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1 **Review**

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3 **Pteridophyte fungal associations: current knowledge and future perspectives**

4

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15 **SHORT RUNNING TITLE:** Fungal associations in pteridophytes

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18 **Abstract**

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

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

40

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

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44

45

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

56 The distribution and morphology of the fungal associations in extant pteridophytes
57 and their fossil ancestors is summarized in Strullu-Derrien et al. (2014), though the main
58 content of this account is new data and interpretation of fossils (Boullard & Lemoine, 1971;
59 Remy et al., 1994; Taylor et al., 1995; Redecker et al., 2000; Karatygin et al., 2006; Krings et
60 al., 2007a, 2007b). A second recent review focuses mainly on bryophytes (Rimington et al.,
61 2016). Rather than simply reiterate the information in these accounts here we focus on the
62 current state of knowledge of fungal associations in *extant* pteridophytes; we highlight
63 highly significant recent advances, give critical assessments of shortcomings in published
64 accounts to date and point out exciting avenues for future studies. Apart from a handful of
65 electron microscope studies and even fewer molecular investigations, our knowledge of the
66 occurrence of mycorrhizas across pteridophytes is based solely on light microscope
67 observations. The reviews by Rayner (1927) and Burgeff (1938) and more recently by Wang
68 & Qiu (2006) and Lehnert et al. (2016) together with the exhaustive survey of 420 taxa by
69 Boullard (1957), check lists for the British flora (Harley & Harley, 1987; Newman & Reddell,
70 1987), and field surveys in countries across the world - for example: China (Zhang et al.,
71 2004; Zhao, 2000; Zhi-wei, 2000), Costa Rica (Lesica & Antibus, 1990), Ecuador (Lehnert et
72 al., 2009; Kessler et al., 2014), Honduras (Zubek et al., 2010), India (Muthakumar & Udaiyan,
73 2000; Muthakumar & Prabia, 2012, 2013; Muthuraja et al., 2014; Sudha & Ammani, 2010),
74 Lesotho (Moteetee et al., 1996), Mexico (Lara-Pérez et al., 2015), New Zealand (Cooper,
75 1976), Pakistan (Iqbal et al., 1981), Malaysia and Indonesia (Nadarajah & Nawawi, 1993;

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

92 These provisos aside, the vast majority if not all of the likely symbiotic fungi found in
93 pteridophytes fall into the arbuscular mycorrhizal (AM) category characterised by
94 intracellular hyphal coils +/- fine arbuscular hyphae and vesicles. Less frequent are dark
95 septate hyphae often associated with pseudosclerotia. By extrapolation from their well-
96 documented occurrence in seed plants (Jumpponen, 2001; Jumpponen & Trappe, 1998;
97 Mandyam & Jumpponen, 2005; Newsham, 2011; Newsham et al., 2014; Schmid et al., 1995)
98 it is reasonable to assume that these are ascomycetous. Conspicuously absent are any *bona*
99 *fide* records of basidiomycetes. The recent report that the main endophyte in
100 gametophytes of *Lycopodium alpinum* is a basidiomycete (Horn et al., 2013), despite
101 compelling cytological evidence (Burgeff, 1938; Bruchmann, 1898; Campbell, 1908; Duckett
102 & Ligrone, 1992; Lang, 1899; Schmid & Oberwinkler, 1993) and molecular data (Winther &
103 Friedman, 2007a) to the contrary in this and other lycopod gametophytes, is almost
104 certainly due to flawed analysis procedures (see Rimington et al., 2014 for a full critique).
105 Since the symbiotic status of AM fungi in seed plants and liverworts is beyond question, it
106 seems reasonable to assume the same for pteridophytes as is borne out with transmission
107 electron microscopy (TEM) studies that have invariably shown apparently healthy

108 interactions between the partners (Duckett & Ligrone, 1992; Kovács et al., 2003; Schmid &
109 Oberwinkler, 1993; 1994; 1995; 1996; Turnau et al., 1993). However, such studies have to
110 date been limited to pteridophytes where a fungus is invariably present and those where
111 such colonisations appear more sporadic, e.g., the sporophytes of the vast majority of
112 leptosporangiate ferns, have yet to be investigated. Indeed the study by Turnau et al.
113 (1993) on *Pteridium* contains the only published transmission electron micrographs of which
114 we are aware of a typical AM association in the roots of a polypod (Polypodiales) fern.

