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Cite this article: Desirò A, Duckett JG, Pressel S, Villarreal JC, Bidartondo MI. 2013 Fungal symbioses in hornworts: a chequered history. Proc R Soc B 280: 20130207.
http://dx.doi.org/10.1098/rspb.2013.0207

Received: 28 January 2013
Accepted: 28 February 2013

Subject Areas:
evolution, ecology, molecular biology

Keywords:
arbuscular mycorrhizas, *Endogone*, evolution, fungi, plants, symbiosis

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2013.0207 or via http://rspb.royalsocietypublishing.org.

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Fungal symbioses in hornworts: a chequered history

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Hornworts are considered the sister group to vascular plants, but their fungal associations remain largely unexplored. The ancestral symbiotic condition for all plants is, nonetheless, widely assumed to be arbuscular mycorrhizal with Glomeromycota fungi. Owing to a recent report of other fungi in some non-vascular plants, here we investigate the fungi associated with diverse hornworts worldwide, using electron microscopy and molecular phylogenetics. We found that both Glomeromycota and Mucoromycotina fungi can form symbioses with most hornworts, often simultaneously. This discovery indicates that ancient terrestrial plants relied on a wider and more versatile symbiotic repertoire than previously thought, and it highlights the so far unappreciated ecological and evolutionary role of Mucoromycotina fungi.

1. Introduction

Hornworts (Anthocerotophyta) are an ancient phylum, approximately 300–400 million years old, now considered sister to the earliest vascular plants [1–6]. They have a worldwide distribution in moist temperate and tropical habitats as pioneer colonizers of nutrient-poor substrates. Having thalloid gametophytes and persistent sporophytes, hornworts are key to understanding the transformation from the gametophyte-dominated life cycles of non-vascular plants to the sporophyte-dominated life cycles of vascular plants [7].

Arbuscular mycorrhizal fungi (Glomeromycota) [8] are the prevalent symbionts of extant vascular plants and the early-diverging lineages of the major clades (simple and complex thalloid liverworts, lycophytes and ferns). Thus, identification of mycorrhizal fungi within plants relies routinely on Glomeromycota-specific detection. Glomeromycota were firmly regarded as the ancestral mycorrhizal type [9,10] until the recent discovery of *Endogone*-like Mucoromycotina fungi in the earliest liverwort lineage (Haplomitriopsida) and in some early simple and complex thalloid liverworts questioned that idea [11]. Glomeromycota fungi are reported to associate with five hornworts, based on electron microscope [12], *in vitro* [13] and molecular studies [11]. However, two hornworts are reported to harbour *Endogone*-like fungi [11] and two others are considered non-symbiotic [14,15]. We ignore the fungal symbioses of the vast majority of the 200–220 hornwort species, though the scanty information to date hints at some parallels with liverworts. Furthermore, while knowledge of Glomeromycota arbuscular mycorrhizal fungi has blossomed ever since they were taxonomically separated from *Endogone* [16], the latter have become grossly neglected. Because the information available is insufficient to understand the symbiotic past and present of fungi, hornworts and vascular plants, here we carry out the first global molecular and cytological analysis of hornworts (table 1; electronic supplementary material, tables S1 and S2) designed to test their symbioses.

2. Material and methods

(a) Sampling

At each of nearly 200 sites, we collected at least one colony of each hornwort species. Each collection was subsampled within one week, cleaned with forceps and rinsed

Table 1. Summary of the hornworts sampled, subdivided by species and the fungal status of each species. The number of samples within each fungal clade is in brackets.

organism	location	sample number	Glomeromycota	Mucoromycotina
Anthoceros sp.	Ascension Island, Malaysia, South Africa	5	Archaeosporaceae (1), Claroideoglomeraceae (1), Paraglomeraceae (1)	group A (3), B (1), D (1), Mucoromycotina (1)
Anthoceros agrestis	China, England, Scotland	4	Acaulosporaceae (1), undescribed Archaeosporales (2), Claroideoglomeraceae (3)	group A (3)
Anthoceros fusiformis	USA	2	n.d.	group B (1), D (1)
Anthoceros lamellatus	Panama	1	Archaeosporaceae (1)	group D (1)
Anthoceros laminiferus	New Zealand	16	Acaulosporaceae (1), Archaeosporaceae (1), Claroideoglomeraceae (1)	group A (2), C (1), I (1), Mucoromycotina (1)
Anthoceros punctatus	Scotland, South Africa, USA, Wales	7	Acaulosporaceae (2), Archaeosporaceae (1), Archaeosporales (1), Claroideoglomeraceae (1), Glomeraceae (3)	group A (2)
Dendroceros crispus	Panama	1	n.d.	n.d.
Dendroceros validus	Malaysia	1	n.d.	n.d.
Folioceros sp.	Malaysia	3	Claroideoglomeraceae (1), Glomeraceae (2)	group E (1)
Folioceros fuciformis	China, Malaysia	6	undescribed Archaeosporales (2), Glomeraceae (3)	n.d.
Leiosporoceros dussii	Panama	1	n.d.	n.d.
Megaceros sp.	New Zealand	9	Archaeosporaceae (1), Glomeraceae (1)	group B (1), C (1), Mucoromycotina (2)
Megaceros flagellaris	Australia, Malaysia	4	n.d.	n.d.
Megaceros leptohymenius	New Zealand	18	undescribed Archaeosporales (1), Claroideoglomeraceae (1), Glomeraceae (2)	group A (1), C (3), Mucoromycotina (2)
Megaceros pellucidus	New Zealand	9	undescribed Archaeosporales (1)	group C (1), F (1)
Nothoceros giganteus	New Zealand	3	n.d.	n.d.
Nothoceros vincentianus	Panama	4	Acaulosporaceae (1), Glomeraceae (1)	group F (1), Mucoromycotina (1)
Notothylas javanica	Panama	1	Diversisporaceae (1)	n.d.
Notothylas orbicularis	Panama	1	Glomeraceae (1)	n.d.
Phaeoceros sp.	China	1	Glomeraceae (1)	group B (1)
Phaeoceros carolinianus	Ascension Island, Australia, China, Italy, Malaysia, New Zealand, Panama, South Africa, USA	58	Acaulosporaceae (9), Archaeosporaceae (9), undescribed Archaeosporales (4), Claroideoglomeraceae (10), Glomeraceae (21)	group A (10), B (9), C (4), D (5), F (1), G (1), Mucoromycotina (5)
Phaeoceros dendroceroides	Panama	2	Archaeosporaceae (1), Claroideoglomeraceae (1)	group B (1), E (1)
Phaeoceros laevis	China, England, Falkland Islands, Scotland, Wales	13	Archaeosporaceae (3), Claroideoglomeraceae (1), Glomeraceae (2)	group A (4), B (2), I (1)
Phaeoceros (*Paraphymatoceros*) *pearsonii*	USA	1	n.d.	n.d.
Phaeomegaceros sp.	Australia, Malaysia	2	Glomeraceae (1)	group D (1)
Phaeomegaceros coriaceus	New Zealand	19	Acaulosporaceae (3), Archaeosporaceae (1), undescribed Archaeosporales (1), Claroideoglomeraceae (1), Glomeraceae (5)	group A (5), B (3), C (2), D (1), E (1), F (1), G (2), H (2), I (1), L (1), Mucoromycotina (3)
Phaeomegaceros hirticalyx	New Zealand	7	n.d.	group C (1)

