Letters

Fungal associations of basal vascular plants: reopening a closed book?

Introduction

The widely held hypothesis that Glomeromycota fungi alone formed the ancestral land plant–fungus symbiosis (Pirozynski & Dalpé, 1989; Selosse & Le Tacon, 1998; Wang & Qiu, 2006; Parniske, 2008) has recently been challenged by new lines of evidence from molecular, cytological, functional and palaeontological studies. First, liverworts of the earliest divergent clade, the Haplomitriopsida, form a mutualistic mycorrhiza-like relationship, whereby there is reciprocal exchange of plant carbon (C) for fungal nitrogen (N) and phosphorus (P), with members of the Mucoromycotina (Bidartondo *et al.*, 2011; Field *et al.*, 2014), a fungal lineage considered basal or sister to the Glomeromycota (James *et al.*, 2006; Lin *et al.*, 2014). Second, other basal plants, including complex and simple thalloid liverworts and hornworts, enter into associations with both Mucoromycotina and Glomeromycota fungi, sometimes simultaneously (Bidartondo *et al.*, 2011; Desirò *et al.*, 2013). Third, dual partnerships involving fungi with affinities to Glomeromycota and Mucoromycotina have been reported in fossils of early vascular plants from the Devonian (Strullu-Derrien *et al.*, 2014).

Turning to the fungal associations of the extant representatives of the early diverging vascular plant lineages, the glomeromycete identity of fungi in ferns (Monilophyta) has never been questioned – a consensus borne out by cytology and limited DNA sequencing data (Wang & Qiu, 2006; Ogura-Tsujita *et al.*, 2013). By contrast, the unusual cytology of fungal colonization in lycopods (Lycopodiophyta), highly reminiscent of the cytology reported in the Haplomitriopsida genus *Treubia* (Duckett *et al.*, 2006), suggested unique fungal partnerships or 'lycopodioid mycothallus interactions' (Duckett & Ligrone, 1992; Schmid & Oberwinkler, 1993) until a molecular study detected Glomeromycota in this group (Winther & Friedman, 2008), thus 'laying to rest over a century of speculations and uncertainty' surrounding their identity (Leake *et al.*, 2008). However, Winther & Friedman's study, and a more recent investigation proposing a basidiomycete as the main symbiont in a member of the Lycopodiaceae (Horn *et al.*, 2013; but see rebuttal in Strullu-Derrien *et al.*, 2014 criticizing their limited molecular and microscopical data), used methods that do not detect Mucoromycotina fungi. Therefore, it remains to be determined whether members of the Mucoromycotina related to the fungi known to enter into mutualism with basal liverworts (Field *et al.*, 2014) also form associations with vascular plants. To test this possibility, we carried out molecular and microscopical analyses of the fungal associations of all the major lineages of lycopods and ferns.

Materials and Methods

Sampling sites were globally distributed (Supporting Information Table S1). At least one mature plant colony was collected from each site. Plants were processed for cytological and molecular analyses within 1 wk of collection by removing and cleaning roots with forceps and sterile water. Roots were prepared for scanning and transmission electron microscopy as previously described (Pressel *et al.*, 2010; Desirò *et al.*, 2013). Extraction and sequencing of genomic fungal DNA were performed using the method of Bidartondo *et al.* (2011). In brief, the universal fungal 18S primer combination NS1 (White *et al.*, 1990) and EF3 (Smit *et al.*, 1999) was used to amplify DNA which was cloned (TOPO TA; Invitrogen) and sequenced using an Applied Biosystems Genetic Analyser 3730 (Waltham, MA, USA). Between four and eight clones were sequenced for each sample and identified using NCBI BLAST (Altschul *et al.*, 1997). Sequence editing and assembly were performed in Geneious v5.6 (http://www.geneious.com). The alignment algorithms of MUSCLE were used within MEGA v5.1 (Tamura *et al.*, 2011), with reference sequences from GenBank. Using UCHIME (Edgar *et al.*, 2011) within MOTHUR (http://www.mothur.org), confirmed sequences were not chimeric. Evolutionary models were tested in MEGA. Bayesian inference was carried out using MrBayes (Huelsenbeck & Ronquist, 2001) and FigTree v1.4 (http://tree.bio.ed.ac.uk) for visualization and editing. Representative DNA sequences have been deposited in GenBank (KJ952212–KJ952241).

