Glomeromycota and/or Mucoromycotina associations in this group. Similarly, reports of dark septate, assumingly ascomycetous, hyphae in a range of pteridophytes, advocating a mutualistic relationship, are not backed by functional evidence and the fact that the fungus invariably occupies dead host tissue points to saprotrophy and not mutualism. The best conclusion that can be reached based on current evidence is that the fungal symbionts of pteridophytes belong to the two fungal lineages Mucoromycotina and Glomeromycota. Do symbiotic fungi and host pteridophytes engage in mutually beneficial partnerships? To date only two, pioneering studies have addressed this key question demonstrating reciprocal exchange of nutrients between the sporophytes of *Ophioglossum vulgatum* and *Osmunda regalis* and their fungal symbionts. There is a pressing need for more functional investigations also extending to the gametophyte generation and coupled with *in vitro* isolation and resynthesis studies to unravel the effect of the fungi on their host.

Key words: functional studies, fungal associations, Glomeromycota, Mucoromycotina, mutualisms, mycorrhizas, pteridophytes.

Whereas several past decades up to the present have witnessed a wealth of morphological, functional and molecular studies on seed plant mycorrhizas (Smith & Read, 2008) together with seminal advances this century on mutually beneficial fungal associations in liverworts (Field et al., 2014; 2015b), investigations of mycorrhizas in spore–bearing vascular plants lag far behind (see Mehltreter, 2010 for a recent critical summary). This is all the more surprising since knowledge of the nature and biology of fungal associations in extant pteridophytes are keys to understanding the evolution of fungal symbioses, a phenomenon widely recognised as a major innovation that drove plant terrestrialization around 460-480 MYA (Pirozynski & Malloch, 1975; Selosse & Le Tacon, 1998; Bonfante & Genre, 2008; Parniske, 2008).

The distribution and morphology of the fungal associations in extant pteridophytes and their fossil ancestors is summarized in Strullu-Derrien et al. (2014), though the main content of this account is new data and interpretation of fossils (Boullard & Lemoine, 1971; Remy et al., 1994; Taylor et al., 1995; Redecker et al., 2000; Karatygin et al., 2006; Krings et al., 2007a, 2007b). A second recent review focuses mainly on bryophytes (Rimington et al., 2016). Rather than simply reiterate the information in these accounts here we focus on the current state of knowledge of fungal associations in *extant* pteridophytes; we highlight highly significant recent advances, give critical assessments of shortcomings in published accounts to date and point out exciting avenues for future studies. Apart from a handful of electron microscope studies and even fewer molecular investigations, our knowledge of the occurrence of mycorrhizas across pteridophytes is based solely on light microscope observations. The reviews by Rayner (1927) and Burgeff (1938) and more recently by Wang & Qiu (2006) and Lehnert et al. (2016) together with the exhaustive survey of 420 taxa by Boullard (1957), check lists for the British flora (Harley & Harley,1987; Newman & Reddell, 1987), and field surveys in countries across the world - for example: China (Zhang et al., 2004; Zhao, 2000; Zhi-wei, 2000), Costa Rica (Lesica & Antibus, 1990), Ecuador (Lehnert et al., 2009; Kessler et al.,2014), Honduras (Zubek et al.,2010), India (Muthakumar & Udaiyan, 2000; Muthukumar & Prabia, 2012, 2013; Muthuraja et al., 2014; Sudha & Ammani , 2010), Lesotho (Moteetee et al., 1996), Mexico (Lara-Pérez et al., 2015), New Zealand (Cooper, 1976), Pakistan (Iqbal et al., 1981), Malaysia and Indonesia (Nadarajah & Nawawi, 1993;

Kessler et al., 2010a), Reunion (Kessler et al., 2010b), USA (Berch & Kendrick,1982; Gemma & Koske, 1995; Gemma et al., 1992; Laferrière & Koske, 1981), all report a high incidence of mycorrhizas but perhaps lower than for seed plants. These listings have serious failings. Apart from some of the data coming from unverified secondary sources (all in fact in Lehnert et al., 2016), many of the sampled species comprised roots and rhizomes from dried herbarium specimens (over 75% in the case of Boullard, 1957). In addition, these listings give but scant attention to the vital status of the host organs (see Moteetee et al., 1996 for detailed critique) and thus it is very difficult to glean precise information about the status of the symbiotic fungi as either mutualistic, saprophytic or parasitic (Mehltreter, 2010). We do know for certain however, that mycoheterotrophic gametophytes must be parasitic on their fungi (Leake et al., 2008). The frequent occurrence of two very different fungi side by side in the same host points strongly to a mixture of trophic categories. In the absence of rigorous sampling procedures that pay careful attention to the vital status of the fungus-containing organs, broad generalizations and detailed analyses in the literature to date about the overall incidence of mycorrhization in pteridophytes with inferences about phylogeny and ecology (e.g., Lehnert et al., 2016) should be viewed with considerable caution.

