# Mycoheterotrophic interactions are not limited to a narrow phylogenetic range of arbuscular mycorrhizal fungi

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# Mycoheterotrophic interactions are not limited to a narrow phylogenetic range of arbuscular mycorrhizal fungi

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## Abstract

**The majority of achlorophyllous mycoheterotrophic plant species associate with arbuscular mycorrhizal fungi (AMF). Previous studies have shown that some species are highly specialized towards narrow lineages of AMF and have suggested that only particular lineages of these fungi are targeted by mycoheterotrophic plants. To test this hypothesis, we analyzed all available partial SSU sequences of AMF associated with mycoheterotrophic plants including data from 13 additional specimens from French Guiana, Gabon and Australia. Sequences were assigned to 'virtual taxa' (VT) according to the Maarj*AM* database. We found that 20% of all known Glomeromycota VT are involved in mycoheterotrophic interactions and the majority of associations involve Glomeraceae (*Glomus* Group A) fungi. While some mycoheterotrophic plant species have been found growing with only a single VT, many species are able to associate with a wide range of AMF. We calculated significant phylogenetic clustering of Glomeromycota VT involved in mycoheterotrophic interactions, suggesting that associations between mycoheterotrophic plants and AMF are influenced by the phylogenetic relationships of the fungi. Our results demonstrate that many lineages of AMF are prone to exploitation by mycoheterotrophic plants. However, mycoheterotrophs from different plant lineages and different geographical regions tend to be dependent on lineages of AMF that are phylogenetically related.**

*Keywords*: arbuscular mycorrhizal symbiosis, cheating, Glomeromycota, *Glomus*, mycoheterotrophy

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## Introduction

Arbuscular mycorrhizal fungi (AMF) are part of the most common mycorrhizal symbiosis between plants and fungi. Representatives of the fungal phylum Glomeromycota form symbiotic arbuscular mycorrhizas with the roots of 70–90% of land plant species (Parniske 2008; Smith & Read 2008). These AMF facilitate the uptake of soil nutrients by plants in exchange for photosynthetically fixed carbon. Because a single fungus can simultaneously associate with multiple unrelated host plants, this allows for the formation of mycorrhizal networks, linking plants of the same or different species (Giovannetti *et al.* 2004). Reciprocal nutrient supply is an important factor for the stability of the arbuscular mycorrhizal mutualism but cannot solely explain its evolutionary persistence (Kiers *et al.* 2011; Selosse & Rousset 2011).

Among plants that associate with AMF, over 220 species of achlorophyllous flowering plants are entirely dependent on arbuscular mycorrhizal networks for their carbon supply (Merckx *et al.* 2009) and are referred to as 'mycoheterotrophs' (Leake 1994). While mycoheterotrophy

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on AMF only occurs in eight angiosperm families, it is not restricted to flowering plants. The gametophytes of some club mosses and ferns are nonphotosynthetic and associate with AMF, pointing towards mycoheterotrophy at this stage of their life cycle (Winther & Friedman 2009; Merckx & Freudenstein 2010). Over the past decade, molecular techniques have been used to identify the AMF in roots of mycoheterotrophic plants. These studies indicate that arbuscular mycorrhizal mycoheterotrophs appear to be specialized on 'narrow' clades of fungi, although the degree of specialization is somewhat variable. Extreme cases of fungal specialization have been reported in the angiosperm genera *Arachnitis* (Corsiaceae), *Voyriella* (Gentianaceae) (Bidartondo *et al.* 2002), *Afrothismia* (Thismiaceae) (Merckx & Bidartondo 2008) and *Petrosavia* (Petrosaviaceae) (Yamato *et al.* 2011b), and to a lesser extent in the mycoheterotrophic interactions of *Sciaphila* (Triuridaceae) (Yamato *et al.* 2011a), *Botrychium* (Ophioglossaceae) (Winther & Friedman 2007) and *Huperzia* (Lycopodiaceae) (Winther & Friedman 2008). However, other mycoheterotrophs have been found to associate with multiple distantly related Glomalean fungi (Bidartondo *et al.* 2002; Franke *et al.* 2006; Winther & Friedman 2009; Merckx *et al.* 2010; Courty *et al.* 2011). Phylogenetic reconstruction of the AMF hosts of mycoheterotrophs has led to the identification of restricted clades of fungi that are involved in mycoheterotrophic interactions (Winther & Friedman 2007, 2008). The finding that particular clades of AMF have been infiltrated by mycoheterotrophs has prompted the hypothesis that '[…] it may be that only a few fungal species or sequence variants have proved amenable to involvement in the necessary tripartite symbiosis between a mycoheterotroph and an autotrophic host […]' (Smith & Read 2008, p. 37). To test this hypothesis, we combined data from mycoheterotrophic plants and related species from French Guiana, Gabon and Australia with all AMF sequences previously obtained from mycoheterotrophic plants. Our goal was to examine the phylogenetic host range of AMF that associate with mycoheterotrophs.

