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REGULAR PAPER

# Phylogenetic affinity of arbuscular mycorrhizal symbionts in *Psilotum nudum*

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**Abstract** Many lineages of land plants (from lycopsids to angiosperms) have non-photosynthetic life cycle phases that involve obligate mycoheterotrophic arbuscular mycorrhizal (AM) associations where the plant host gains organic carbon through glomalean symbionts. Our goal was to isolate and phylogenetically identify the AM fungi associated with both the autotrophic and underground mycoheterotrophic life cycle phases of *Psilotum nudum*. Phylogenetic analyses recovered 11 fungal phylotypes in four diverse clades of *Glomus A* that form AM associations with *P. nudum* mycoheterotrophic gametophytes and autotrophic sporophytes, and angiosperm roots found in the same greenhouse pots. The correspondence of identities of AM symbionts in *P. nudum* sporophytes, gametophytes and neighboring angiosperms provides compelling evidence that photosynthetic heterospecific and conspecific plants can serve as the ultimate sources of fixed carbon for mycoheterotrophic gametophytes of *P. nudum*, and that the transfer of carbon occurs via shared fungal networks. Moreover, broader phylogenetic analyses suggest greenhouse *Psilotum* populations, like field-surveyed populations of mycoheterotrophic plants, form AM associations with restricted clades of *Glomus A*. The phylogenetic affinities and distribution of *Glomus A* symbionts indicate that *P. nudum* greenhouse populations have the potential to be exploited as an experimental system to further study the physiology, ecology and evolution of mycoheterotrophic AM associations.

**Keywords** Arbuscular mycorrhizal associations · *Psilotum nudum* · Mycoheterotrophy · *Glomus*

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## Introduction

Arbuscular mycorrhizal (AM) symbioses are the most common type of underground plant–fungal association found among land plants, and are characterized by the presence of branching hyphae (arbuscules) that penetrate the cell walls of the host plant (Smith and Read 2008). The majority of AM symbioses are mutualistic interactions between photosynthetic plants and fungi in Glomeromycota (glomalean fungi), where the plant host gains essential mineral nutrients from the fungal symbiont in exchange for fixed carbon (Smith and Read 2008; Schussler et al. 2001; Brundrett 2004). To date, most research has focused on the ecology, development, physiology and evolution of the "typical" mutualistic AM association between a glomalean fungus and a photosynthetic host plant (reviewed in Smith and Read 2008; Smith and Smith 1997; Harrison 1998, 1999; Brundrett 2002, 2004; Hause and Fester 2005; Helgason and Fitter 2005).

More than 1,000 species of land plants have obligate mycoheterotrophic AM associations where the plant host most likely gains all (or most) of its organic carbon from glomalean symbionts (Leake 1993, 2004; Wang and Qiu 2006). These plant taxa, which are either entirely non-photosynthetic or pass through a significant period of their life cycle in a non-photosynthetic condition, can be found

among liverworts, lycopsids, ferns (monilophytes), and angiosperms (reviewed in Bower 1935; Bierhorst 1971; Smith and Smith 1996; Read et al. 2000; Brundrett 2002, 2004). Establishing the molecular phylogenetic affinity of AM symbionts in diverse mycoheterotrophic plants is fundamental to understanding the broader evolutionary and ecological history of AM associations over the nearly 500 million year history of land plants.

For more than a century, the presence of AM fungal symbionts in mycoheterotrophic plants has been extensively studied by microscopy (reviewed in Bower 1935; Bierhorst 1971; Leake 1993, 2004; Read et al. 2000; recent reports include Duckett and Ligrone 1992; Schmid and Oberwinkler 1994, 1996; Imhof 1999a, 1999b, 2004; Carafa et al. 2003; Bidartondo (2005); Dominguez et al. 2005; Leake et al. 2008; Winther and Friedman 2007, 2008). Although most AM fungi in mycoheterotrophic plants have been reported to have intracellular, aseptate hyphae that form coil-like arbuscules (as opposed to the more common branching arbuscules), it is not possible to determine the phylogenetic affinity of the AM symboints based on morphology (Smith and Read 2008; Anderson and Cairney 2004). The morphology of glomalean fungi within the cells of a plant host may be affected by the phylogenetic identity of the host, as well as the physiological relationship between the fungus and the plant (for example, see Smith and Read 2008). DNA sequence-based approaches to identifying AM fungi have been available for decades (reviewed in Brundrett 2002, 2004; Anderson and Cairney 2004) but, until recently, molecular phylogenetic studies had overlooked the ecologically and phylogenetically widespread mycoheterotrophic AM plant–fungal associations.

In the past 6 years, a small number of studies have examined the molecular phylogenetic affinities of AM symbionts in lineages with mycoheterotrophic life cycle stages, including extant members of Lycopodiaceae (Winther and Friedman 2008), eusporangiate ferns (Kovacs et al. 2007; Winther and Friedman 2007), and angiosperms (Bidartondo et al. 2002; Franke et al. 2006). These studies demonstrated that most mycoheterotrophic plants form AM associations with glomalean fungi in the *Glomus A* clade (Bidartondo et al. 2002; Franke et al. 2006; Winther and Friedman 2007, 2008), one of the major groups within the Glomaceae as circumscribed by Schussler et al. (2001). In each of these studies, the *Glomus A* fungi recovered from mycoheterotrophic plants represent previously unknown glomalean diversity based on GenBank searches and phylogenetic analyses.