115 Whether or not dark septate hyphae (see Boullard, 1957; Burgeff, 1938; Dhillon,
116 1993; Fernández et al., 2008; Iqbal et al., 1981; Lara-Pérez et al., 2015; Lehnert et al., 2009;
117 Mandyam & Jumpponen, 2005; Moteetee et al., 1996; Muthukumar & Prabia, 2012;
118 Muthuraja et al., 2014; Nadarajah & Nawawi, 1993; Sudová et al., 2011, for examples) form
119 any kind of mutualistic relationship with pteridophytes has not been explored, but on the
120 evidence to date this would seem unlikely. We are not aware of any published
121 ultrastructural study showing such hyphae in a host cell with healthy cytoplasm in any land
122 plant let alone a pteridophyte, and definitive evidence for a function in seed plants has not
123 yet been forthcoming (Jumpponen, 2001; Jumpponen & Trappe, 1998; Newsham, 2011).
124 Our own observations on the subterranean parts of a wide range of pteridophytes, not to
125 mention bryophytes, point most strongly to saprotrophism rather than any kind of
126 mutualistic relationships. Thus, a thorough light microscope examination will reveal their
127 presence in and along the surface of the older parts of virtually any fern gametophyte, root
128 or rhizome system (see for example Muthuraja et al., 2014), just as it does for older
129 bryophyte rhizoids, thalli and stem tissues. In fact, dark septate hyphae in bryophytes are
130 just as frequent on surfaces of taxa with well characterized symbionts, be these AM fungi,
131 the ascomycete *Pezoloma ericae* or basidiomycetes as those where these symbionts are
132 absent, e.g., all mosses (Field et al., 2015b; Pressel et al., 2010).

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

139 Against this picture of seemingly abundant mycorrhizas in pteridophytes why then
140 are there not more studies? What in particular has hampered functional studies? Two
141 major contributory factors are that some of the most interesting pteridophytes are rare, for
142 example *Stromatopteris* is a New Caledonian endemic (Bierhorst, 1971), and fungus-
143 containing structures like subterranean gametophytes are rarely produced by plants in
144 cultivation, with the notable exception of *Psilotum* (Winther & Friedman, 2009), and are
145 hard to find in nature. The facts that mycoheterotrophic gametophytes are difficult to
146 culture axenically (see Whittier, 1975, 1981, 1998, 2003, 2005, 2011; Whittier & Braggins,
147 2000; Whittier & Carter, 2007a,b; Whittier et al., 2005, for special protocols) and that
148 glomeromycote fungi cannot be cultured axenically (Field et al., 2014) severely restrict the
149 scope of functional studies—for example, fulfilling Koch’s postulates and thus dissecting
150 host growth response to the presence of symbionts. Further impediments are that wiry
151 monilophyte roots are extremely difficult to infiltrate with resins for transmission electron
152 microscopy (Duckett et al., 1988) and fern roots generally often fix suboptimally due to their
153 high content of phenolics (see for example the micrographs in Peterson & Brisson, 1977;
154 Berch & Kendrick, 1982; Makgomol & Sheffield, 2001; Kovács et al., 2007). High phenolic
155 content might also challenge the accessibility of fern roots to fungi (Schneider, 1996).

156

157 **Systematic Survey**

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

160

161 **Lycopsida**

162

163 Gametophytes

164 The gametophytes of every *Lycopodium* species (here used *sensu lato* to include
165 *Diphasiastrum*, *Huperzia* plus *Phylloglossum*, *Lycopodium* and *Lycopodiella*) in the
166 Lycopodiaceae investigated to date, whether totally subterranean or partially surface-living,
167 contain fungi with a well- defined distribution and highly distinctive cytology (Treub, 1884;
168 Burgeff, 1938; Bruchmann, 1898, 1908, 1910; Campbell, 1908; Duckett & Ligrone, 1992;
169 Lang, 1899; Schmid & Oberwinkler, 1993; Winther & Friedman, 2007a) (Figs. 1a, 1b). The