## Materials and methods

### *Sampling*

Root samples of the mycoheterotrophic species *Apteria aphylla, Dictyostega orobanchoides, Gymnosiphon capitatus, Gymnosiphon divaricatus* and *Hexapterella gentianoides* (Burmanniaceae) were collected at various sites in French Guiana in August 2008. Roots of *Campylosiphon congestus* (previously in *Burmannia* (Maas & Maas-van de Kamer 2010), *Gymnosiphon longistylus* (Burmanniaceae) and *Sciaphila ledermannii* (Triuridaceae) were sampled in Gabon in February 2008, and two *Thismia rodwayi* (Thismiaceae) specimens were collected at a site in Tasmania, Australia, in December 2008. For collection details see Table S1 (Supporting information). Root samples were washed with tap water immediately after collection and stored on 2% CTAB Buffer until further processing. DNA was extracted from root samples using methods described elsewhere (Gardes & Bruns 1993). For most species, DNA was extracted from entire root systems. For species with larger root systems, a DNA sample contained a few roots taken from the same specimen. Partial SSU rDNA fragments from the associated AMF were amplified following previously described methods (Schechter & Bruns 2008) with primers NS31 (Simon *et al.* 1992) and AM1 (Helgason *et al.* 1998). All PCR products were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). For each PCR product, eight clones were unidirectionally sequenced using the plasmid primer T7. Sequencing reactions were performed by the Macrogen sequencing facilities (Seoul, South Korea).

### *Data*

In addition to the sequences obtained from root samples, we downloaded all available partial SSU rDNA data of AMF from mycoheterotrophic species of Burmanniaceae, Corsiaceae, Gentianaceae, Thismiaceae and Triuridaceae (Bidartondo *et al.* 2002; Franke *et al.* 2006; Merckx & Bidartondo 2008; Merckx *et al.* 2010; Yamato *et al.* 2011a,b), and AMF sequences from the mycoheterotrophic gametophytes of Lycopodiaceae, Psilotaceae and Ophioglossaceae (Winther & Friedman 2007, 2008, 2009) from GenBank. In total this, data set contained AMF sequences from 30 fully mycoheterotrophic flowering plant species and mycoheterotrophic gametophytes of four fern and lycophyte species. For details see Table S1 (Supporting information). All sequences were assigned to virtual taxa ('VT') based on sequence similarity and phylogenetic relationships. In Glomeromycota, VT are sequence groups based on bootstrap support and ≥97% sequence similarity of NS31/AM1 sequences (Öpik *et al.* 2009). There are currently 282 VT described for Glomeromycota (Öpik *et al.* 2010). Sequence similarity information was obtained by BLAST search against the Maarj*AM* database (Öpik *et al.* 2010). Phylogenetic relationships were inferred by adding one SSU rDNA sequence of each Glomeromycota virtual taxon from the Maarj*AM* database to our data set (thus adding 282 sequences). The resulting data set was aligned with the MAFFT (ver. 6.814b) alignment tool (Katoh *et al.* 2002), implemented in Geneious Pro (ver. 5.4.6) (Drummond *et al.* 2011). Duplicate sequences were removed from the alignment using

RAxML (ver. 7.0.4) (Stamatakis 2006) reducing the data set to 408 unique sequences of 662 bp each. A phylogenetic tree was calculated under maximum likelihood (ML) with RAxML using the GTR + Γ + I model of molecular evolution as selected with jModeltest (ver. 0.1.1) (Posada 2008). Based on this information, the SSU rDNA sequences of AMF from mycoheterotrophic plants were each assigned to its closest related VT if sequence similarity with this VT was ≥97% (following Öpik *et al.* 2010). Using these criteria, a few sequences could not be assigned to existing VT and therefore four new VT were created, labelled 'VTX A' to 'VTX D'.