Here, we report on AM symbionts throughout the life cycle in *Psilotum nudum* (Psilotaceae). Historically, there has been much debate and confusion over the phylogenetic positioning of *Psilotum* (Bierhorst 1953, 1977; Kaplan 1977; Takiguchi et al. 1997). The most recent phylogenetic analyses place Psilotaceae (*Psilotum* and *Tmesipteris*) as the sister group to Ophioglossaceae (*Ophioglossum*, *Botrychium*, and *Helminthostachys*), and the Psilotaceae + Ophioglossaceae clade as sister to the rest of extant ferns (monilophytes) (Pryer et al. 2001, 2004). *P. nudum*, like previously studied species of *Botrychium*, *Huperzia*, and *Lycopodium* (Winther and Friedman 2007, 2008), is characterized by a life cycle with long-lived photosynthetic sporophytes and long-lived (several years) mycoheterotrophic gametophytes (Bierhorst 1954a, b, 1955, 1968, 1971) (Fig. 1).

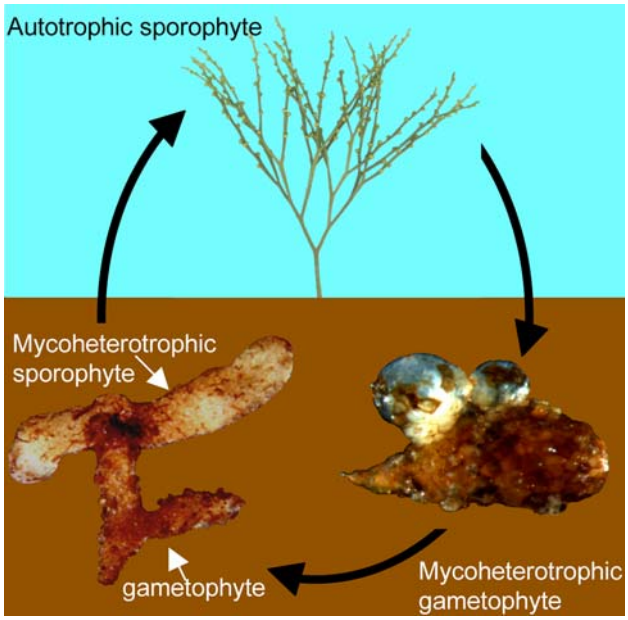


**Fig. 1** *Psilotum nudum* life cycle within a pot. Meiotically produced haploid spores are shed from the diploid sporophyte. Each haploid spore develops underground into a multicellular mycoheterotrophic gametophyte. After fertilization, a diploid mycoheterotrophic sporophyte develops underground and is attached initially to the gametophyte. Eventually, the sporophyte emerges above ground and becomes autotrophic

AM associations can be found throughout the life cycle of *P. nudum*: in the underground non-photosynthetic gametophyte, the young underground non-photosynthetic sporophyte attached to the gametophyte, and the mature photosynthetic sporophyte (Bierhorst 1971) (Figs. 1, 2). Subterranean *P. nudum* gametophytes and other subterranean Psilotaceae gametophytes (*Tmesipteris*) appear to require AM symbionts for growth and the production of gametes (Holloway 1918, 1939; Bierhorst 1954a, b, 1955, 1968, 1971; Whittier and Peterson 1980; Peterson et al. 1981; Duckett and Ligrone 2005). *P. nudum* spores and gametophytes grown axenically require sugars in order to grow and produce gametes without a fungal symbiont (Whittier 1973, 1985, 1988, 1990; Whittier and Braggins 1994; Whittier and Given 1987). The *P. nudum*