170 presence of several unique features, in particular an intercellular phase of fungal
171 proliferation (Fig. 1b), led Schmid & Oberwinkler (1993) to coin the term 'lycopodioid
172 mycothallus' interaction. The first sequencing study on two gametophytes of *Lycopodium*
173 *hypogaeae* identified the fungus as a member of the Glomeraceae (following Redecker et al.,
174 2013 for the classification of arbuscular mycorrhizal fungi), a clade also found in other
175 mycoheterotrophic lineages (Merckx et al., 2009; Merckx, 2013). In contrast, a second
176 molecular study found that both ITS and LSU sequences identified the fungus in the
177 gametophytes of *Lycopodium alpinum* as Sebaciniales group B, a basal clade of the
178 agaricomycetes (Basidiomycota) (Horn et al., 2013).

179

180 **Sporophytes**

181 Turning to the sporophytes, light microscope surveys indicate that possible symbiotic
182 associations appear to be somewhat sporadic in the thin wiry roots of both Lycopodiaceae
183 and Selaginellaceae and at best are confined to a minority of the species studied (Boullard,
184 1957). Morphologically the fungi appear to be AM with large trunk hyphae, finer hyphal
185 coils and/ or arbuscules and vesicles.

186 By analogy with monilophytes (see below), the fatter and fleshier roots of *Isoëtes*
187 appear to be far better candidates for mycorrhization than their narrow wiry counterparts in
188 *Lycopodium* and *Selaginella*. However, Boullard (1957) found fungi in just one out of the 12
189 both terrestrial and aquatic species he examined, and none were found in *I. lacustris* by
190 Søndergaard & Laegaard (1977). The sole exception was *I. engelmannii*, a species of
191 transient pools, whereas *I. transvaalensis* from the same kind of habitat appears to be
192 fungus-free (Moteetee et al., 1996). Subsequently, three light microscope studies have
193 revealed AM fungi together with dark septate hyphae in the roots of the two completely
194 submerged aquatic species *I. lacustris* and *I. echinospora* in Europe (Sudová et al., 2011) and
195 several terrestrial plus two aquatic species from India (Sharma, 1998; Radhika & Rodrigues
196 2007). Sudová et al. (2011) are at pains to point out that the precise identity and function of
197 the fungi remains to be elucidated. The likely absence of mycorrhizas in *Isoëtes* most likely
198 reflects a primarily aquatic ancestry since most taxa are restricted to aquatic or semiaquatic
199 habitats.

200 The first molecular study of the symbionts in lycophyte roots yielded results that
201 have shattered the long held notion that the glomeromycetes alone were the primeval

202 vascular land plant fungal symbionts (Rimington et al., 2014). Though confirming the pre-
203 existing picture that fungal colonization appears to be less frequent than in ferns (lycophods
204 with fungi in 7 of 20 species from 17 of the 101 samples versus ferns with fungi in 13 of 18
205 species from 33 out of 58 samples—Rimington et al., 2014), Glomeromycota fungi (all in the
206 Glomeraceae) were present in only three of the lycophyte species while the other four
207 contained diverse Mucoromycotina, including six new clades. These Mucoromycotina fungi
208 belonging to different clades sometimes occurred within the same species, and even the
209 same plant.

210

211 **Monilophytes**

212

213 **Gametophytes**

214 The few electron microscope studies to date of subterranean mycoheterotrophic fern
215 gametophytes (*Botrychium* (Kovács et al., 2003; Schmid & Oberwinkler, 1994),
216 *Ophioglossum* (Schmid & Oberwinkler, 1996), *Psilotum* and *Tmesipteris* (Duckett & Ligrone,
217 2005)) have revealed that the exclusively intracellular symbionts comprise hyphal coils with
218 arbuscule-like side branches and vesicles, i.e., they are typical Glomeromycota (Figs. 1c-1f).
219 The analysis of DNA sequences confirms the fungi in *Botrychium* (Winther & Friedman,
220 2007b) and *Psilotum* (Winther & Friedman, 2009; Rimington et al., 2014) and *Tmesipteris*
221 (Rimington et al., 2014) as Glomeraceae. At the other extreme, fungi are absent from the
222 endosporic gametophytes in heterosporous ferns and lycophytes. Whether or not this is
223 also the case in *Playtzoma microphyllum*, the only fern with exosporic free-living
224 photosynthetic gametophytes (Duckett & Pang, 1984), has yet to be investigated.