### *Phylogenetic community structure*

To examine patterns of phylogenetic community structure, the relationships between all Glomeromycota VT, including the four new VT found in this study, were calculated on a data set containing one SSU sequence for each VT and aligned using MAFFT (286 taxa, 610 bp). See Table S2 (Supporting information) for a complete list of sequences used. Phylogenetic inference was conducted with RAxML using the GTR + Γ + I model of molecular evolution as selected using jModeltest. The resulting highest-likelihood tree was rooted by selecting the Archaeosporales and Paraglomerales as the outgroup. This tree was used to investigate potential phylogenetic clustering of the VT involved in mycoheterotrophic interactions by calculating the nearest taxon index (NTI) and the net relatedness index (NRI) with the R package PICANTE (Kembel *et al.* 2010). The NTI and NRI indices are standardized effect sizes indicating phylogenetic clustering or overdispersion (Gotelli & Rhode 2002). NTI is a measure of the phylogenetic distance to the nearest taxon for each taxon in the sample and quantifies the extent of terminal clustering, independent of deep-level clustering. NRI is a standardized measure of the mean pairwise phylogenetic distance of taxa in a sample, relative to a phylogeny of an appropriate species pool, and quantifies overall clustering of taxa on a tree (Webb *et al.* 2002). Positive NTI and NRI values indicate phylogenetically clustered communities. Negative values indicate overdispersed communities (phylogenetic evenness). NTI and NRI values were calculated on the Glomeromycota phylogeny for all VT detected in mycoheterotrophic plant roots, and for sets of VT detected in each plant family separately. In addition, NTI and NRI values for the same sets of VT were also calculated on a Glomeromycota phylogeny that was pruned to include only Glomeraceae and Diversisporales. Calculations were performed with 999 randomizations using two different null models. Choosing an appropriate null model and species pool to measure phylogenetic community structure requires careful consideration. Every null model makes different assumptions, and using two null models or different species pools to analyze the same data can give radically different results (Webb *et al.* 2011). We opted to perform the calculations with these null models: (i) the sample.labels model in which the distance matrix labels are shuffled across all taxa and (ii) the phylogeny.pool model in which the community data matrix is randomized by drawing species from the pool of species occurring in the distance matrix with equal probability.

## Results

Root samples of fully mycoheterotrophic species collected in Gabon and French Guiana each contained several different fungal partial 18S rDNA sequences. In contrast, two specimens of *Thismia rodwayi* from Tasmania were found to associate with AMF for which identical partial 18S rDNA sequences were obtained. In total, 58 different VT were found to associate with arbuscular mycorrhizal mycoheterotrophic plants from various habitats around the world (Fig. 1). Eight of these VT belong to Diversisporales; the rest are Glomeraceae (*Glomus* Group A). Diversisporales VT were only detected in tropical species of Burmanniaceae, Gentianaceae and Triuridaceae. Öpik *et al.* (2010) described 282 Glomeromycota VT, and we found four new VT. These new VT were found in *Hexapterella gentianoides, Dictyostega orobanchoides, Gymnosiphon capitatus, Gymnosiphon divaricatus* (Burmanniaceae) and *Sciaphila ledermannii* (Triuridaceae). All new VT were part of Glomeraceae except for 'VT D', which is placed in Diversisporales.

Twenty-two VT were detected in roots of mycoheterotrophic Burmanniaceae (20 specimens sampled from nine species), 19 VT in Triuridaceae (35 specimens from four species), seven VT in Gentianaceae (12 specimens from six species), seven VT in Thismiaceae (25 specimens from eight species), one VT in Corsiaceae (3 specimens from one species), one in Petrosaviaceae (17 specimens from one species), four VT in Psilotaceae (16 specimens from one species), six VT in Ophioglossaceae (16 specimens from two species) and one VT in Lycopodiaceae (two specimens from one species).

Overlap in VT occurs in mycoheterotrophic Burmanniaceae, Gentianaceae, Thismiaceae and Triuridaceae (Fig. 1). No overlap in VT between the other families was found, nor between mycoheterotrophic angiosperms, ferns and lycophytes (Fig. 2).

Calculation of NTI and NRI values on the Glomeromycota phylogeny (Fig. 2) using two different null models resulted in very similar values (Table 1). Across the Glomeromycota phylogeny, significant positive NTI values were obtained for these groups of VT: all VT detected