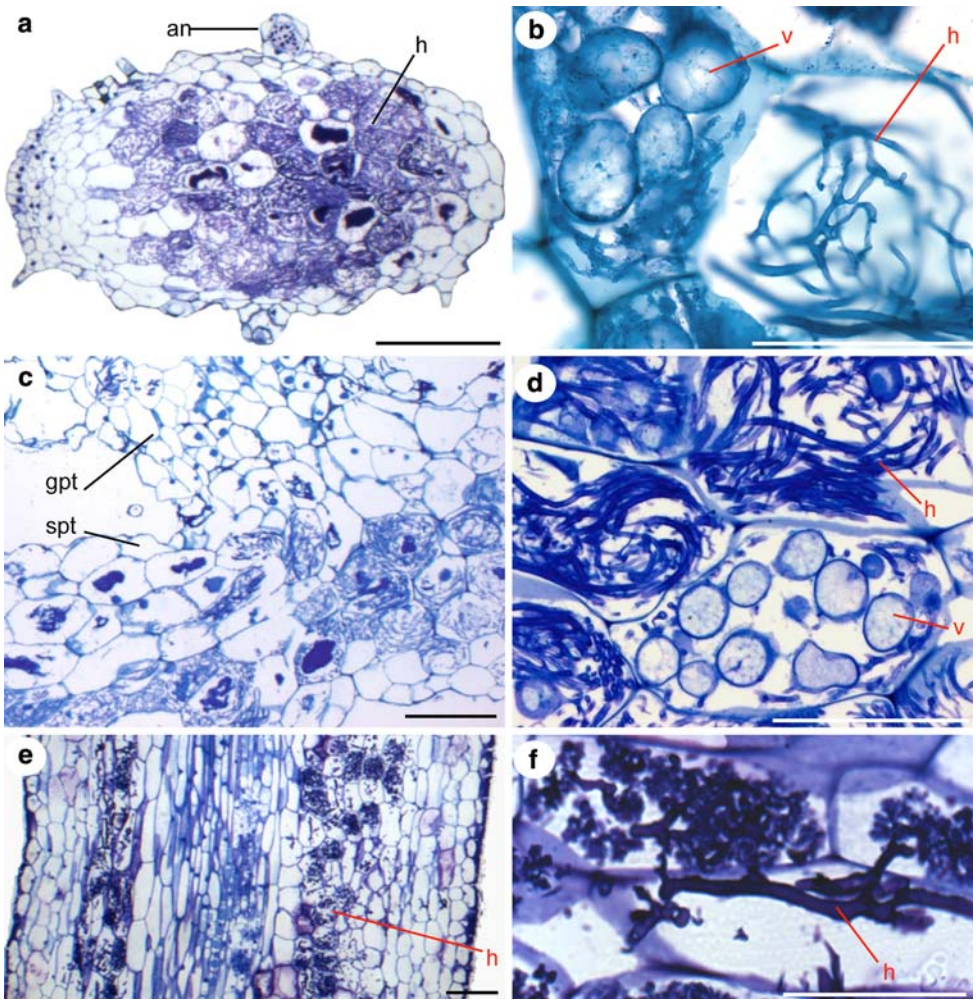


**Fig. 2** Arbuscular mycorrhizal (AM) associations in *P. nudum* gametophytes and sporophytes, and neighboring plant, *Austrobaileya scandens*. **a** Longitudinal section of *P. nudum* gametophyte with AM hyphae (*h*) and antheridia (*an*). **b** Magnified view of AM hyphae in *P. nudum* gametophyte with fungal vesicles (*v*) and hyphae. **c** Longitudinal section of gametophyte (*gpt*) sporophyte (*spt*) junction with no transfer of fungi. **d** Magnified view of AM hyphae in *P. nudum* photosynthetic sporophyte. **e** Longitudinal section of *A. scandens* root with AM fungal hyphae. **f** Magnified view of AM hyphae in *A. scandens*. *Bars* **a**, **c**, **e** 250 μm; **b**, **d**, **f** 50 μm

photosynthetic sporophyte, like most autotrophic land plants, has AM symbionts (Fig. 2) that are assumed to engage in a mutualistic relationship with the host plant.

In order to further characterize the potential network of glomalean partners of the mycoheterotrophic life cycle stages, we employed a molecular phylogenetic approach to identify and determine the distribution of AM symbionts in *P. nudum* mycoheterotrophic gametophytes, *P. nudum* photosynthetic sporophytes and co-occurring flowering plants from greenhouse pots (Figs. 1, 2). We conducted phylogenetic analyses in order to determine how AM symbionts isolated from *P. nudum* plants are phylogenetically related to other Glomeromycota taxa, especially those known to engage in AM associations with other mycoheterotrophic plant species. We compared AM associates of greenhouse mycoheterotrophic populations to those previously reported from field-collected mycoheterotrophic plant communities in order to determine if similar patterns are found in both field and greenhouse conditions.

## Materials and methods

### Collection

In the summers of 2003 and 2004, collections of *P. nudum* sporophytes and surrounding soil were sent overnight from greenhouses at the University of Massachusetts, Amherst. *P. nudum* was collected from pots containing either the angiosperm *Austrobaileya scandens* (Austrobaileyaceae) or *Swietenia macrophylla* (Meliaceae). *P. nudum* gametophytes and heterotrophic sporophytes were isolated by sieving soil under a dissecting scope with United States Standard Testing Sieves No. 80 and No. 20. A total of 16 gametophytes (eight from *Austrobaileya* samples and eight from *Swietenia* samples) from the Massachusetts greenhouse were collected and used for DNA sequencing. Putative heterotrophic sporophytes were isolated but were not used in phylogenetic analyses because of the difficulty involved in determining if the material was a heterotrophic sporophyte or a gametophyte with no gametangia. Only

gametophytes with visible gametangia were used for DNA analysis. Additional gametophytes were collected for sectioning. Rhizomes from photosynthetic *P. nudum* aboveground sporophytes were collected and processed for DNA sequencing and sectioning. Roots were also collected from *Austrobaileya scandens* and *Swietenia macrophylla* for DNA sequencing and histological sectioning.

### Light microscopy

Tissue was fixed in a 4% acrolein solution in 1% PIPES buffer. The tissue was dehydrated in an ethanol series to 95% and then infiltrated and embedded in the plastic monomer glycol methacrylate (JB-4 embedding kit, Polysciences, Warrington, PA). Serial sections (5 μm) of plastic-embedded specimens were made using a Leica rotary microtome (Leica Microsystems, Bannockburn, IL) and glass knives. Serial sections were mounted on slides and stained with 0.1% toluidine blue. Slides showing important features were photographed digitally using a Zeiss Axiophot microscope equipped with a Zeiss Axiocam digital camera (Zeiss MicroImaging, Thornwood, NY).

### Sequence amplification

DNA was extracted from surface-sterilized (as described in Winther and Friedman 2008) *Psilotum* gametophytes and rhizomes, *A. scandens* roots, and *S. macrophylla* roots using a standard phenol-chloroform extraction method (DeSalle et al. 2002). Most of the nuclear small subunit ribosomal DNA (18S) sequences were amplified using GEOA2-Geo11 and GEOA2-SS1492 (Simon et al. 1993; Schwarzott and Schussler 2001)—primers that have previously been used in the amplification of glomalean fungi from plant roots and isolated spores (although only in a nested reaction). Through trial and error we found we did not have to perform the nested procedure described in Schwarzott and Schussler (2001) to achieve amplification of fungal 18S DNA in plant material. All plant-fungal DNA samples were amplified with both primer pairs. Although most 18S sequences used in our analyses were obtained using the above primer pairs, initial PCR reactions were carried out using different primer sets that amplified *Glomus* sequences phylogenetically similar to those subsequently recovered in the above reactions. These 18S sequences were amplified using a nested PCR protocol with universal primers pairs NS1-NS8 (White et al. 1990) followed by VANS1-NS4 or VANS1-NS21 (Simon et al. 1992) for shorter sequences not used in analyses, or *Glomus*-specific primers VANS1-GLOMR1311 (Bidartondo et al. 2002) or VANS1-SS1492. It should be noted that the primers used are not necessarily specific to glomalean fungi and recent papers have developed primers that are more specific to Glomeromycota (Anderson and Cairney 2004; Hijri et al. 2006). PCR reactions used Eppendorf MasterTaq and followed instructions provided in the manual with the inclusion of 2.5 μl bovine serum albumin (BSA) 10 mg/ml and 1 μl of each 10 mM primer to each reaction. The PCR program was 95°C for 1 min 30 s, 35 cycles of 94°C for 45 s, 56.5°C for 55 s, 72°C for 2 min 30 s + 1 s per cycle, hold 72°C for 10 min.