These provisos aside, the vast majority if not all of the likely symbiotic fungi found in pteridophytes fall into the arbuscular mycorrhizal (AM) category characterised by intracellular hyphal coils +/- fine arbuscular hyphae and vesicles. Less frequent are dark septate hyphae often associated with pseudosclerotia. By extrapolation from their well-documented occurrence in seed plants (Jumpponen, 2001; Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2005; Newsham, 2011; Newsham et al., 2014; Schmid et al., 1995) it is reasonable to assume that these are ascomycetous. Conspicuously absent are any *bona fide* records of basidiomycetes. The recent report that the main endophyte in gametophytes of *Lycopodium alpinum* is a basidiomycete (Horn et al., 2013), despite compelling cytological evidence (Burgeff, 1938; Bruchmann, 1898; Campbell, 1908; Duckett & Ligrone, 1992; Lang, 1899; Schmid & Oberwinkler, 1993) and molecular data (Winther & Friedman, 2007a) to the contrary in this and other lycopod gametophytes, is almost certainly due to flawed analysis procedures (see Rimington et al., 2014 for a full critique). Since the symbiotic status of AM fungi in seed plants and liverworts is beyond question, it seems reasonable to assume the same for pteridophytes as is borne out with transmission electron microscopy (TEM) studies that have invariably shown apparently healthy interactions between the partners (Duckett & Ligrone, 1992; Kovács et al., 2003; Schmid & Oberwinkler, 1993; 1994; 1995; 1996; Turnau et al., 1993). However, such studies have to date been limited to pteridophytes where a fungus is invariably present and those where such colonisations appear more sporadic, e.g., the sporophytes of the vast majority of leptosporangiate ferns, have yet to be investigated. Indeed the study by Turnau et al. (1993) on *Pteridium* contains the only published transmission electron micrographs of which we are aware of a typical AM association in the roots of a polypod (Polypodiales) fern.

Whether or not dark septate hyphae (see Boullard, 1957; Burgeff, 1938; Dhillon, 1993; Fernández et al., 2008; Iqbal et al., 1981; Lara-Pérez et al., 2015; Lehnert et al., 2009; Mandyam & Jumpponen, 2005; Moteetee et al., 1996; Muthukumar & Prabia, 2012; Muthuraja et al., 2014; Nadarajah & Nawawi, 1993; Sudová et al., 2011, for examples) form any kind of mutualistic relationship with pteridophytes has not been explored, but on the evidence to date this would seem unlikely. We are not aware of any published ultrastructural study showing such hyphae in a host cell with healthy cytoplasm in any land plant let alone a pteridophyte, and definitive evidence for a function in seed plants has not yet been forthcoming (Jumpponen, 2001; Jumpponen & Trappe, 1998; Newsham, 2011). Our own observations on the subterranean parts of a wide range of pteridophytes, not to mention bryophytes, point most strongly to saprotrophism rather than any kind of mutualistic relationships. Thus, a thorough light microscope examination will reveal their presence in and along the surface of the older parts of virtually any fern gametophyte, root or rhizome system (see for example Muthuraja et al., 2014), just as it does for older bryophyte rhizoids, thalli and stem tissues. In fact, dark septate hyphae in bryophytes are just as frequent on surfaces of taxa with well characterized symbionts, be these AM fungi, the ascomycete *Pezoloma ericae* or basidiomycetes as those where these symbionts are absent, e.g., all mosses (Field et al., 2015b; Pressel et al., 2010).

In addition to the likely AM status of most pteridophyte symbionts, a further very common feature is that root hairs and rhizoids are the major sites of direct fungal entry. Direct entry into the epidermal cells is also likely in taxa with very few root hairs, e.g., Marattiales (Bierhorst, 1971). As in liverworts (Duckett & Read, 1995; Kowal et al., 2016), colonized rhizoids and root hairs frequently have malformed tips (Boullard, 1957; Moteetee et al., 1996).

Against this picture of seemingly abundant mycorrhizas in pteridophytes why then are there not more studies? What in particular has hampered functional studies? Two major contributory factors are that some of the most interesting pteridophytes are rare, for example *Stromatopteris* is a New Caledonian endemic (Bierhorst, 1971), and fungus-containing structures like subterranean gametophytes are rarely produced by plants in cultivation, with the notable exception of *Psilotum* (Winther & Friedman, 2009), and are hard to find in nature. The facts that mycoheterotrophic gametophytes are difficult to culture axenically (see Whittier, 1975, 1981, 1998, 2003, 2005, 2011; Whittier & Braggins, 2000; Whittier & Carter, 2007a,b; Whittier et al., 2005, for special protocols) and that glomeromycote fungi cannot be cultured axenically (Field et al., 2014) severely restrict the scope of functional studies—for example, fulfilling Koch's postulates and thus dissecting host growth response to the presence of symbionts. Further impediments are that wiry monilophyte roots are extremely difficult to infiltrate with resins for transmission electron microscopy (Duckett et al., 1988) and fern roots generally often fix suboptimally due to their high content of phenolics (see for example the micrographs in Peterson & Brisson, 1977; Berch & Kendrick, 1982; Makgomol & Sheffield, 2001; Kovács et al., 2007). High phenolic content might also challenge the accessibility of fern roots to fungi (Schneider, 1996).