in distilled water. Fungal fruitbodies were obtained from public and personal collections. Plant vouchers are in the herbarium of the Natural History Museum.

(b) Ultrastructural analysis

Preparation of samples for transmission electron microscopy followed Ligrone & Duckett [17]. Healthy thalli were fixed in 3 per cent glutaraldehyde, 1 per cent fresh formaldehyde and 0.75 per cent tannic acid in 0.05 M Na–cacodylate buffer, pH 7, for 3 h at room temperature. After rinses in 0.1 M buffer, the samples were postfixed in buffered (0.1 M, pH 6.8) 1 per cent osmium tetroxide overnight at 4°C, dehydrated in an ethanol series and embedded in Spurr's resin via ethanol. Thin sections were cut with a diamond knife, stained with methanolic uranyl acetate for 15 min and in Reynolds' lead citrate for 10 min, and observed with a Hitachi H-7100 transmission electron microscope at 100 kV. For cryo-scanning electron microscopy, the protocol of Duckett *et al*. [18] was followed. For light microscopy, 0.5 µm thick sections were cut with a diamond histo-knife, stained with 0.5 per cent toluidine blue and photographed with a Zeiss Axioskop light microscope fitted with an MRc Axiocam digital camera.

(c) Fungal detection and identification

A 2–3 mm section of the colonized part of each thallus was placed in 300 µl cetyltrimethylammonium bromide (CTAB) extraction buffer and stored at −80°C until use. Genomic DNA was extracted from one to three thallus fragments from each collection with the method of Gardes & Bruns [19] but using GeneClean (QBioGene) for purification. The same protocol was used for 1–2 mm inner fragments of each fungal fruitbody. Fungal 18S ribosomal DNA was amplified using JumpStart (Sigma) with primers NS1 [20] and EF3 [21] by 94°C for 2 min, 35 cycles of 94°C for 40 s, 54°C for 30 s, 72 min for 1 min 45 s and a final step at 72°C for 7 min. Universal fungal primers failed to amplify most samples; thus, we applied nested PCR with additional 18S primer sets: AML1–AML2 [22] to detect Glomeromycota, and EndAD1f (5′-GTAGTTGAATTTTAGCCYTGGCT-3′) and EndAD2r (5′-ACCTTCCG GCCAAGGTTATARAC-3′) to detect Endogonales. For nested PCR, Glomeromycota 18S was first amplified using PicoMaxx high fidelity (Agilent Technologies) with NS1 [20] and EF3 [21] by 95°C for 2 min, 30 cycles of 95°C for 40 s, 58°C for 30 s, 72°C for 1 min 45 s and a final step at 72°C for 7 min. Subsequently, products were nested using JumpStart with AML1–AML2 [22] by 94°C for 4 min, 28 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 50 s with a final extension step at 72°C for 7 min. Endogonales 18S was first amplified using PicoMaxx with EndAD1f and NS8 [20]. The cycling conditions were as mentioned earlier with annealing at 63°C for 30 s and extension at 72°C for 1 min 20 s. Products were then nested using JumpStart with EndAD1f and EndAD2r (approx. 890 bp) by 94°C for 4 min, 27 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min and a final step of 72°C for 7 min. All products from fungal, Glomeromycota and Endogonales amplifications were cloned with TOPO TA cloning kit for sequencing (Invitrogen), and at least four clones, for each sample, were sequenced with BigDye v. 3.1 on an ABI3730 genetic analyser (Applied Biosystems).

(d) Bioinformatics

The DNA sequences were assembled and curated using BioEdit v. 7.1.3 [23] and Mega v. 5 [24], aligned using MUSCLE [25] or webPrank [26], and compared by BLAST [27] against the INSD [28]. Phylogenetic analyses were conducted separately for Glomeromycota and Mucoromycotina. Phylogenies were inferred with RaxmlGUI v. 1.3 [29] and MrBayes v. 3.1.2 [30], using jModelTest v. 2.1.1 [31] to choose best-fit models of nucleotide substitution. The GTR + G model was chosen for both analyses. A total of 1000 bootstrap replicates were performed for trees constructed with RaxmlGUI, whereas the Markov chain Monte Carlo was run for 10 million generations. For both Glomeromycota and Mucoromycotina, two phylogenetic analyses were conducted: the first using representative DNA sequences of each clade and the second using all sequences. Alignments and trees are available in TreeBASE (submission 13945) [32]. Representative DNA sequences are in GenBank (KC708342–KC708444).