### Sequencing

All amplified DNA samples were cloned using TOPO TA cloning kits (Invitrogen, Carlsbad, CA). A minimum of 12, and usually 20, colonies were chosen from each reaction for sequencing. Colonies were isolated and grown in TSB broth at 35°C for 18 h. Plasmid DNA was extracted using the Mini Wizard Prep Plasmid Kit (Promega, Madison, WI). Extracted plasmid DNA was used directly in a cycle sequence reaction using the DYEnamic ET Terminator Cycle Sequencing Kit from Amersham Biosciences (Amersham Biosciences, Piscataway, NJ). The cycle sequence reaction mixture was 2 μl sequencing reagent premix, 2 μl template, 0.33 μl primer, 7 μl water, and 0.75 μl 5X sequencing buffer. The cycle sequence reaction was 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, repeated 25 times. The cycle sequence reactions were cleaned using Sephadex Column System from ABI sciences. Samples were run at the University of Colorado Sequencing Facility in the Department of Ecology and Evolutionary Biology on an MJ Research Base Station 51. 18S sequences (EF558776-EF558800) have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/).

### Phylogenetic analyses

Each sequence was run through a BlastN search (http://www.ncbi.nlm.nih.gov/blast/) on GenBank to confirm it as glomalean. The 25 closest blast hits to each sequence were included in our analyses. Sequences were first aligned in Sequencher version 2 (Gene Codes Corporation, Ann Arbor, MI), and then exported to Clustal X (Thompson et al. 1997) for further alignment. The Clustal X alignment was then edited by hand using MacClade version 4.6 OS X (Sinauer Associates, Sunderland, MA). Due to high variability in Glomeromycota 18S sequences including 6–10 base pair insertions and deletions, only those bases that could be unambiguously aligned are included in our analyses [see electronic supplementary material (ESM; online supplement 1) for sequence alignment]. Neighbor joining, maximum parsimony and Bayesian analyses were conducted in PAUP v.2b. (Swofford 2002) and MrBayes (Huelsenbeck and Ronquist 2001). Bayesian analyses are based on sampling 5 million generations using the GTR

model of evolution. Neighbor joining and maximum parsimony analyses were run to 10,000 generations.

Previously published Glomeromycota 18S sequences from the families Glomaceae, Diversisporaceae, Acaulosporaceae, Gigasporaceae, Archeosporaceae, Geosiphonaceae and Paraglomaceae used in our phylogenetic analyses are from Schwarzott et al. (2001) (see ESM supplement 2 for list of species and accession numbers). Sequences isolated from the mycoheterotrophic angiosperms *Arachnitis uniflora*, *Voyria corymbosa*, *Voyriella parviflora* (Bidartondo et al. 2002) were included in our analysis. Sequences isolated from the mycoheterotrophic and photosynthetic life cycle stages of *Botrychium lanceolatum*, *Botrychium crenulatum* and neighboring plant *Caltha palustris* (Winther and Friedman 2007), seven species of Lycopodiaceae photosynthetic sporophytes (*H. hypogeae*, *L. clavatum* ssp. contiguum, *H. tetragona*, *H. crassa* var *crassa*, *H. affinis*, *H. hypogeae*, *L. calvatum* ssp. *clavatum*, and *H. urbanii*), and the gametophytes of one species of lycopsid (*H. hypogeae*) (Winther and Friedman 2008) were included in phylogenetic analyses. In addition, sequences recovered recently from photosynthetic sporophytes of *Botrychium virginianum* (Kovacs et al. 2007) were included in our analyses. It should be noted that although Kovacs et al. (2007) use *Botrychium virginianum*, current phylogenetic hypotheses restrict the genus "*Botrychium*" to moonworts (*Botrychium* subgenus *Botrychium*) and *Botrychium virginianum* is now *Botrypus virginianus* (Hauk et al. 2003). Most of the sequences previously recovered from mycoheterotrophic plants and from cultivated Glomeromycota species represent sequences from GenBank Blast searches that were among the 25 sequences most similar to each of the isolated *Psilotum* sequences.

## Results

### *Glomus* AM diversity in *P. nudum*

We isolated a total of 30 unique 18S sequences from *P. nudum* mycoheterotrophic gametophytes and photosynthetic sporophytes, and from roots of the neighboring angiosperms *A. scandens* and *S. macrophylla* (Fig. 3; Table 1). All recovered sequences are in the Glomaceae, specifically in *Glomus group A* as circumscribed by Schussler et al. (2001). The recovered sequences represent previously unknown glomalean sequence diversity based on phylogenetic analyses and GenBank searches.

The diversity of glomalean symbionts within a plant can be characterized phylogenetically via the number of recovered phylotypes, e.g., clades of closely related sequences with high statistical support. Previous studies have identified *Glomus* phylotypes with bootstrap values from 77 to 100% and sequence affinity within phylotypes that ranges from 96.5 to 100% (Vandenkoornhuyse et al. 2002; Gollotte et al. 2004; Opik et al. 2003; Rosendahl and Stukenbrock 2004; Winther and Friedman 2007, 2008). Our phylogenetic analyses partition the 30 18S sequences isolated from the greenhouse population of *P. nudum* into 11 phylotypes (A–K) that are supported by ≥90% maximum parsimony (MP) and neighbor joining (NJ) bootstrap values and Bayesian posterior probability (BPP) values (Table 1; Fig. 3). Sequence affinity within the phylotypes ranges from 96 to 99.8% (Fig. 3).