Systematic Survey

Nomenclature for the higher orders follows Christenhusz et al., (2011) and the phylogeny Knie et al., (2015) modified after Pryer et al., (2004).

Lycopsida

Gametophytes

The gametophytes of every *Lycopodium* species (here used *sensu lato* to include *Diphasiastrum, Huperzia* plus *Phylloglossum, Lycopodium* and *Lycopodiella*) in the Lycopodiaceae investigated to date, whether totally subterranean or partially surface-living, contain fungi with a well- defined distribution and highly distinctive cytology (Treub, 1884; Burgeff, 1938; Bruchmann, 1898, 1908, 1910; Campbell, 1908; Duckett & Ligrone, 1992; Lang, 1899; Schmid & Oberwinkler, 1993; Winther & Friedman, 2007a) (Figs. 1a, 1b). The presence of several unique features, in particular an intercellular phase of fungal proliferation (Fig. 1b), led Schmid & Oberwinkler (1993) to coin the term 'lycopodioid mycothallus' interaction. The first sequencing study on two gametophytes of *Lycopodium hypogeae* identified the fungus as a member of the Glomeraceae (following Redecker et al., 2013 for the classification of arbuscular mycorrhizal fungi)*,* a clade also found in other mycoheterotrophic lineages (Merckx et al., 2009; Merckx, 2013). In contrast, a second molecular study found that both ITS and LSU sequences identified the fungus in the gametophytes *of Lycopodium alpinum* as Sebacinales group B, a basal clade of the agaricomycetes (Basidiomycota) (Horn et al., 2013).

Sporophytes

Turning to the sporophytes, light microscope surveys indicate that possible symbiotic associations appear to be somewhat sporadic in the thin wiry roots of both Lycopodiaceae and Selaginellaceae and at best are confined to a minority of the species studied (Boullard, 1957). Morphologically the fungi appear to be AM with large trunk hyphae, finer hyphal coils and/ or arbuscules and vesicles.

By analogy with monilophytes (see below), the fatter and fleshier roots of *Isoëtes* appear to be far better candidates for mycorrhization than their narrow wiry counterparts in *Lycopodium* and *Selaginella.* However, Boullard (1957) found fungi in just one out of the 12 both terrestrial and aquatic species he examined, and none were found in *I. lacustris* by Søndergaard & Laegaard (1977). The sole exception was *I. engelmannii,* a species of transient pools, whereas *I. transvaalensis* from the same kind of habitat appears to be fungus-free (Moteetee et al., 1996). Subsequently, three light microscope studies have revealed AM fungi together with dark septate hyphae in the roots of the two completely submerged aquatic species *I. lacustris* and *I. echinospora* in Europe (Sudová et al., 2011) and several terrestrial plus two aquatic species from India (Sharma, 1998; Radhika & Rodrigues 2007). Sudová et al. (2011) are at pains to point out that the precise identity and function of the fungi remains to be elucidated. The likely absence of mycorrhizas in *Isoëtes* most likely reflects a primarily aquatic ancestry since most taxa are restricted to aquatic or semiaquatic habitats.

The first molecular study of the symbionts in lycophyte roots yielded results that have shattered the long held notion that the glomeromycotes alone were the primeval vascular land plant fungal symbionts (Rimington et al., 2014). Though confirming the pre-existing picture that fungal colonization appears to be less frequent than in ferns (lycopods with fungi in 7 of 20 species from 17 of the 101 samples versus ferns with fungi in 13 of 18 species from 33 out of 58 samples—Rimington et al., 2014), Glomeromycota fungi (all in the Glomeraceae) were present in only three of the lycophyte species while the other four contained diverse Mucoromycotina, including six new clades. These Mucoromycotina fungi belonging to different clades sometimes occurred within the same species, and even the same plant.

Monilophytes

Gametophytes

The few electron microscope studies to date of subterranean mycoheterotrophic fern gametophytes (*Botrychium* (Kovács et al., 2003; Schmid & Oberwinkler, 1994), *Ophioglossum* (Schmid & Oberwinkler, 1996), *Psilotum* and *Tmesipteris* (Duckett & Ligrone, 2005)) have revealed that the exclusively intracellular symbionts comprise hyphal coils with arbuscule-like side branches and vesicles, i.e., they are typical Glomeromycota (Figs. 1c-1f). The analysis of DNA sequences confirms the fungi in *Botrychium* (Winther & Friedman, 2007b) and *Psilotum* (Winther & Friedman, 2009; Rimington et al., 2014) and *Tmesipteris* (Rimington et al., 2014) as Glomeraceae. At the other extreme, fungi are absent from the endosporic gametophytes in heterosporous ferns and lycophytes. Whether or not this is also the case in *Playtzoma microphyllum,* the only fern with exosporic free-living photosynthetic gametophytes (Duckett & Pang, 1984), has yet to be investigated.