(e) Inferring symbiotic history

We sampled 26 hornwort DNA accessions (see the electronic supplementary material, table S1). As outgroups, we included five taxa representing early land plants (liverworts, mosses) and seedless vascular plants (lycopods, ferns; electronic supplementary material, table S1). Whenever possible, we used the collection from which fungal data were obtained. Plant genomic DNA isolation followed protocols described by Duff *et al*. [33,34]; newly generated DNA sequences are in GenBank (JX885632–JX885643). To infer phylogenetic relationships, we used the plastid gene *rbc*L. Sequence editing and alignment were carried out in Geneious v. 5.5.6 and alignments are in TreeBASE (submission 13455) [32]. Phylogenetic analyses were performed under likelihood (ML) optimization and the GTR + G substitution model using RAxML [35]. Statistical support was assessed via 100 ML bootstrap replicates and the same substitution model. Each tree tip was scored for fungal absence or presence based on molecular and microscopy data. Ancestral reconstructions of Glomeromycota and Mucoromycotina were carried out separately relying on ML as implemented in Mesquite using the Markov 1-parameter model [36], the highest likelihood tree from RAxML and parameters estimated from data. An asymmetrical two-rate parameter model was also used with similar results.

3. Results

(a) Fungal colonization of hornworts

We collected 199 hornworts of over 20 species, in 10 of the 12 described genera, from six continents (see the electronic supplementary material, table S2). Fungal hyphae are inter- and intracellular in the central parts of thalli that bear numerous rhizoids, from above the ventral epidermis to the base of the large genus-diagnostic mucilage chambers (figure 1*a*), and cyanobacterial colonies (figure 1*b–d*; electronic supplementary material, figure S1*a–c*). Intracellularly, thin-walled and often branching hyphae form swellings and vesicles (figure 1*e*,*d*; electronic supplementary material, figure S1*e–g*); cells just below the mucilage chambers mainly contain collapsed fungal remains (figure 1*g*,*h*). In the intercellular spaces, in close proximity to host cell walls, both thin-walled hyphae (see the electronic supplementary material, figure S2*b*) and fungal structures with thick multi-layered walls are present (figure 1*g*,*i*,*j*; electronic supplementary material, figure S2*a*). The latter often have thin-walled hyphae in their lumina (figure 1*i*,*j*). Intracellular hyphae intimately associated with plant cell walls (see the electronic supplementary material, figure S2*c*) or penetrating cells (see the electronic supplementary material, figure S2*d*) are common.

(b) Identification of fungi

We found that 121 of 199 samples were colonized by Glomeromycota and/or Mucoromycotina. Fifty samples were associated with both, whereas 42 and 29 samples harboured only Glomeromycota or Mucoromycotina,