Results

Molecular and cytological analyses showed that both Mucoromycotina and Glomeromycota fungi associate with lycopods and ferns (Figs 1, 2). We examined samples from 20 lycopod and 18 fern species, and detected fungi in seven and 13 species, respectively (Table S1). Glomeromycota fungi were present in three lycopod species while Mucoromycotina were found in four. Fungal colonization was detected in only 17 of the 101 lycopod samples analysed. Diverse Mucoromycotina fungi colonized lycopods, sometimes occurring within the same species, and even the same plant, and six new Mucoromycotina clades were discovered (Fig. S2). Colonization rates in ferns were higher (33 out of 58 samples) and showed specificity to Glomeromycota (Fig. S1). Ferns exclusively contained members of the order Glomerales, with the exception of one *Ophioglossum* (Diversisporales), one *Psilotum* (Archaeosporales), one *Tmesipteris* (Archaeosporales), and three

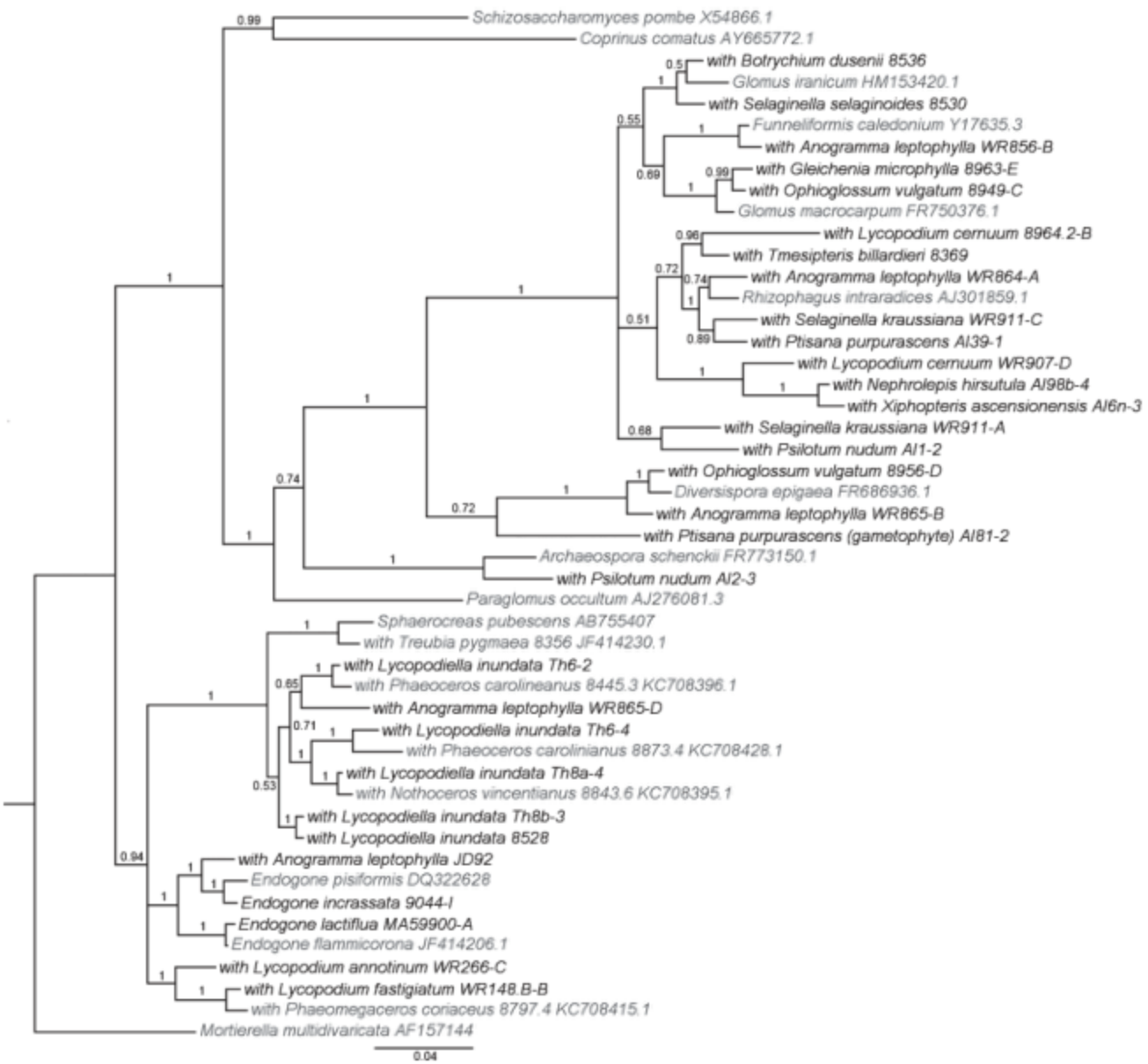

Fig. 1 Representative fungal associates of basal vascular plants in a Bayesian full 18S nrDNA analysis. Both lycopods and ferns harbour diverse Mucoromycotina and Glomeromycota fungi. Reference sequences from GenBank are highlighted in grey. Analysis was performed using an HKY85 model (nst = 2) and invgamma rates. Four heated chains were run simultaneously with a chain length of 1.1×10^6.