Glomeraceae have now been confirmed in the cordate photosynthetic gametophytes of *Angiopteris* in the sister eusporangiate lineage to the Marattiales and in *Osmunda* at the base of the leptosporangiate tree (Ogura-Tsujita et al., 2013). However, in a second marattioid genus, *Ptisana*, the gametophyte fungus is a member of the Diversisporaceae (Rimington et al., 2014). In all three genera the distribution and morphology of the fungi in the ventral midrib region of the cordate gametophytes mirrors that in many thalloid liverworts (Ligrone et al., 2007) and is repeated throughout the leptosporangiate ferns (Ogura-Tsujita et al., 2016). Widely different sporophyte and gametophyte morphologies now rest comfortably with the recent placement of horsetails

(Equisetales) as sister to all other monilophytes (Knie et al., 2015) rather than as a sister clade to the Marattiaceae (Pryer et al., 2004). A further difference is that symbionts are absent in *Equisetum* gametophytes although their multicellular ventral cushions attached to the substratum would appear to be preadapted, at least structurally, for fungal colonisation. This absence is most likely linked to their ecology. Whereas superficial fern gametophytes may be terrestrial on mineral or peaty soils, epilithic or epiphytic (Farrar et al., 2008) and often grow adjacent to endophyte-containing bryophytes, those of *Equisetum* have only been found in habitats like lake, reservoir and river margins (Duckett & Duckett, 1980). These are transient, nutrient-rich habitats and all the associated liverworts also lack fungi.

With a few notable exceptions discussed below, viz., Hymenophyllaceae, *Stromatopteris* (Gleicheniaceae), *Schizaea* and *Actinostachys* (Schizaeaceae) and Vittariaceae, the gametophytes of most leptosporangiate ferns and the Marattiales grow above ground, are green and photosynthetic and usually cordate in form. The central cushion is distinctly thicker and more frequently colonized by fungi in the Marattiaceae and Osmundaceae than in more derived families. General statements about the incidence of possible symbiotic fungi range from somewhat common to absent (Bell & Helmsley, 2000; Ogura-Tsujita et al., 2016). Most studies on wild fern gametophytes have focused on their ecology and reproductive biology (Farrar et al., 2008), with the difficulty of identifying these down to the species or even genus level (Farrar, 2003) further contributing to the lack of data on fungi. Whatever the present gaps in overall coverage of the ferns, two features do appear to be constant: rhizoids are the major routes of fungal entry and *bona fide* symbionts are invariably present in the ventral cell layers in the central cushion region, but are much less frequent in the unistratose wings (Ogura-Tsujita et al., 2013; 2016).

Extending their morphological and molecular study on *Angiopteris* and *Osmunda* (Ogura-Tsujita et al., 2013) to a range of pre-Polypodiales leptosporangiate ferns to include two species in the Gleicheniales and four in the Cyatheales, Ogura-Tsujita et al. (2016) found that not only were 58-97% of the gametophytes colonized by AM fungi but that these also belonged to a wide range of Glomeromycota fungi. In addition to Glomeraceae, they also found members of the Claroideoglomeraceae, Gigasporaceae, Acaulosporaceae, and Archaeosporales fungi previously unknown in ferns but which are widespread in thalloid liverworts and hornworts (Bidartondo et al., 2011; Desirò et al., 2013; Field et al., 2015b). There is now a pressing need to extend these molecular studies to Polypodiales since recent light microscope studies indicate the presence of similar associations in a range of genera; *Adiantum, Pellaea* (Turnau et al., 2005), *Dryopteris* (Reyes-Jaramillo et al., 2008), *Nephrolepis* (Muthukumar & Prabia , 2012) and *Pteris* (Martinez et al., 2012), and particularly since the discovery of both Glomeromycota (Glomeraceae and Diversisporaceae) and Mucororomycotina in *Anogramma leptophylla* from the only fungal DNA sequencing study to date on the sporophytes of a member of the Polypodiales (Figs. 2c, 2d) (Rimington et al., 2014).

In contrast to the widespread and likely obligate occurrence of symbiotic fungi in cordate gametophytes, the asexually-reproducing long-lived, independent, strap-shaped gametophytes of the Vittarioideae and the filamentous gametophytes in the filmy ferns are almost certainly fungus-free (Duffy et al., 2015; Farrar, 1974, 2003; Farrar et al., 2008; Rumsey et al., 1990, 1993). This may reflect the fact that these are predominantly epiphytic lineages (Nayar & Kaur, 1971) with ecology paralleling that of the fungus-free Porellales in the liverworts (Pressel et al., 2010).