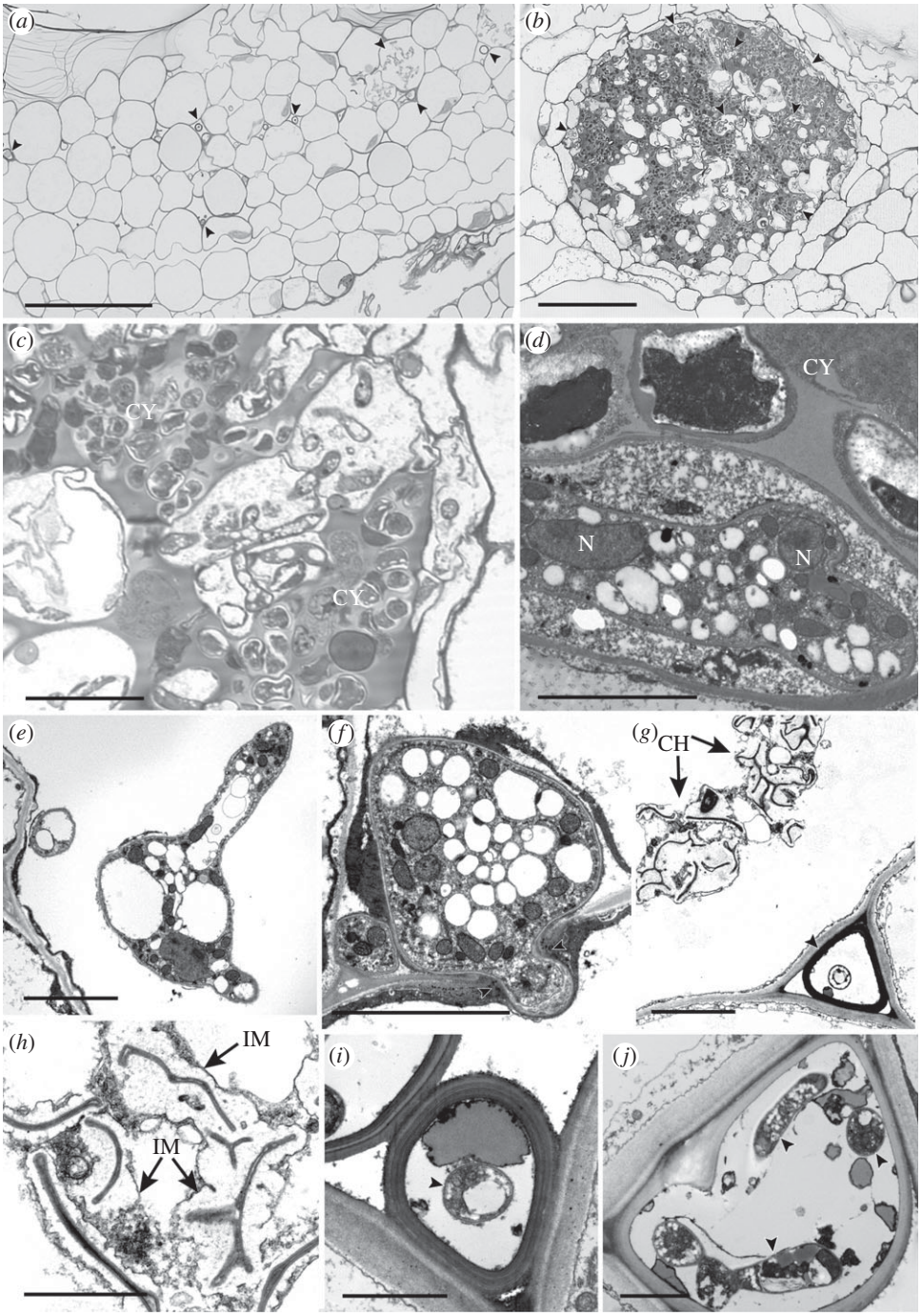

Figure 1. (*a–c*) Light and (*d–j*) transmission electron micrographs of fungal endophytes in (*a*,*g*,*h*) *Folioceros* (MA33) and in (*b–f*) *Anthoceros* (MA29). (*a–c*) Fungal hyphae occur either (*a*) scattered in the central region of the thallus (arrowed) or (*b*) in close association with cyanobacterial colonies (CY; arrowed, enlarged in *c*). (*d*) Multi-nucleate hypha (N, nucleus) in cell adjacent to a cyanobacterial colony (CY). (*e*,*f*) Intracellular hyphae; (*e*) branched hypha and (*f*) hypha bridging the walls (arrowed) of two adjacent host cells. (*g*) Thick-walled fungal structure in mucilage-filled intercellular space (arrowed) adjacent to intracellular collapsed hyphae (CH). (*h*) Detail of collapsed intracellular hyphae, note the extensive interfacial matrix (IM). (*g*,*j*) Intercellular thick-walled fungal structures with internal thin-walled hyphae (arrowed). Scale bars: (*a*,*b*) 100 µm, (*c*) 20 µm, (*d–g*) 5 µm, (*h–j*) 2 µm.

respectively (see the electronic supplementary material, table S2). *Anthoceros*, *Folioceros*, *Notothylas*, *Phaeoceros* and *Phaeomegaceros* were abundantly colonized by fungi, *Megaceros* and *Nothoceros* were occasionally colonized, and *Dendroceros validus*, *D. crispus*, *Leiosporoceros dussii*, *Megaceros flagellaris*, *Nothoceros giganteus* and *Phaeoceros* (*Paraphymatoceros*) *pearsonii* were not colonized.

We detected fungi from each of the four Glomeromycota orders (figure 2; electronic supplementary material, figure S3, and tables S1 and S2). The fungi in most hornwort samples (65 samples) belonged to Glomerales in each of its five genera [37]. The fungi from 30 hornwort samples were Archaeosporales, in the *Archaeospora* clade, and in a clade sister to *Ambispora* and *Geosiphon* [37]. The fungi from 18 hornwort samples were Diversisporales (17 in the Acaulosporaceae and one in the Diversisporaceae). Paraglomerales were detected in one sample.

We detected 13 Mucoromycotina clades supported by strong bootstrap/posterior probability values and with sequences from at least three different samples (figure 3; electronic supplementary material, figure S4, and tables S1 and S2). These comprise the *Endogone*-like clades detected