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

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

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

257 Extending their morphological and molecular study on *Angiopteris* and *Osmunda*
258 (Ogura-Tsujita et al., 2013) to a range of pre-Polypodiales leptosporangiate ferns to include
259 two species in the Gleicheniales and four in the Cyatheales, Ogura-Tsujita et al. (2016) found
260 that not only were 58-97% of the gametophytes colonized by AM fungi but that these also
261 belonged to a wide range of Glomeromycota fungi. In addition to Glomeraceae, they also
262 found members of the Claroideoglomeraceae, Gigasporaceae, Acaulosporaceae, and
263 Archaeosporales fungi previously unknown in ferns but which are widespread in thalloid
264 liverworts and hornworts (Bidartondo et al., 2011; Desirò et al., 2013; Field et al., 2015b).
265 There is now a pressing need to extend these molecular studies to Polypodiales since recent

266 light microscope studies indicate the presence of similar associations in a range of genera;
267 *Adiantum*, *Pellaea* (Turnau et al., 2005), *Dryopteris* (Reyes-Jaramillo et al., 2008),
268 *Nephrolepis* (Muthukumar & Prabia, 2012) and *Pteris* (Martinez et al., 2012), and
269 particularly since the discovery of both Glomeromycota (Glomeraceae and
270 Diversisporaceae) and Mucoromycotina in *Anogramma leptophylla* from the only fungal
271 DNA sequencing study to date on the sporophytes of a member of the Polypodiales (Figs.
272 2c, 2d) (Rimington et al., 2014).

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

280 The other leptosporangiate ferns with axial and filamentous gametophytes are
281 *Actinostachys* and *Schizaea* in the Schizaeales and *Stromatopteris* in the Gleicheniales (Lang,
282 1902; Bierhorst, 1966, 1967, 1968a, 1968b, 1971; Britton & Taylor, 1901; Kiss & Swatzell,
283 1996; Pryer et al., 2004; Raghavan, 1989; Swatzell et al., 1996; von Anderkas & Raghavan,
284 1985). In these three genera the gametophytes are either partly (*Schizaea*) or totally
285 subterranean (*Actinostachys*, *Stromatopteris*) and therefore mycoheterotrophic. Virtually
286 every cell, including the multicellular rhizoids and epidermis in the multiseriate filaments in
287 *Stromatopteris* and *Schizaea* (Bierhorst, 1966, 1967, 1968b, 1971) are packed with hyphae.
288 In the tuberous axes with septate rhizoids in *Actinostachys* (Bierhorst, 1968a), the fungus
289 has a similar distribution to that in *Psilotum* and *Tmesipteris* (Duckett & Ligrone, 2005) in
290 that many of the epidermal cells are fungus-free. In addition to their multicellular rhizoids,
291 a further unusual feature in *Schizaea* is that the gametophytes develop so called
292 rhizoidophores. These are large, highly vacuolated spherical cells which develop two to
293 three rhizoids (von Aderkas & Raghavan, 1985) and form receptacles for a symbiotic fungus
294 (Britton & Taylor 1901, Kiss & Swatzell, 1996; Swatzell et al., 1996) which, from published
295 light micrographs and illustrations, appears to be AM as is the case for the symbionts
296 throughout the gametophytes of all three genera. The swollen rhizoidophores and septate
297 rhizoids in these fern gametophytes are strikingly reminiscent of the rhizoid modifications

298 associated with fungi in leafy liverworts (Kowal et al., 2016; Pressel et al., 2008b, 2010; Read
299 et al., 2000) and in particularly their septation in the Schistochiaceae (Pressel et al., 2008a).
300 However, the liverwort fungus here is invariably the ascomycete *Pezoloma ericae*.