The other leptosporangiate ferns with axial and filamentous gametophytes are *Actinostachys* and *Schizaea* in the Schizaeales and *Stromatopteris* in the Gleicheniales (Lang, 1902; Bierhorst, 1966, 1967, 1968a, 1968b, 1971; Britton & Taylor, 1901; Kiss & Swatzell, 1996; Pryer et al., 2004; Raghavan, 1989; Swatzell et al., 1996; von Anderkas & Raghavan, 1985). In these three genera the gametophytes are either partly (*Schizaea*) or totally subterranean (*Actinostachys, Stromatopteris*) and therefore mycoheterotrophic. Virtually every cell, including the multicellular rhizoids and epidermis in the multiseriate filaments in *Stromatopteris* and *Schizaea* (Bierhorst, 1966, 1967, 1968b, 1971) are packed with hyphae. In the tuberous axes with septate rhizoids in *Actinostachys* (Bierhorst, 1968a), the fungus has a similar distribution to that in *Psilotum* and *Tmesipteris* (Duckett & Ligrone, 2005) in that many of the epidermal cells are fungus-free. In addition to their multicellular rhizoids, a further unusual feature in *Schizaea* is that the gametophytes develop so called rhizoidophores. These are large, highly vacuolated spherical cells which develop two to three rhizoids (von Aderkas & Raghavan, 1985) and form receptacles for a symbiotic fungus (Britton & Taylor 1901, Kiss & Swatzell, 1996; Swatzell et al., 1996) which, from published light micrographs and illustrations, appears to be AM as is the case for the symbionts throughout the gametophytes of all three genera. The swollen rhizoidophores and septate rhizoids in these fern gametophytes are strikingly reminiscent of the rhizoid modifications associated with fungi in leafy liverworts (Kowal et al., 2016; Pressel et al., 2008b, 2010; Read et al., 2000) and in particularly their septation in the Schistochiaceae (Pressel et al., 2008a). However, the liverwort fungus here is invariably the ascomycete *Pezoloma ericae.*

Sporophytes

In terms of gross morphology, fern roots fall into two categories: fat and fleshy, 2 or more mm in diameter, and often lacking thickened walls and phenolic deposits versus thin and wiry, only ca. 1mm in diameter with phenolic compounds impregnating the cortical cells and/or thickened walls (Schneider, 1996, 2000). The former features are the rule in the Ophioglossales and Marattiales and to some extent the Osmundales whilst the latter are typical of most leptosporangiate clades with the exception of the rootless Salviniales. The rhizomes in the rootless members of the Hymenophyllales (Duckett et al., 1996; Schneider, 1996; Schneider et al., 2002; Ebihara et al., 2007) have a similar overall structure. The roots of horsetails are similarly thin and wiry. The rhizomes in the rootless whisk ferns resemble fleshy roots anatomically (Schneider, 1996, 2000) and the shoot system in *Stromatopteri*s, where roots are rare, functions in a similar manner.

Fungi appear to be ubiquitous in all the taxa with fleshy roots where they occupy several layers of cortical cells. Perhaps unique to *Ophioglossum* is its absence of root hairs (Scheider et al., 2002, 2009) recalling the fungus-colonised subterranean gametophytic axes in the liverwort *Haplomitrium* (Carafa et al., 2003). Ultrastructural studies on *Psilotum* and *Tmesipteris* (Duckett & Ligrone, 2005), *Ophioglossum* (Schmid & Oberwinkler, 1996), *Botrychium* (Kovács et al., 2003) and the marattioid fern *Ptisana* (Rimington et al., 2014) have shown that the host-fungus relationships appear to be the same in both generations of the same species. Typical AM ultrastructure has now been confirmed in all these five genera (Rimington et al., 2014) but further work is needed to establish whether this is the case in *Helmintostachys* and in the other five genera in the Marattiales as would appear from Boullard's (1957) light microscope observations.

For the reasons noted previously, published data on the distribution of possible symbiotic fungi in ferns with wiry roots are highly problematic (Figs. 2a, 2b). The situation is not helped by the extreme paucity of ultrastructural studies. We are aware of only a single paper (Turnau et al., 1993) that shows a typical AM association in a fern with wiry roots, *Pteridium*. A further ultrastructural study on *Gleichenia* by Schmid et al. (1995) only features electron micrographs of the gametophytes. Two other electron microscope studies show ascomycetes (simple septa and Woronin bodies) in *Loxsomopsis* (Cyatheales) (Lehnert et al., 2009) and epiphytes in the genera *Elaphaglossum*, *Hymenophyllum*, *Grammitis* and *Lellingeria.* However, none of the micrographs show the ascomycetous symbionts surrounded by healthy host cytoplasm thus calling into question the existence of fern mycorrhizas, discussed as a feature possibly more beneficial in epiphytes by Mehltreter (2010). This, together with the unlikely symbiotic status of dark septate hyphae as illustrated in Boullard (1957), Fernández et al. (2008), Muthukumar & Prabia (2012) and Muthuraja et al. (2014), indicates that it is highly unlikely that pteridophytes form mutualistic associations with ascomycetes.