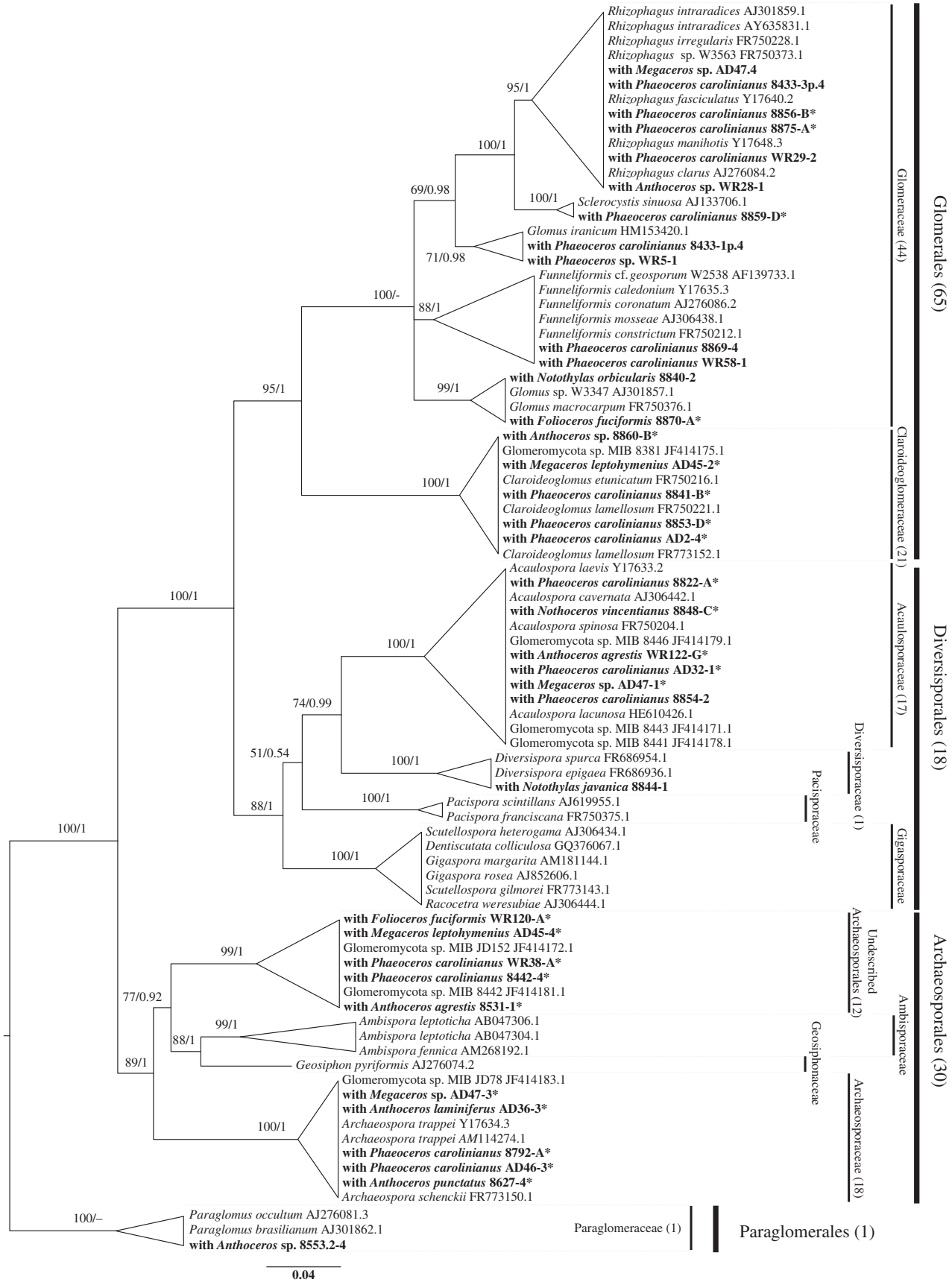

Figure 2. Phylogenetic placement of representative Glomeromycota fungi retrieved from hornworts. The tree encompasses different subclades, as in the study by Krüger *et al.* [37]. The DNA sequences retrieved from hornworts are in bold. Support values are from maximum-likelihood/Bayesian analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis. Numbers of retrieved sequences belonging to each Glomeromycota family/order are in brackets. The sequences marked with an asterisk were retrieved using fungal primers NS1-EF3 [20,21].

by Bidartondo *et al.* [11] and three new clades (B, G and I). Three clades include fruitbodies of described *Endogone*: *E. aggregata*/*E. pisiformis*, *E. flammicorona*/*E. lactiflua* and *E. oregonensis*. Only two other *Endogone* sp. fruitbodies (W5994 and T14506) are present in the tree, in clades E and G, respectively. The remaining eight clades have only fungal DNA sequences retrieved from plants (figure 3; electronic supplementary material, figure S4). The new