We are not equating the 11 18S glomalean phylotypes with glomalean species. Glomalean fungi are asexual, multinucleate organisms, and 18S and ITS sequences isolated from single spores have been shown to vary 0.5–3 and 6–18% (Clapp et al. 1995; Kjoller and Rosendahl 2001; Antoniolli et al. 2000; Jansa et al. 2002). As a result, circumscription of glomalean species based on molecular data can be difficult. The isolated 18S phylotypes represent discrete evolutionary units that are statistically robust phylogenetic groupings of genotypes that share an evolutionary history (Winther and Friedman 2007, 2008).

### Distribution of *Glomus* AM diversity in *P. nudum*

Of the 11 18S phylotypes recovered from the greenhouse *Psilotum* plants, 8 were isolated from mycoheterotrophic gametophytes. Each individual gametophyte yielded a single phylotype, and gametophytes from the same pot did not necessarily have the same fungal phylotype. However, every phylotype isolated from a gametophyte was always recovered from a photosynthetic plant residing in the same pot (Table 1).

All 11 18S phylotypes were recovered from photosynthetic life cycles stages of *P. nudum* and/or neighboring flowering plants (*A. scandens* and *S. macrophylla*). Each *P. nudum* sporophyte or neighboring angiosperm yielded between two and four phylotypes. At least one of the phylotypes found in a photosynthetic sporophyte was also recovered from a mycoheterotrophic gametophyte of Psilotum occupying the same pot. Two *Glomus* phylotypes were recovered only from *P. nudum* photosynthetic sporophytes and one phylotype was recovered only from neighboring flowering plants. It should be noted that the same two phylotypes were recovered from both angiosperms *A. scandens* and *S. macrophylla* (Table 1).

### Phylogenetic relationships of *Glomus* phylotypes isolated from *P. nudum*

All 18S phylogenetic analyses strongly support the identification of 11 18S phylotypes from *P. nudum* that group

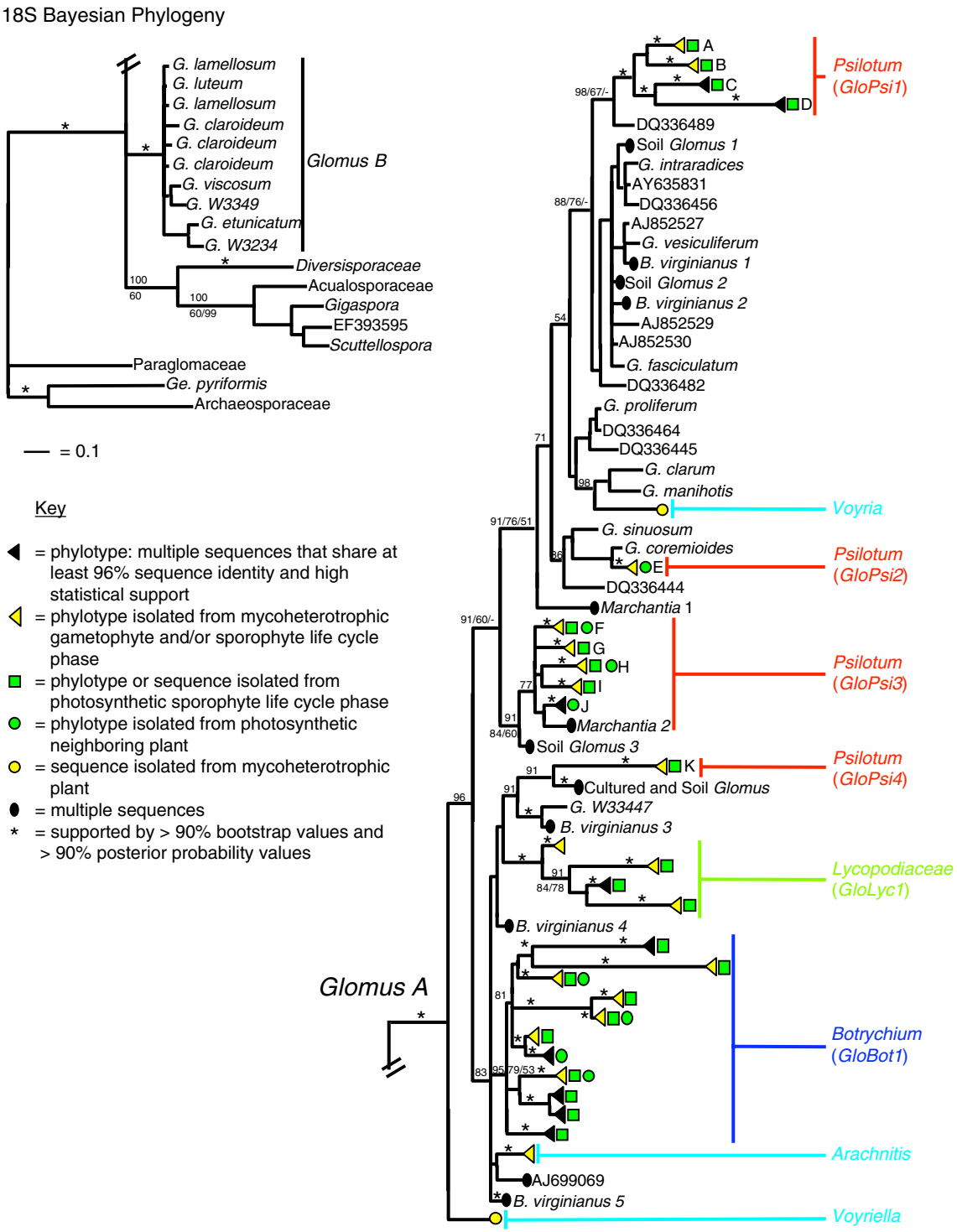


**Fig. 3** Bayesian 18S phylogeny based on 976 characters and isolated sequences of glomalean AM fungi from mycoheterophic ferns (*Psilotum* and *Botrychium*), Lycopodiaceae (*Huperzia and Lycopodium*) and angiosperms (*Voyria*, *Voyriella*, and *Arachnitis*). Bayesian posterior probabilities are indicated above the branch. Neighboring-joining and parsimony bootstrap values are also indicated above the branch when bootstrap support is greater than 50%. Phylotypes *A–K* correspond to those in Table 1. *Horizontal bar* Genetic distance. Accession numbers for sequences in GloBot1, GloLyc1, *Arachnitis*, *Voyria*, *Voyriella*, Soil *Glomus* 1, Soil *Glomus* 2, Soil *Glomus* 3, Cultured and Soil *Glomus*, *B. virginianus* 1-5, and *Marchantia* 1-2 can be found in ESM supplement 3