Three groups where fungi are almost certainly absent are the freshwater genus *Ceratopteris* (Hickok et al., 1995; Renzaglia & Warne, 1995), the heterosporous water ferns (Salviniales) and Equisetales. However, in order to clarify conflicting evidence for symbionts we made our own critical observations. As reported by previous authors (Boullard, 1957; Dhillon, 1993; Fernández et al., 2008), we found both AM fungi with vesicles and dark septate hyphae in old roots of six species of *Equisetum* from different habitats, viz., *E. arvense, E. fluviatile, E. giganteum, E. hyemale, E. telmateia* and *E. variegatum.* Fungi were never observed in young roots with intact apices and DNA sequencing produced negative results (Rimington et al., unpublished data). We suggest that a similar study of *Marsilea* will reveal that the AM fungal structures described to date (Bhat & Kaveriappa, 2003) are confined to necrotic roots. Similar scrutiny of roots in the Hymenophyllaceae (Fig. 2b), where Boullard (1979) found a high incidence of septate hyphae, and the trichomes in rootless species of *Trichomanes* which lack root hairs/rhizoids (Schneider, 2000; Duckett et al., 1996) yielded identical results: we never saw fungi in healthy roots nor in their trichomes. We suggest, with the hindsight of extensive molecular and ultrastructural sampling of liverwort and hornwort fungi (Bidartondo & Duckett, 2010, Desirò et al., 2013; Pressel et al., 2010; Ligrone et al., 2007) that, were similar critical studies extended to ferns from a wide range of habitats, symbionts would be less frequent in extreme epiliths, epiphytes and in tree ferns with aerial roots than in taxa growing through some soil at least. Ferns, liverworts and hornworts also share a paucity or absence of fungi in aquatic taxa. The very limited sequencing data published to date have revealed members of the Glomeraceae in several genera (*Botrychium, Ophioglossum, Gleichenia, Psilotum,* *Tmesipteris , Pitsana, Xiphopteris, Nephrolepis, Anogramma, Osmunda*), Diversisporaceae in two (*Anogramma* and *Ophioglossum*) and Mucoromycotina in just one *Anogramma* (Kovács et al.,2007; Winther & Friedman, 2007b; 2009; Rimington et al., 2014; Field et al., 2012; 2015a) .

Functional considerations

Green chlorophyllous monilophyte gametophytes can be readily cultured axenically (Raghavan, 1989) and the same is true for those of lycophytes, though most of these are extremely slow growing and require more selective methods, particularly those that lack chlorophyll (Whittier, 1981, 1998, 2003, 2005, 2011; Whittier & Braggins, 2000; Whittier & Carter, 2007a,b; Whittier et al., 2005). Thus, they would appear to be highly suitable material for investigations into the effects of the fungi on the hosts. Such studies have yet to be attempted; however, a recent paper by Martinez et al. (2012) clearly demonstrates their feasibility. When grown on a substrate inoculated with *Rhizophagus irregularis,* gametophytes of *Pteris vittata* displayed *Paris*-type recolonization whilst the roots had *Arum*-type colonisation. Unfortunately, the substrate used in the experiments was a perlite, peat and soil mixture and some of the published images show other infections with dark aseptate hyphae. We now need similar recolonization experiments performed under axenic conditions using either *Glomus* spores, as that inoculum as has been used to colonise hornwort thalli (Schüßler, 2000), or colonised seedlings of flowering plants. Since DNA sequencing studies are now revealing an increasing range of glomeromycete fungi in pteridophytes (Field et al., 2015a,b; Ogura-Tsujita et al., 2016), not to mention mucoromycetes (Rimington et al., 2014) which can be grown axenically (Field et al., 2014) and are thus much more convenient inocula, an exciting future beckons.

In planning experiments considerable thought also needs to be given to the choice of the best host plants. Ideally, we need model taxa which are readily cultured, have short life cycles and where fungal associations are ubiquitous in nature and thus have functional signalling network pathways (Wang et al., 2010). Looking at cryptogams the only one meeting these criteria as a model to date, is the hornwort *Anthoceros agrestis* (Szövényi et al., 2015). *Ceratopteris thalictroides* (Hickok et al., 1995; Renzaglia & Warne, 1995), the moss *Physcomitrella patens* (Lang et al., 2016) and the liverwort *Marchantia polymorpha*

(Alam & Pandey, 2016; Bowman et al., 2016; Ishikazi et al., 2016), not to mention *Arabidopsis,* are all symbiont-free. Though these absences are almost certainly secondary losses, recent in *Marchantia* and more ancient in *Ceratopteris* and *Physcomitrella*, they are far from ideal for studying the function of signalling network pathways that were present from the dawn of terrestrialization (Wang et al., 2010). For monilophytes, we suggest *Anogramma leptophylla* with its short lived sporophytes and perennial gametophytes (Goebel, 1905) as a new option for fungal functional studies. In the lycopods, whether the model species *Selaginella apoda* (Schulz et al., 2010) is regularly colonized by endophytes requires further study. For homosporous taxa, we single out *Lycopodiella inundata* because of its short-lived sporophytes, surface-living and more readily cultured photosynthetic gametophytes (Whittier, 2005; Whittier & Carter, 2007a,b) as the best choice.