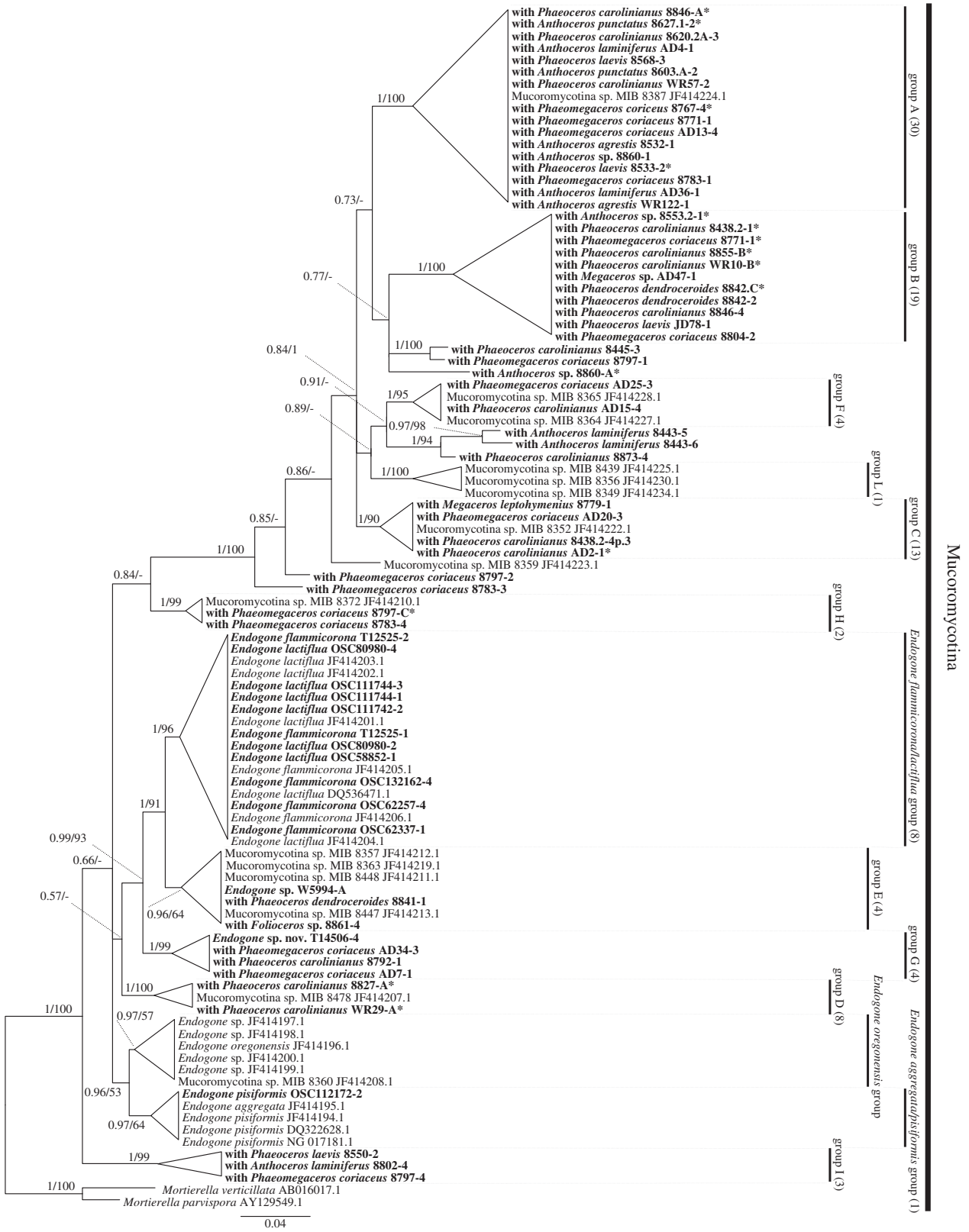

Figure 3. Phylogenetic placement of representative *Endogone*-like fungi detected in hornworts within the Mucoromycotina clade. The tree has 13 clades. The DNA sequences retrieved from hornworts are in bold. Support values are from Bayesian/maximum-likelihood analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis. Numbers of retrieved sequences belonging to each group are in brackets. Sequences marked with an asterisk were retrieved using fungal primers NS1-EF3 [20,21].

primers turned out to be also effective for the amplification of 10 decades-old *Endogone* fruitbodies.

(c) Symbiotic history of fungi and hornworts

Uncertainty (ML prob. = 0.50) in ancestral reconstruction analyses does not allow us to state conclusively whether Glomeromycota or Mucoromycotina (see the electronic supplementary material, figure S5 and table S3) were present in the ancestor of hornworts. Maximum-likelihood reconstruction (see the electronic supplementary material, figure S5 and table S3) moderately supports the presence of Glomeromycota in the ancestor of Anthocerotaceae (ML prob. = 0.56), Dendrocerotaceae (ML prob. = 0.73), *Megaceros* (ML prob. = 0.62) and

Phaeoceros (ML prob. = 0.99). If Glomeromycota were ancestral in hornworts, they were lost at least five times. In turn, colonization by Mucoromycotina may have evolved three to four times from a non-symbiotic plant ancestor. Similarly, our analyses indicate Mucoromycotina were acquired independently in *Anthoceros*, excluding *Folioceros* (ML prob. = 0.72; but higher in the ancestor of the *A. agrestis–punctatus* group, ML prob. = 0.99), *Phaeoceros* (ML prob. = 0.99) and the ancestor of Dendrocerotaceae with moderate support (ML prob. = 0.74; electronic supplementary material, table S3). The alternative scenario would be ancestral Mucoromycotina with subsequent losses in *Leiosporoceros*, *Nothoceros*, *Dendroceros*, *Megaceros flagellaris*, *Notothylas* and *P. pearsonii*.

4. Discussion

The first systematic study of fungi in hornworts reveals that this ancient plant lineage has developed a variety of symbiotic strategies. Hornworts form symbioses with (i) nearly all clades of the arbuscular mycorrhizal phylum Glomeromycota, (ii) unexpectedly diverse members of the unplaced subdivision Mucoromycotina, both closely and distantly related to *Endogone*, (iii) both Glomeromycota and Mucoromycotina, or (iv) neither Glomeromycota nor Mucoromycotina. This shifting symbiotic scenario contrasts with the conservatism of liverwort–basidiomycete symbioses [38], liverwort–Mucoromycotina symbioses in the earliest land plant lineages [11], and with the symbioses of later thalloid liverworts and vascular plants, where mycorrhizal symbioses with Glomeromycota are nearly ubiquitous and only a few recent lineages have switched to Basidiomycota, Ascomycota or to being non-mycorrhizal. Trappe [39] first reported that arbuscular mycorrhizas are the ancestral mycorrhiza in angiosperms. A vast survey by Wang & Qiu [9] confidently extended this notion to all land plants. However, the results presented here further reinforce the recent discovery that Glomeromycota were not the only symbiotic fungi involved in early plant evolution [11]. Mucoromycotina probably preceded the Glomeromycota in liverworts, and in hornworts either group may have been ancestral.

Cytological features characteristic of Glomeromycota and Mucoromycotina agree with molecular data. Large vesicles filling plant cells are diagnostic of Glomeromycota. Intracellular fungal swellings plus thin-walled hyphae and thick-walled fungal structures in small mucilage-filled intercellular spaces are typical not only of Mucoromycotina in the basal liverwort *Treubia* [40,41] but also of fungi in the Devonian fossil plant *Nothia* [42,43]. The ultrastructure of hornwort symbionts is also similar to that of the spores of *Endogone flammicorona* sporocarps [44]. The principal mode of fungal entry in hornworts may well be via mucilage clefts [12], in contrast to rhizoidal entry in the majority of liverworts. It is noteworthy that, as in liverworts, hornworts that are either epiphytic and epiphyllous (*Dendroceros*) or with thalli growing either over other bryophytes (*Nothoceros*) or in very wet places (*Megaceros*) usually lack fungi [41].

In liverworts, fungal symbioses are either obligate or absent in different taxa, and fungi occupy specific regions of the thalli or stems [41]. By contrast, fungi in hornworts are more capricious; inter- and intracellular hyphae are usually present in the central parts of thalli in regions with numerous rhizoids and extend from subepidermal layers to the base, but not into large mucilage cavities, when present (e.g. *Anthoceros*). Hyphae are also closely associated with cyanobacteria, suggesting a relationship resembling that of the only non-mycorrhizal glomeromycete, *Geosiphon*, which is symbiotic with cyanobacteria [45]. A functional relationship between fungi and cyanobacteria may also explain the absence of the former in some hornwort samples. There are indications that the more abundant the cyanobacteria, the less likely are hornworts to harbour fungi. Thus, *Nostoc* cyanobacteria are most extensive in the thallus channels of fungus-free *Leiosporoceros* [15], and cyanobacterial chambers are most prominent in *P. hirticalyx* (J. G. Duckett & S. Pressel 2012, unpublished data), which frequently lacks a fungus. In Blasiales, the only liverworts with cyanobacteria, *Nostoc* are more numerous than in hornworts, and fungi are absent [46]. As in liverworts, hornwort sporophytes are fungus-free, and hyphae never occur in the placental region despite its extensive mucilage-filled intercellular spaces [47].