**Table 1** Plant species, life cycle phases and recovered phylotypes in *Psilotum Glomus A* clades

| Clade | Plant species *Glomus* phylotypes isolated from | Life cycle phase | Isolated phylotype(s) |
|---|---|---|---|
| GloPsi1 | *Psilotum nudum* | Photosynthetic sporophytes | A, B, C, D |
| | | Mycoheterotrophic gametophytes | A, B |
| GloPsi2 | *P. nudum* | Mycoheterotrophic gametophytes | E |
| | *Austrobaileya scandens* | Photosynthetic sporophytes | E |
| | *Swientiena macrophylla* | Photosynthetic sporophytes | E |
| GloPsi3 | *P. nudum* | Photosynthetic sporophytes | F, G, H, I, J |
| | | Mycoheterotrophic gametophytes | F, G, H, I, J |
| | *A. scandens* | Photosynthetic sporophytes | F, H, J |
| | *S. macrophylla* | Photosynthetic sporophytes | F, H, J |
| | *Marchantia* | Photosynthetic gametophyte | *Marchantia* 2 |
| GloPsi4 | *P. nudum* | Photosynthetic sporophyte | K |
| | | Mycoheterotrophic gametophytes | K |

into four well-supported clades in *Glomus A*: GloPsi1, GloPsi2, GloPsi3, and GloPsi4 (Fig. 3; Table 1). GloPsi1 (>90% BPP, NJ, MP) is well resolved and composed of two phylotypes isolated from *P. nudum* gametophytes and sporophytes as well as two phylotypes isolated only from *P. nudum* sporophytes. GloPsi2 (>90% BPP, NJ, MP) is sister to the morphologically and molecularly characterized *Glomus* species *G. coremioides* and consists of a single phylotype isolated from *P. nudum* gametophytes and neighboring angiosperms. GloPsi3 is a reasonably well supported (83% BPP, 70 NJ) but poorly resolved clade of five phylotypes isolated from *P. nudum* gametophytes and sporophytes, as well as neighboring angiosperms. GloPsi3 also includes *Glomus* sequences previously isolated from the liverwort *Marchantia foliaceae* (Russell and Bulman 2005). GloPsi4 (>90% BPP, NJ, MP) is a single phylotype isolated from *P. nudum* gametophytes and sporophytes and is sister to a clade of previously cultured morphologically and molecularly characterized *Glomus* species from photosynthetic plants and environmental soil samples (see ESM supplement 3 for table of accession numbers). The four clades isolated from *P. nudum* are not more closely related to each other than to other *Glomus A* sequences and clades.

## Broader phylogenetic relationships of *Glomus* phylotypes isolated from ferns, lycopods and angiosperms with mycoheterotrophic life cycle phases

Our 18S phylogenetic analyses recovered phylogenetic relationships within the order Glomeromycota that are fundamentally similar to those found in the past (Schussler et al. 2001; Bidartondo et al. 2002; Walker and Schussler 2004; Winther and Friedman 2007, 2008). Our analyses support the monophyly and interrelationships of *Glomus A* and *Glomus B*, the two major groups of Glomaceae as circumscribed by Schussler et al. (2001) (and revised by Walker and Schussler 2004; Redecker and Raab 2006). In addition, our analyses confirmed support for six other previously recognized families in Glomeromycota: Diversiporaceae, Acualosporaceae, Gigasporaceae, Geosiphonaceae, Archeosporaceae, and Paraglomaceae.

Our broad-scale phylogenetic analyses recovered a clade of fungi (GloBot1) that includes the same 11 phylotypes previously isolated from field-collected underground mycoheterotrophic gametophytes, mycoheterotrophic sporophytes, autotrophic sporophytes and neighboring plants of *B. crenulatum* and *B. lanecolatum* (Winther and Friedman 2007) (see ESM Table S1 for accession numbers and lifecycle information). GloBot1 had previously been found to nest in a clade (MH1) that included sequences isolated from the mycoheterotrophic angiosperm *Arachnitis uniflora* (GloAra1) (Winther and Friedman 2007, 2008). With the addition of more *Glomus A* sequences, the relationship of sequences in GloBot1 to GloAra1 has become ambiguous. However, it should be noted that in all previous analyses, GloBot1 has been supported as a clade.

GloLyc1 is a well-supported (>90% BPP, NJ, MP) and resolved clade that includes four phylotypes previously isolated from seven species of *Huperzia* and *Lycopodium* (Lycopodiaceae) collected from the Páramos of Ecuador (Fig. 3, ESM Table S1). GloLyc1, formerly identified as MH3 (Winther and Friedman 2008), includes three phylotypes isolated from underground mycoheterotrophic gametophytes and photosynthetic sporophytes of *H. hypogeae*. The relationship of GloLyc1 to other *Glomus A* sequences in unresolved.