With the recent demonstration that pteridophytes contain both Mucoromycotina and a range of Glomeromycota fungi (Rimington et al., 2014) there is now a pressing need to carry out functional studies using isotope tracers (^{13}C, ^{33}P and ^{15}N) like those recently carried out on liverworts (Field et al., 2014, 2015a, 2015b) and extend the pioneering work by Field et al. (5a) on *Ophioglossum* and *Osmunda* . These, the only studies to date on mycorrhizal functioning in pteridophytes, clearly demonstrated the reciprocal exchange of plant-C for fungal–acquired N and P between the green sporophytes of *Ophioglossum vulgatum* and *Osmunda regalis* and their fungal symbionts. In the case of *O. vulgatum,* nutritional mutualism was demonstrated between the fern sporophytes and a highly specific fungal partner *Glomus macrocarpum*, a derived taxon in the Glomeraceae.

In addition to showing mutualistic and specific symbiosis between this eusporangiate fern and Glomeromycota fungus, the Field et al. (2015a,b) study raises the questions of fungal specificity and intergenerational fidelity (Leake et al., 2008) and the precise nature of the relationships between the fully mycoheterotrophic subterranean gametophyes and the early achlorophyllous sporophytic stages (Boullard, 1979; Bruchmann, 1908; 1910) followed by the formation of the photosynthetic above ground fronds that supply organic carbon to the fungus. The authors propose that the symbiosis may operate a ’take-now, pay -later strategy’ (Cameron et al., 2008) and also raise the possibility that the sporophytes revert to mycoheterotrophy during the below ground dormancy period from mid-summer to the following spring. Unfortunately, Field et al. (2012a) were unable to locate gametophytes in their study and thus investigate whether the gametophytes acquire all their carbon from the sporophytes via a common symbiont. In support of intergenerational fidelity was the demonstration that the symbiotic relationship in their *Ophioglossum* plants was highly specific, as is the case in *Huperzia* (Winther & Friedman, 2007a) where both gametophytes and sporophytes share the same three AM phylotypes. Whilst all the evidence to date indicates that pteridophyte gametophytes appear to have high fungal specificity, a general feature of mycoheterotrophy (Bidartondo et al., 2003; Merckx & Freudenstein, 2010), the fact that in *Botrychium crenulatum* fungal diversity increases through the transition from mycoheterotrophy to autotrophy (Winther & Friedman, 2007a) and Kovács et al. (2007) found between five and seven AM fungi in sporophytes of *B. virginianum* suggests that pteridophyte sporophytes probably benefit from a wider range of AM fungi. This premise is borne out by subsequent DNA sequencing studies revealing an increasing number of Glomeromycota plus Mucoromycotina, sometimes together in the same plants (Rimington et al., 2014).

A further factor to be added to the functional debate is that in all the ultrastructural studies on pteridophytes with subterranean gametophytes to date there is remarkable congruence in the host-fungal cytology between the two generations (Duckett & Ligrone, 2005). Since the gametophytes have ‘cheating’ associations where only the host receives benefits (Bidartondo et al., 2003; Brundrett, 2002, 2004), how far then this might also be true for the sporophytes? Duckett & Ligrone (2005) point out that coiling AM mycorrhizas are a feature of exploitative associations in angiosperms (Brundrett, 2004) and that the multiple waves of colonisation that are outlived by the host cells bear a striking resemblance to the fate of the fungi in orchid mycorrhizas, in the mycoheterotrophic liverwort *Aneura* (*Cryptothallus*) *mirabilis* and in closely related *Aneura s*pecies (Ligrone & Duckett, 1993; Duckett & Ligrone, 2008). Further isotope studies like those by Field et al. (2015a) are now needed to establish just how far the fungal associations in pteridophytes fall into the category of balanced versus exploitative (Bidartondo et al., 2003; Brundrett, 2002, 2004).

Evolutionary perspectives

The discovery of an increasing range of symbionts belonging to both the Mucoromycotina and the Glomeromycota (Rimington et al., 2014) and the presence of fungi with characteristics of both groups in Devonian plants (Strullu-Derrien et al., 2014) has now overturned the long held view that the Glomeromycota alone formed the ancestral land-plant fungus symbiosis (Leake et al., 2008). The presence of both groups of fungi in lycopods and the predominance of a range of Glomeromycota in later diverging ferns closely fit the phylogenetic distribution of these fungi in thalloid liverworts with dual partnerships in basal clades and Glomeromycota alone in more derived groups (Bidartondo et al., 2011; Field et al., 2016). Whilst most of the associations in extant pteridophytes almost certainly have ancient origins, the presence of Mucoromycotina in *Anogramma* may be a much more recent acquisition associated with this fern's unique life history (Goebel, 1905). These discoveries clearly emphasise the novel emerging notion that fungal symbioses at the dawn of plant terrestrialization were much more diverse than hitherto assumed (Field et al., 2015b).