Anogramma (Mucoromycotina and Diversisporales) specimens; *Anogramma* was the only fern genus harbouring Mucoromycotina fungi. All samples analysed were sporophytes, with the exception of one fern gametophyte (*Ptisana* sp.), which contained Gigasporaceae fungi. This investigation added two new samples to the still limited database of *Endogone* fruiting body DNA sequences (including the first *E. incrassata*) and supported the placement of *Sphaerocreas pubescens* (Hirose *et al.*, 2014) in Mucoromycotina Group L (*sensu* Desirò *et al.*, 2013).

The cytology of fern–fungal associations hitherto undescribed is illustrated in Fig. 2. In *Anogramma* colonized by Mucoromycotina (Fig. 2a,b), the exclusively intracellular fungus produces large hyphae, finer short-lived coils and vesicles (Fig. 2b). Fungal structures are surrounded by host plasma membrane and healthy host cytoplasm packed with mitochondria (Fig. 2a). Fungal associations in both the roots and gametophytes of *Ptisana* (Fig. 2c–g) comprise structures typical of Glomeromycota colonization, including arbuscules, large vesicles and hyphal coils, which are intimately associated with the plant cell wall.

Discussion

This study demonstrates for the first time that the extant representatives of the earliest diverging clades of vascular plants, lycopods and ferns, form associations with both Mucoromycotina and Glomeromycota fungi. Lycopod sporophytes rely on a variety of strategies, entering into partnership with either Glomeromycota or Mucoromycotina, both or often neither. By contrast, all the ferns