Three diverse *Glomus A* clades form AM associations with non-photosynthetic angiosperms. A single sequence isolated from *Voyriella parviflora* (Bidartondo et al. 2002) is sister to all other *Glomus* A sequences. A single sequence isolated from *Voyria corymbosa* (Bidartondo

et al. 2002) is nested within a larger clade that includes sequences isolated from environmental soil samples and cultured *Glomus* species (Fig. 1). A single clade representing six sequences isolated from *Arachnitis uniflora* (Bidartondo et al. 2002) has previously been grouped with phylotypes isolated from *B. crenulatum* and *B. lanceolatum* (Winther and Friedman 2007, 2008). It should be noted that *Glomus* sequences isolated from *Arachnitis* have always been supported as a clade in phylogenetic analyses (Bidartondo et al. 2002; Winther and Friedman 2007, 2008). With the addition of more sequence diversity in our analyses, the phylogenetic relationships of *Arachnitis Glomus A* symbionts to other *Glomus A* clades and sequences is unresolved.

Although the phylogenetic relationships of *Glomus A* clades that form AM associations with plants with mycoheterotrophic life cycle phases are poorly resolved, it is apparent that these clades are distributed across the phylogenetic breadth of *Glomus A*. GloPsi1, GloPsi2, and *Voyria* are nested within a moderately supported clade (98% BPP, 76% NJ, 51% MP) that includes cultured *Glomus* species *G. intraradices*, *G. manihotis* and *G. sinuosum.* GloPsi3 is in a moderately supported (91% BPP, 84% NJ, 60% MP) clade that includes uncultured *Glomus A* (Soil *Glomus* 3) and soil cultured *Glomus* species including *G. mosseae*, *G. caledonium*, and *G. fragilistratum*. The remaining mycoheterotrophic phylotype clades including GloPsi1, GloBot1, GloLyc1, *Voyriella*, *Arachnitis*, and *Voyria* remain unresolved within the general topology of *Glomus A* (given the limited branch support across the different tree building methods).

## Discussion

### Phylogenetic affinities of AM symbionts in *P. nudum*

Although the presence of fungal symbionts had been well documented by microscopy in gametophytes and sporophytes of *P. nudum* (reviewed in Bierhorst 1971; Read et al. 2000), nothing was known of the phylogenetic affinities of the fungal partners. Our broad phylogenetic analyses provide compelling evidence for the discovery of four distinct *Glomus A* clades (GloPsi1, GloPsi2, GloPsi3, GloPsi4) capable of forming mycoheterotrophic AM associations with *P. nudum*. Establishing the molecular phylogenetic affinities of AM symbionts throughout the life cycle of *P. nudum* builds upon our previous research on the *Glomus* symbionts in mycoheterotrophic ferns and lycopsids (Winther and Friedman 2007, 2008) and provides an emerging picture of the fungal diversity, specificity, ecology and evolution involved in mycoheterotrophic AM plant-fungal symbioses.

The four *Psilotum Glomus A* clades, as well as the five other *Glomus A* clades previously recovered from mycoheterotrophic plants (GloBot1 (Winther and Friedman 2007), GloLyc1 (Winther and Friedman 2008), *Arachnitis*, *Voyria*, and *Voyriella* (Bidartondo et al. 2002) are dispersed throughout the *Glomus A* phylogeny (Fig. 3). Our sequence data from *P. nudum* AM symbionts further supports the hypothesis (Winther and Friedman 2007, 2008) that many, if not all, members of *Glomus A* may be able to form mycoheterotrophic AM associations under the appropriate conditions, and that *Glomus A* may represent a fungal clade with the ability to form complex labile AM networks where the fungus may receive or transfer carbon to the plant.

To date, each *Glomus A* clade found in association with a mycoheterotrophic plant has been recovered only from a single plant family: one Ophioglossaceae clade (GloBot1 from *B. lanceolatum,* and *B. crenulatum*), four Psilotaceae clades (GloPsi1-GloPsi4 from *P. nudum*), two Gentianaceae clades (from *Voyria* and *Voyriella*), one Corsiaceae clade (*Arachnitis*) and one Lycopodiaceae clade (GloLyc1 from *Huperzia* and *Lycopodium*). It is distinctly possible that particular lineages of plants engage in AM associations only with a certain subset of *Glomus A*. If true, this could be the result of the coevolution of mycoheterotrophic plant lineages with a certain subset of *Glomus A* and/or specificity based upon ecological and physiological differences between individual *Glomus A* phylotypes. Alternatively, future sampling of many mycoheterotrophic species from the same location might reveal that the same *Glomus A* clades can coexist and interact with different families of mycoheterotrophic plants.

Each *Psilotum* gametophyte harbored a single phylotype while the photosynthetic sporophyte had as at least two and as many as four phylotypes. Although *Psilotum* sporophytes harbored a greater number of phylotypes than the gametophytes, all recovered phylotypes were restricted to the same four *Glomus A* clades (GloPsi1, GloPsi2, GloPsi3, GloPsi4) that were found in their mycoheterotrophic life cycle phases. A similar pattern has been reported in *Botrychium crenulatum*, *Botrychium lanceolateum* (Winther and Friedman 2007), and *Huperzia hypogeae* (Winther and Friedman 2008), where the multiple phylotypes recovered from photosynthetic sporophytes were restricted to the same *Glomus A* clades (GloBot1 and GloLyc1) found in their mycoheterotrophic life cycle phases (Fig. 3). As has been suggested previously by the authors (Winther and Friedman 2008), it may be that photosynthetic sporophytes in plant lineages with an alternation of generations characterized by free-living photosynthetic sporophytes and mycoheterotrophic gametophytes are constrained to form AM associations with small clades of *Glomus A*. This is potentially due to the fact that the sporophyte is the

ultimate source of fixed carbon for the gametophytes via shared fungal hyphae.