301

302 **Sporophytes**

303 In terms of gross morphology, fern roots fall into two categories: fat and fleshy, 2 or more
304 mm in diameter, and often lacking thickened walls and phenolic deposits versus thin and
305 wiry, only ca. 1mm in diameter with phenolic compounds impregnating the cortical cells
306 and/or thickened walls (Schneider, 1996, 2000). The former features are the rule in the
307 Ophioglossales and Marattiales and to some extent the Osmundales whilst the latter are
308 typical of most leptosporangiate clades with the exception of the rootless Salviniiales. The
309 rhizomes in the rootless members of the Hymenophyllales (Duckett et al., 1996; Schneider,
310 1996; Schneider et al., 2002; Ebihara et al., 2007) have a similar overall structure. The roots
311 of horsetails are similarly thin and wiry. The rhizomes in the rootless whisk ferns resemble
312 fleshy roots anatomically (Schneider, 1996, 2000) and the shoot system in *Stromatopteris*,
313 where roots are rare, functions in a similar manner.

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

325 For the reasons noted previously, published data on the distribution of possible
326 symbiotic fungi in ferns with wiry roots are highly problematic (Figs. 2a, 2b). The situation is
327 not helped by the extreme paucity of ultrastructural studies. We are aware of only a single
328 paper (Turnau et al., 1993) that shows a typical AM association in a fern with wiry roots,
329 *Pteridium*. A further ultrastructural study on *Gleichenia* by Schmid et al. (1995) only

330 features electron micrographs of the gametophytes. Two other electron microscope studies
331 show ascomycetes (simple septa and Woronin bodies) in *Loxosomopsis* (Cyatheales) (Lehnert
332 et al., 2009) and epiphytes in the genera *Elaphaglossum*, *Hymenophyllum*, *Grammitis* and
333 *Lellingeria*. However, none of the micrographs show the ascomycetous symbionts
334 surrounded by healthy host cytoplasm thus calling into question the existence of fern
335 mycorrhizas, discussed as a feature possibly more beneficial in epiphytes by Mehltreter
336 (2010). This, together with the unlikely symbiotic status of dark septate hyphae as
337 illustrated in Boullard (1957), Fernández et al. (2008), Muthukumar & Prabia (2012) and
338 Muthuraja et al. (2014), indicates that it is highly unlikely that pteridophytes form
339 mutualistic associations with ascomycetes.

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

362 *Tmesipteris*, *Pitsana*, *Xiphopteris*, *Nephrolepis*, *Anogramma*, *Osmunda*), Diversisporaceae in
363 two (*Anogramma* and *Ophioglossum*) and Mucoromycotina in just one *Anogramma* (Kovács
364 et al., 2007; Winther & Friedman, 2007b; 2009; Rimington et al., 2014; Field et al., 2012;
365 2015a).

366

367 **Functional considerations**

368

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

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

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

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

415 In addition to showing mutualistic and specific symbiosis between this eusporangiate
416 fern and Glomeromycota fungus, the Field et al. (2015a,b) study raises the questions of
417 fungal specificity and intergenerational fidelity (Leake et al., 2008) and the precise nature of
418 the relationships between the fully mycoheterotrophic subterranean gametophytes and the
419 early achlorophyllous sporophytic stages (Boullard, 1979; Bruchmann, 1908; 1910) followed
420 by the formation of the photosynthetic above ground fronds that supply organic carbon to
421 the fungus. The authors propose that the symbiosis may operate a 'take-now, pay -later
422 strategy' (Cameron et al., 2008) and also raise the possibility that the sporophytes revert to
423 mycoheterotrophy during the below ground dormancy period from mid-summer to the
424 following spring. Unfortunately, Field et al. (2012a) were unable to locate gametophytes in
425 their study and thus investigate whether the gametophytes acquire all their carbon from the

426 sporophytes via a common symbiont. In support of intergenerational fidelity was the
427 demonstration that the symbiotic relationship in their *Ophioglossum* plants was highly
428 specific, as is the case in *Huperzia* (Winther & Friedman, 2007a) where both gametophytes
429 and sporophytes share the same three AM phylotypes. Whilst all the evidence to date
430 indicates that pteridophyte gametophytes appear to have high fungal specificity, a general
431 feature of mycoheterotrophy (Bidartondo et al., 2003; Merckx & Freudenstein, 2010), the
432 fact that in *Botrychium crenulatum* fungal diversity increases through the transition from
433 mycoheterotrophy to autotrophy (Winther & Friedman, 2007a) and Kovács et al. (2007)
434 found between five and seven AM fungi in sporophytes of *B. virginianum* suggests that
435 pteridophyte sporophytes probably benefit from a wider range of AM fungi. This premise is
436 borne out by subsequent DNA sequencing studies revealing an increasing number of
437 Glomeromycota plus Mucoromycotina, sometimes together in the same plants (Rimington
438 et al., 2014).