Several features mark out pteridophyte-fungus relationships as highly distinct from those in both liverworts and seed plants. Whereas in liverworts there have been successive waves of fungal colonization and losses (from Mucoromycotina alone in the Haplomitriopsida to fungus-free *Blasia* at the foot of the thalloid phylogeny to re-acquisition of both fungal lineages in the complex and simple thalloid lineages), there is no similar clear pattern in pteridophytes (See Fig. 3) apart from a likely loss of AM from the lycophytes to the horsetails, consequence of their recent reassignment to the base of the monilophyte tree (Knie et al., 2015) from sister to the Marattiaceae (Pryer et al., 2004), and their reacquisition in eusporangiate ferns. Fungus-free early-branching horsetails are also in line with the notion of increasing mycorrhizal dependency as a putative apomorphy in the Ophioglossales (Schneider et al., 2009). Moreover, in liverworts the AM fungi were subsequently replaced by basidiomycetes (Bidartondo & Duckett, 2010) and the ascomycete *Pezoloma ericae* (Duckett & Read, 1995; Pressel et al., 2010; Kowal et al., 2016), whereas there is no good evidence of symbioses with either of these fungi in pteridophytes. Similarly, in seed plants there are repeated incidences of losses and gains of diverse fungi (Smith & Read, 2008). Until there is unequivocal evidence for a physiological role for ascomycetes and particularly dark septate hyphae, pteridophytes are best regarded as containing Glomeromycota and Mucoromycotina symbionts alone.

Fungal associations appear to have been progressively lost through monilophyte evolution. Fungi are obligate and ubiquitous in the earlier lineages but their incidence become far more capricious in polypod ferns. This trend is very clearly contrary to species richness; whereas the Polypodiales number thousands of species, the numbers of species are much lower for earlier groups; *Ophioglossum* 25-30, *Botrychium* 50-60, Marattiales 135, Osmundales 25 and Schizaeales 190 (Christenhusz et al., 2016). Two possible explanations come to mind. One is a switch in root anatomy from fleshy to wiry which accompanied the evolution of the leptosporangiate ferns. The second are the radiations of leptosporangiate ferns as epiphytes (Schuettpelz & Pryer, 2009). This is paralleled by the loss of symbionts in epiphytic liverwort clades (Pressel et al., 2010), whilst their absence in water ferns and *Isoëtes* recalls the paucity of mycorrhizas in aquatic seed plants (Søndergaard & Laegaard, 1977; Shah, 2014). It is also interesting to note that fungi are also absent from the crown group liverwort family Ricciaceae (Ligrone et al., 2007) many of which grow alongside the *Isoëtes* species of ephemeral pools.

Conclusions

Recent discoveries demonstrating the occurrence not only of Glomeromycota but also of Mucoromycotina fungi in pteridophytes coupled with all but two pioneering studies providing the first compelling evidence for mutualistic nutrient exchange between *Ophioglossum*, *Osmunda* and their fungal symbiont are now paving the way towards an exciting new era in pteridophyte-fungal association research. Given the key position of pteridophytes in land plant evolution, a better understanding of the nature and biology of the interactions between pteridophytes and their fungal symbionts has major implications for unravelling key events at the dawn of plant terrestrialization and the evolutionary history of mycorrhizas. Targeted molecular investigations, and functional studies using isotope tracers coupled with *in vitro* isolation and recolonization experiments will go a long way toward elucidating the nature and dynamics of these key interactions. Turning to model organisms, current cryptogam model organisms with the exception of the hornwort *Anthoceros agrestis* (and extending to the seed plants—see *Arabidopsis*) are unsuitable for mycorrhizal research, given that they are all asymbiotic. We propose the fern *Anogramma leptophylla* and the lycophyte *Lycopodiella inundata* as more suitable alternatives.

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Figure legends

Fig. 1. a, b, Semi-thin sections of the fungal zone of the chlorophyllous surface living gametophyte of *Lycopodiella cernua*; (**a**) intracellular hyphal coils; (**b**) hyphae (arrowed) in the mucilage-filled intracellular spaces. **c, d,** Transmission electron micrographs of a *Psilotum nudum* rhizome showing waves of fungal colonisation. V, vesicle; D, degraded hyphal masses; and, arrowed, fine coiled hyphae. **e, f,** Scanning electron micrographs of *Botrychium virginianum* root showing fungal zone in the cortex (arrowed in e) and intracellular hyphal coils (f). Scale bars: 500 µm (e); 50 µm (a, f); 20 µm (b, c); 10 µm (d).

Fig. 2. a, b, Light micrographs of living root apices of (**a**) *Schizaea dichotoma* and (**b**) *Hymenophyllum tanbrigense*. Note the fungus-free rhizoids of these wiry roots. In (b) arrow points to a mucilage papilla. **c, d,** Semi-thin sections of the overwintering tuber of *Anogramma leptophylla*: (c) peripheral cells packed with mucoromycete symbionts; (d) central cells packed with lipid reserves and lacking fungal colonisation. Scale bars: 500 µm (a); 200 µm (b); 20 µm (c, d).

Fig. 3. Phylogram (after Knie et al., 2015) showing the distribution of mutualistic fungal associations in pteridophytes. Note the increasing uncertainty ascending the tree. At present Mucoromycotina fungi are only known from *Lycopodium* sporophytes and *Anogramma*, both generations.