How the newly discovered intimate symbioses, or lack thereof, between hornworts and fungi from Glomeromycota and/or Mucoromycotina facilitate or constrain establishment and growth under different environmental conditions is unknown. Sources of uncertainty include (i) the evolutionary relationships and ecological niche of Mucoromycotina, a group of fungi where taxon sampling remains severely limited but which it is becoming increasingly clear harbours extensive phylogenetic diversity and was instrumental in the origin and diversification of land plants; (ii) that the fungi of only a few ferns, lycopods and liverworts are known; (iii) that some early branching events during terrestrial plant evolution are poorly supported by current analyses; and (iv) that the functional significance of the symbioses is untested. Here, we opened new windows into the history of plants and fungi in terrestrial ecosystems, and the current distribution and ecology of plant–fungal symbioses.

We thank J. Trappe, P. McGee, C. Walker and Oregon State University Herbarium for fungal specimens, Z. Ludlinska (Nanovision Centre, Queen Mary University of London) for her invaluable assistance in operating the cryo-SEM, DoC New Zealand and Fairy Lake Botanical Garden, China, for collecting permits, C. Walker for comments on the manuscript, and W. Rimington for help generating molecular data. A.D. was funded by the University of Turin and Bando ad Alta Formazione of the Regione Piemonte. J.G.D. thanks the Leverhulme Trust for an Emeritus Fellowship that enabled this study. J.C.V. was supported by DFG grant no. RE-603/14-1.

References

1. Groth-Malonek M, Pruchner D, Grewe F, Knoop V. 2004 Ancestors of trans-splicing mitochondrial introns support serial sister relationships of hornworts and mosses with vascular plants. *Mol. Biol. Evol.* **22**, 117–125. (doi:10.1093/molbev/msh259)
2. Qiu Y-L *et al*. 2006 The deepest divergences in land plants inferred from phylogenomic evidence. *Proc. Natl Acad. Sci. USA* **103**, 15 511–15 516. (doi:10.1073/pnas.0603335103)
3. Renzaglia KS, Villarreal JC, Duff RJ. 2009 New insights into morphology, anatomy, and systematics of hornworts. In *Bryophyte biology* (eds B Goffinet, AJ Shaw), pp. 139–172, 2nd. edn. Cambridge, UK: Cambridge University Press.