### Distribution of *Glomus* symbionts in *P. nudum*

The phylogenetic identities of *Glomus* symbionts associated with various phases of the life cycle of *Psilotum* suggest complex and interdependent biological relationships between the mycoheterotrophic gametophytes and neighboring conspecific and heterospecific photosynthetic plants through shared fungal networks. Each of the four *Psilotum Glomus* clades were recovered from *P. nudum* mycoheterotrophic gametophytes as wells as photosynthetic *P. nudum* sporophytes and/or photosynthetic angiosperms located in the same pot (Fig. 3; Table 1). Previous studies have established that *P. nudum* gametophytes require either AM symbionts (in nature) or a source of fixed carbon (in axenic cultures) for proper development (Holloway 1918, 1939; Bierhorst 1954a, b, 1955, 1968, 1971; Whittier 1973; Whittier and Given 1987; Whittier and Peterson 1980; Peterson et al. 1981; Whittier and Braggins 1994). Our finding that the fungal phylotype found in each mycoheterotrophic gametophyte always matches one of the phylotypes in a neighboring photosynthetic sporophyte within the same greenhouse pot indicates that underground *P. nudum* gametophytes obtain carbon via epiparasitism through shared fungal networks from photosynthetic conspecific and/or heterospecific neighboring plants (Table 1).

Currently, there is a debate in the literature as to whether glomalean AM associations facilitate carbon flow and uptake between plants via shared glomalean networks (Fitter et al. 1998; Wu et al. 2001, 2002; Carey et al. 2004; Pfeffer et al. 2004) as has been demonstrated in ectomycorrhizal associations (Simard et al. 1997; Read 1998; Bidartondo et al. 2004). However, our *Psilotum* results are consistent with previous studies in other plant taxa with AM mycoheterotrophic associations such as *Huperzia* (Winther and Friedman 2008), *Botrychium* (Winther and Friedman 2007), *Arachnitis*, *Voyria* and *Voyriella* (Bidartondo et al. 2002), where neighboring photosynthetic conspecific and/or heterospecific plants are likely to contribute organic carbon through shared glomalean networks. By exploiting a controlled greenhouse set of conditions, we are able to conclusively demonstrate that there is an absolute correspondence between the specific glomalean fungal partners of the mycoheterotrophic phase of *Psilotum* and a subset of the fungi found in neighboring photosynthetic plants.

### Greenhouse *P. nudum* populations as an experimental system

The *P. nudum* populations used in this study were from greenhouses. It is of note that the phylogenetic diversity of *Glomus A* recovered here is greater than has been the case in field-collected plants of species with mycoheterotrophic life cycle phases (Bidartondo et al. 2002; Franke et al. 2006; Winther and Friedman 2007, 2008). There is no way of knowing whether the four *Psilotum Glomus* clades (GloPsi1, GloPsi2, GloPsi3, GloPsi4) from our greenhouse samples might be isolated from field-collected *P. nudum* populations.

University greenhouses have plants collected from all over the world and consequently the potential to harbor a greater diversity of *Glomus* than might be found in any one site in nature. As a consequence, it is not surprising that diverse *Glomus A* clades (GloPsi1, GloPsi2, GloPsi3, GloPsi4) were recovered from greenhouse *P. nudum* populations. In fact, the diversity of *Glomus* phylotypes and clades isolated from *P. nudum* suggests that the *Glomus* phylotypes recruited to form AM mycoheterotrophic associations may be more dependent upon the location, environment, and specific availability of *Glomus A* and less so on the phylogenetic affinity of the plant species. At this time it seems reasonable to conclude that plant affinity, plant community, environment and geography probably all contribute to determining the particular *Glomus A* symbiont(s) that are selected (from the immediate environment) to form AM associations with mycoheterotrophic plants.

It is important to draw attention to the fact that, even under greenhouse conditions, only *Glomus A* was recovered from *P. nudum*. Representatives of other Glomeromycota clades were never amplified from our mycoheterotrophic samples. All four *Glomus A* clades recovered from *Psilotum* greenhouse pots are distributed within and between the photosynthetic and mycoheterotrophic life cycle phases and neighboring plants as has been observed in natural populations of plants with a similar life cycle including *Botrychium* (Winther and Friedman 2007) and *Huperzia* (Winther and Friedman 2008). Thus, based on the morphology, identity and distribution of the *Glomus A* symbionts in greenhouse *P. nudum* plants, AM associations in *P. nudum* are similar to associations found in nature. As such, greenhouse populations of *P. nudum* possess many attributes (including being the only plant with mycoheterotrophic life cycle stages that consistently produces all phases of its life cycle under greenhouse conditions) that recommend it as a model species to further study the ecology, evolution and physiology of mycoheterotrophic AM associations under controlled conditions.

## Conclusions

In summary, we have documented that four clades of *Glomus A* are capable of forming mycoheterotrophic associations with *P. nudum*. Furthermore, the distribution

of the four clades throughout the autotrophic and mycoheterotrophic life cycles stages provides further evidence that mycoheterotrophic life cycles stages gain fixed carbon from neighboring heterospecific and/or conspecific plants thorough shared fungal networks.

As data accumulates on the phylogenetic identities of mycoheterotrophic AM symbionts in diverse plant lineages, it is apparent that much more needs to be learned about the ecology, physiology and evolution of the symbiotic relationship between plants and fungi in *Glomus A*. *Glomus A* is the largest and most diverse fungal group known to form AM associations with the majority of land plants in all environments (Harrier 2001; Brundrett 2004; Opik et al. 2006; Ligrone et al. 2007). Given that the fungal symbionts recovered from mycoheterotrophic ferns, lycopsids and angiosperms span the known phylogenetic breadth of *Glomus A*, it is possible *Glomus A* represents a clade of fungi where carbon transfer is widespread and flexible. What we have documented in *Psilotum*, *Botrychium*, and *Huperzia* may represent an extreme form of carbon transfer through mycoheterotrophic and autotrophic AM associations throughout the life cycles of lycopsid, fern, and angiosperm species. Further study of carbon flow dynamics in AM mycoheterotrophic communities could establish a new framework for studying the ecology and evolution of "mutualistic" AM associations where carbon flow between separate plants in a community is potentially widespread through shared *Glomus A* networks.

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