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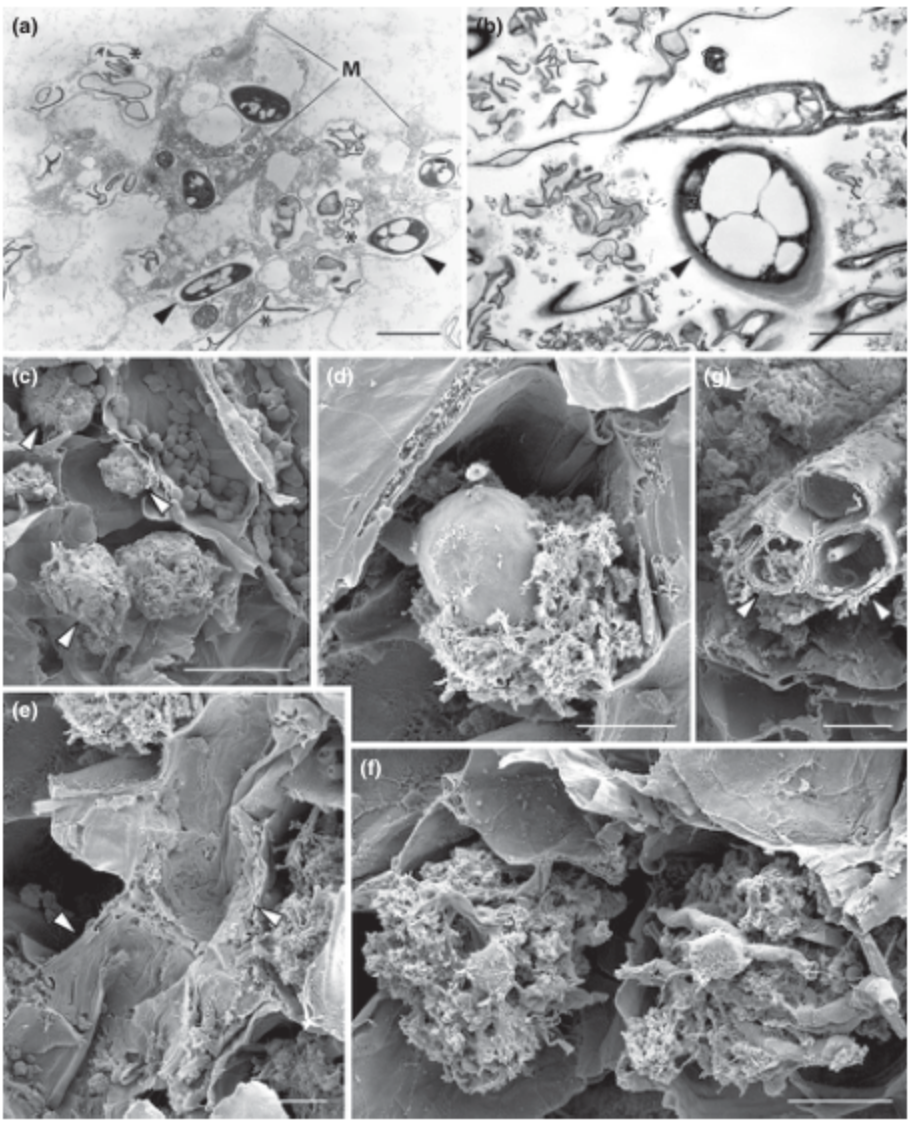

Fig. 2 Fungal colonization in ferns. (a, b) Transmission electron micrographs of *Anogramma leptophylla* colonized by Mucoromycotina fungi. Fungal colonization is largely confined to a zone where the tubers join the main root system and the lipid-filled tubers, as in hornworts and liverworts, are fungus-free. (a) Early stage in fungal colonization showing living (arrowed) and collapsed (*) hyphae surrounded by healthy host cytoplasm packed with mitochondria (M). (b) Later stage of colonization showing a large hypha, clusters of collapsed short-lived hyphae and a vesicle (arrowed). (c–g) Scanning electron micrographs of *Ptisana purpurascens* colonized by Glomeromycota fungi. (c) Fungal structures (indicated by arrows) in root inner cortex cells packed with amyloplasts. (d) Large vesicle and fine hyphal coil. (e) Hyphae tightly appressed to the inner walls of colonized cells (indicated by arrows). (f) Arbuscules. (g) Fungal entry is via the root hairs (indicated by arrows). Bars: (c) 50 μm; (d–g) 20 μm.

sampled associated exclusively with Glomeromycota, with the exception of the derived genus *Anogramma* where dual partnerships were detected. Our discovery finally provides an explanation for the unusual colonization patterns reported before in some lycopods (Duckett & Ligrone, 1992; Schmid & Oberwinkler, 1993), consisting of an intracellular phase and extensive fungal proliferation in gametophytic mucilage-filled intercellular spaces, as also reported in other Mucoromycotina-associated groups: the Haplomiotriopsida liverwort genus *Treubia* (Duckett *et al.*, 2006), several hornwort genera (Desirò *et al.*, 2013), and the Devonian fossil plant *Horneophyton ligneri* (Strullu-Derrien *et al.*, 2014). We hypothesize that the associations between Mucoromycotina fungi and vascular plants are mutualistic. Beyond microscopy, our main line of evidence is the recent demonstration of mutualism between Haplomitriopsida liverworts and Mucoromycotina fungi (Field *et al.*, 2014) closely related to those now detected in vascular plants.