4. Wikström N, He-Nygrén X, Shaw AJ. 2009 Liverworts (Marchantiophyta). In *The time tree of life* (eds SB Hedges, S Kumar), pp. 146–152. New York, NY: Oxford University Press.
5. Chang Y, Graham SW. 2011 Inferring the higher-order phylogeny of mosses (Bryophyta) and relatives using a large, multigene plastid data set. *Am. J. Bot.* **98**, 839–849. (doi:10.3732/ajb.0900384)
6. Villarreal JC, Renner SS. 2012 Hornwort pyrenoids: carbon-concentrating mechanisms evolved and were lost at least five times during the last 100 million years. *Proc. Natl Acad. Sci. USA* **109**, 18 873–18 878. (doi:10.1073/pnas.1213498109)
7. Villarreal JC, Cargill DC, Hagborg A, Söderström L, Renzaglia KS. 2010 A synthesis of hornwort diversity: patterns, causes and future work. *Phytotaxa* **9**, 150–166.
8. Schüßler A, Schwarzott D, Walker C. 2001 A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol. Res.* **105**, 1413–1421. (doi:10.1017/S0953756201005196)
9. Wang B, Qiu Y-L. 2006 Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* **16**, 299–363. (doi:10.1007/s00572-005-0033-6)
10. Smith VSE, Read DJ. 2008 *Mycorrhizal symbiosis*, 3rd edn. San Diego, CA: Academic Press.
11. Bidartondo MI, Read DJ, Trappe JM, Merckx V, Ligrone R, Duckett JG. 2011 The dawn of symbiosis between plants and fungi. *Biol. Lett.* **7**, 574–577. (doi:10.1098/rsbl.2010.1203)
12. Ligrone R. 1988 Ultrastructure of a fungal endophyte in *Phaeoceros laevis* (L.) Prosc. (Anthocerophyta). *Bot. Gaz.* **149**, 92–100. (doi:10.1086/337695)
13. Schüßler A. 2000 *Glomus claroideum* forms an arbuscular mycorrhiza-like symbiosis with the hornwort *Anthoceros punctatus*. *Mycorrhiza* **10**, 15–21. (doi:10.1007/s005720050282)
14. Pocock K, Duckett JG. 1984 A comparative ultrastructural analysis of the fungal endophytes in *Cryptothallus mirabilis* Malm. and other British thalloid hepatics. *J. Bryol.* **13**, 227–233.
15. Villarreal JC, Renzaglia KS. 2006 Structure and development of *Nostoc* strands in *Leiosporoceros dussii* (Anthocerotophyta): a novel symbiosis in land plants. *Am. J. Bot.* **93**, 693–705. (doi:10.3732/ajb.93.5.693)
16. Gerdemann JW, Trappe JM. 1974 The Endogonaceae in the Pacific Northwest. *Mycol. Mem.* **5**, 1–76.
17. Ligrone R, Duckett JG. 1994 Cytoplasmic polarity and endoplasmic microtubules associated with the nucleus and organelles are ubiquitous features of food-conducting cells in bryoid mosses (Bryophyta). *New Phytol.* **127**, 601–614. (doi:10.1111/j.1469-8137.1994.tb03979.x)
18. Duckett JG, Pressel S, P'ng KMY, Renzaglia KS. 2009 Exploding a myth: the capsule dehiscence mechanism and the function of pseudostomata in *Sphagnum*. *New Phytol.* **183**, 1053–1063. (doi:10.1111/j.1469-8137.2009.02905.x)
19. Gardes M, Bruns TD. 1993 ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118. (doi:10.1111/j.1365-294X.1993.tb00005.x)
20. White TJ, Bruns T, Lee S, Taylor JW. 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols* (eds MA Innis, DH Gelfand, JJ Sninsky, TJ White), pp. 315–322. San Diego, CA: Academic Press.
21. Smit E, Leeflang P, Glandorf B, Van Elsas JD, Wernars K. 1999 Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* **65**, 2614–2621.
22. Lee J, Lee S, Young JPW. 2008 Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiol. Ecol.* **65**, 339–349. (doi:10.1111/j.1574-6941.2008.00531.x)
23. Hall TA. 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.
24. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739. (doi:10.1093/molbev/msr121)
25. Edgar RC. 2004 MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**, 113. (doi:10.1186/1471-2105-5-113)
26. Löytynoja A, Goldman N. 2010 webPRANK: a phylogeny-aware multiple sequence aligner with interactive alignment browser. *BMC Bioinformatics* **11**, 579. (doi:10.1186/1471-2105-11-579)
27. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402. (doi:10.1093/nar/25.17.3389)
28. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. 2008 GenBank. *Nucleic Acids Res.* **36**, D25–D30. (doi:10.1093/nar/gkm929)
29. Silvestro D, Michalak I. 2012 RaxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* **12**, 335–337. (doi:10.1007/s13127-011-0056-0)
30. Ronquist F, Huelsenbeck JP. 2003 MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574. (doi:10.1093/bioinformatics/btg180)
31. Darriba D, Taboada GL, Doallo R, Posada D. 2008 jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* **9**, 772. (doi:10.1038/nmeth.2109)
32. Piel WH, Donoghue MJ, Sanderson MJ. 2002 TreeBASE: a database of phylogenetic knowledge. In *To the interoperable 'catalog of life' with partners species 2000 Asia Oceania* (eds J Shimura, KL Wilson, D Gordon), pp. 41–47. Research Report no. 171. Tsukuba, Japan: National Institute for Environmental Studies.
33. Duff RJ, Cargill DC, Villarreal JC, Renzaglia KS. 2004 Phylogenetic relationships of the hornworts based on *rbc*L sequence data: novel relationships and new insights. In *Molecular systematics of bryophytes 98* (eds B Goffinet, V Hollowell, R Magill), pp. 41–58. St Louis, MO: Missouri Botanical Garden Press.
34. Duff RJ, Villarreal JC, Cargill DC, Renzaglia KS. 2007 Progress and challenges in hornwort phylogenetic reconstruction. *Bryologist* **110**, 214–243. (doi:10.1639/0007-2745(2007)110[214:PACTDA])
35. Stamatakis A, Hoover P, Rougemont J. 2008 A rapid bootstrap algorithm for the RAxML web-servers. *Syst. Biol.* **75**, 758–771. (doi:10.1080/10635150802429642)
36. Maddison WP, Maddison DR. 2011 *Mesquite: a modular system for evolutionary analysis*. Version 2.75. See http://mesquiteproject.org.
37. Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. 2012 Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytol.* **193**, 970–984. (doi:10.1111/j.1469-8137.2011.03962.x)
38. Bidartondo MI, Duckett JG. 2010 Conservative ecological and evolutionary patterns in liverwort–fungal symbioses. *Proc. R. Soc. B* **277**, 485–492. (doi:10.1098/rspb.2009.1458)
39. Trappe JM. 1987 Phylogenetic and ecologic aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In *Ecophysiology of VA mycorrhizal plants* (ed. GR Safir), pp. 5–25. Boca Raton, FL: CRC Press.
40. Duckett JG, Carafa A, Ligrone RA. 2006 Highly differentiated glomeromycotean association with the mucilage-secreting, primitive antipodean liverwort *Treubia*: clues to the origins of mycorrhizas. *Am. J. Bot.* **93**, 797–813. (doi:10.3732/ajb.93.6.797)
41. Pressel S, Bidartondo MI, Ligrone R, Duckett JG. 2010 Fungal symbioses in bryophytes: new insights into the twenty first century. *Phytotaxa* **9**, 238–253.
42. Krings M, Taylor TN, Hass H, Kerp H, Dotzler N, Hermsen EJ. 2007 Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *New Phytol.* **174**, 648–657. (doi:10.1111/j.1469-8137.2007.02008.x)
43. Krings M, Taylor TN, Hass H, Kerp H, Dotzler N, Hermsen EJ. 2007 An alternative mode of early land plant colonization by putative endomycorrhizal fungi. *Plant Signal. Behav.* **2**, 125–126. (doi:10.4161/psb.2.2.3970)
44. Bonfante-Fasolo P, Scannerini S. 1976 The ultrastructure of the zygospore in *Endogone flammicorona* Trappe & Gerdemann. *Mycopathologia* **59**, 117–123. (doi:10.1007/BF00493564)
45. Schüßler A, Martin H, Cohen D, Fitz M, Wipf D. 2007 Arbuscular mycorrhiza: studies on the *Geosiphon* symbiosis lead to the characterization of the first glomeromycotean sugar transporter. *Plant Signal. Behav.* **2**, 431–434. (doi:10.4161/psb.2.5.4465)
46. Duckett JG, Prasad AKSK, Davies DA, Walker S. 1977 A cytological analysis of the *Nostoc*–bryophyte relationship. *New Phytol.* **79**, 349–362. (doi:10.1111/j.1469-8137.1977.tb02215.x)
47. Ligrone R, Duckett JG, Renzaglia KS. 1993 The gametophyte–sporophyte junction in land plants. *Adv. Bot. Res.* **19**, 231–317. (doi:10.1016/S0065-2296(08)60206-2)