439 A further factor to be added to the functional debate is that in all the ultrastructural
440 studies on pteridophytes with subterranean gametophytes to date there is remarkable
441 congruence in the host-fungal cytology between the two generations (Duckett & Ligrone,
442 2005). Since the gametophytes have 'cheating' associations where only the host receives
443 benefits (Bidartondo et al., 2003; Brundrett, 2002, 2004), how far then this might also be
444 true for the sporophytes? Duckett & Ligrone (2005) point out that coiling AM mycorrhizas
445 are a feature of exploitative associations in angiosperms (Brundrett, 2004) and that the
446 multiple waves of colonisation that are outlived by the host cells bear a striking resemblance
447 to the fate of the fungi in orchid mycorrhizas, in the mycoheterotrophic liverwort *Aneura*
448 (*Cryptothallus*) *mirabilis* and in closely related *Aneura* species (Ligrone & Duckett, 1993;
449 Duckett & Ligrone, 2008). Further isotope studies like those by Field et al. (2015a) are now
450 needed to establish just how far the fungal associations in pteridophytes fall into the
451 category of balanced versus exploitative (Bidartondo et al., 2003; Brundrett, 2002, 2004).

452

453 **Evolutionary perspectives**

454

455 The discovery of an increasing range of symbionts belonging to both the Mucoromycotina
456 and the Glomeromycota (Rimington et al., 2014) and the presence of fungi with
457 characteristics of both groups in Devonian plants (Strullu-Derrien et al., 2014) has now

458 overturned the long held view that the Glomeromycota alone formed the ancestral land-
459 plant fungus symbiosis (Leake et al., 2008). The presence of both groups of fungi in
460 lycophytes and the predominance of a range of Glomeromycota in later diverging ferns closely
461 fit the phylogenetic distribution of these fungi in thalloid liverworts with dual partnerships
462 in basal clades and Glomeromycota alone in more derived groups (Bidartondo et al., 2011;
463 Field et al., 2016). Whilst most of the associations in extant pteridophytes almost certainly
464 have ancient origins, the presence of Mucoromycotina in *Anogramma* may be a much more
465 recent acquisition associated with this fern's unique life history (Goebel, 1905). These
466 discoveries clearly emphasise the novel emerging notion that fungal symbioses at the dawn
467 of plant terrestrialization were much more diverse than hitherto assumed (Field et al.,
468 2015b).

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

487 Fungal associations appear to have been progressively lost through monilophyte
488 evolution. Fungi are obligate and ubiquitous in the earlier lineages but their incidence
489 become far more capricious in polypod ferns. This trend is very clearly contrary to species

490 richness; whereas the Polypodiales number thousands of species, the numbers of species
491 are much lower for earlier groups; *Ophioglossum* 25-30, *Botrychium* 50-60, Marattiales 135,
492 Osmundales 25 and Schizaeales 190 (Christenhusz et al., 2016). Two possible explanations
493 come to mind. One is a switch in root anatomy from fleshy to wiry which accompanied the
494 evolution of the leptosporangiate ferns. The second are the radiations of leptosporangiate
495 ferns as epiphytes (Schuettpehl & Pryer, 2009). This is paralleled by the loss of symbionts in
496 epiphytic liverwort clades (Pressel et al., 2010), whilst their absence in water ferns and
497 *Isoetes* recalls the paucity of mycorrhizas in aquatic seed plants (Søndergaard & Laegaard,
498 1977; Shah, 2014). It is also interesting to note that fungi are also absent from the crown
499 group liverwort family Ricciaceae (Ligrone et al., 2007) many of which grow alongside the
500 *Isoetes* species of ephemeral pools.

501

502 **Conclusions**

503

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

519

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524

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926

927 **Figure legends**

928

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

936

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

943

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