Our observations demonstrate that intercellular fungal proliferation is a signature of Mucoromycotina colonization, and lend further support to the hypothesis that the early Devonian vascular plant *Nothia*, which also harboured inter- and intracellular fungi (Berbee & Taylor, 2007; Krings *et al.*, 2007a,b), formed associations with Mucoromycotina fungi (Pressel *et al.*, 2010; Strullu-Derrien *et al.*, 2014). Nonetheless, where the fungi are exclusively intracellular (e.g. *Anogramma*), it is impossible to ascertain from cytology alone to which fungal group they belong, as both Glomeromycota and Mucoromycotina produce vesicles and hyphal coils. The short-lived fungal swellings or lumps typical of Mucoromycotina colonization in the Haplomitriopsida (Carafa *et al.*, 2003; Duckett *et al.*, 2006) are unique to this group, the only land plant lineage to date known to associate exclusively with Mucoromycotina fungi (Field *et al.*, 2014). Arbuscules, the signature of Glomeromycota colonization in angiosperms, are produced in some lycopod and fern–Glomeromycota associations (e.g. *Ptisana*, *Angiopteris*, *Osmunda* – Ogura-Tsujita *et al.*, 2013) but are lacking in others (see Strullu-Derrien *et al.*, 2014 and references therein), as is also often the case in liverworts and hornworts.

The presence of Glomeromycota and Mucoromycotina fungi in lycopods and the predominance of Glomeromycota in the later diverging ferns fit the phylogenetic distribution of these fungi in

other 'lower' land plant groups. As such, dual partnerships are the norm in basal thalloid liverworts, while more derived clades have, like ferns, the specificity to Glomeromycota typical of later vascular plant lineages (Smith & Read, 2008). Together with the occurrence of multiple fungal associations in Devonian plants (Strullu-Derrien *et al.*, 2014), this lends further weight to the notion of shifting symbiotic encounters between early land colonists and soil-dwelling fungi before the Glomeromycota became dominant. The presence of Mucoromycotina in *Anogramma* may be a recent reacquisition, on a par with *Endogone* forming ectomycorrhizas with pines (Walker, 1985), and probably relates to its unique life cycle among ferns – comprising short-lived sporophytes and aestivating tubers (Goebel, 1905). It is also possible that associations with Mucoromycotina in lycopods and other plants represent recent acquisitions. However, this seems unlikely, given that the genes required for mycorrhiza formation in angiosperms are highly conserved across major plant lineages and that mycorrhizal genes from Mucoromycotina-associated Haplomitriopsida liverworts recovered the Glomeromycota mycorrhizal phenotype in a transformed mutant of the angiosperm *Medicago truncatula* (Wang *et al.*, 2010). These findings, coupled with the occurrence of Mucoromycotina in extant basal groups of both nonvascular and vascular plants, as well as fossil plants (Strullu-Derrien *et al.*, 2014), indicate that associations between Mucoromycotina and land plants are extremely ancient.

During this investigation, we examined sporophytes only and it would be desirable now to study the cryptic nonphotosynthetic gametophytes of a range of lycopods and ferns, which are expected to be more heavily and consistently colonized by fungi (Read *et al.*, 2000; Ogura-Tsujita *et al.*, 2013). Nevertheless, our discovery that lycopods enter into partnerships with both Mucoromycotina and Glomeromycota fungi opens a new chapter in understanding the origins and evolution of fungal symbioses in vascular plants. Functional studies into the nature of these associations, like those conducted by Field *et al.* (2014) on Haplomitriopsida–Mucoromycotina symbioses, are now needed.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Glomeromycota associates of basal vascular plants in a Bayesian full 18S nrDNA analysis.

Fig. S2 Mucoromycotina associates of basal vascular plants in a Bayesian full 18S nrDNA analysis.

Table S1 Lycopod, fern and fungal fruiting body samples analysed with their origin and fungi detected

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