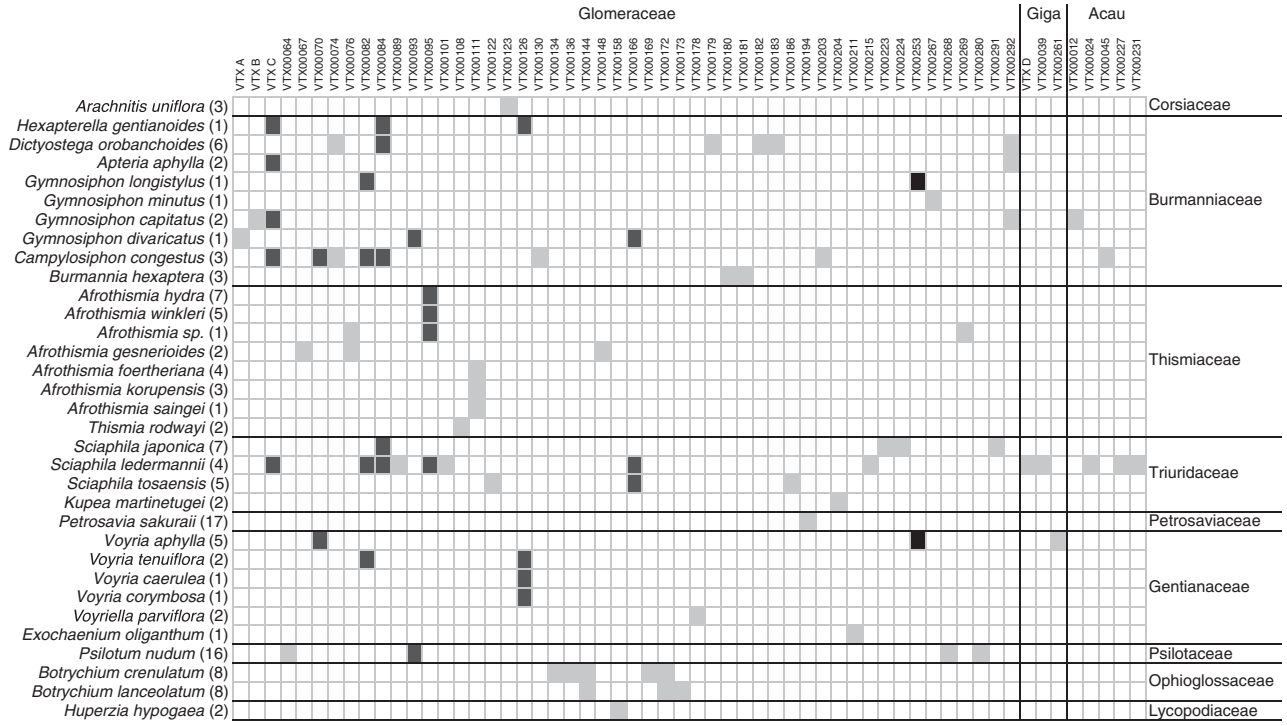


**Fig. 1** Mycoheterotrophic plant species and their associated arbuscular mycorrhizal fungi virtual taxa (VT). Grey boxes indicate associations with VT not found in other mycoheterotrophic plant families; black boxes show associations with VT that are also found in other families. Numbers of investigated specimens for each species are shown between brackets.

in mycoheterotrophic plants, Burmanniaceae, Thismiaceae and Ophioglossaceae. Significant positive NRI values were obtained for VT of these groups: all VT detected in mycoheterotrophic plants, Burmanniaceae, Thismiaceae and Triuridaceae. When the phylogeny was pruned to exclude large clades that have not been detected in mycoheterotrophic plants (i.e. Archaeosporales, Paraglomerales and *Glomus* Group B), significant positive NTI values were obtained for these taxon sets: all VT found in mycoheterotrophic plants, Burmanniaceae, Thismiaceae and Ophioglossaceae. Significant positive NRI values were obtained only for VT found in Thismiaceae.

## Discussion

### *Phylogenetic range of AMF involved in mycoheterotrophy*

Our results show that a wide variety of AMF lineages are involved in mycoheterotrophic interactions, thus rejecting the hypothesis that only a few fungal species or sequence variants are involved in the necessary tripartite symbiosis between a mycoheterotroph and an autotrophic host. Twenty per cent of all known Glomeromycota VT are used for mycoheterotrophic interactions. Most mycoheterotrophs rely on fungi in Glomeraceae. Twenty-six per cent of all described Glomeraceae VT were detected in the roots of a wide range of mycoheterotrophic plant species. Some mycoheterotrophs were found growing with Diversisporales fungi belonging to the families Acaulosporaceae and Gigasporaceae as well. However, for specimens of *Gymnosiphon capitatus*, *Sciaphila ledermannii* and *Campylosiphon congestus*, Acaulosporaceae and Gigasporaceae fungi were always found to co-colonize with Glomeraceae fungi. Thus, it is unclear whether these fungi represent 'facultative' symbionts as suggested by Franke *et al*. (2006). In contrast, in two specimens of *Voyria aphylla*, only Gigasporaceae fungi were found, strongly indicating that these fungi are involved in carbon transfer to the plant. Morphological observations have also suggested that the closely related *Voyria flavescens* harbours Gigasporaceae fungi (Franke 2002). So far no mycoheterotrophs have been found to associate with *Glomus* Group B, Archaeosporales, or Paraglomerales fungi, although this may be influenced by the common use of primer AM1, which has mismatches to these groups (Redecker *et al*. 2000; Husband *et al*. 2002).

Despite the wide range of fungal VT involved in mycoheterotrophic interactions, we found that VT detected in the roots of mycoheterotrophic plants are not randomly distributed across the Glomeromycota

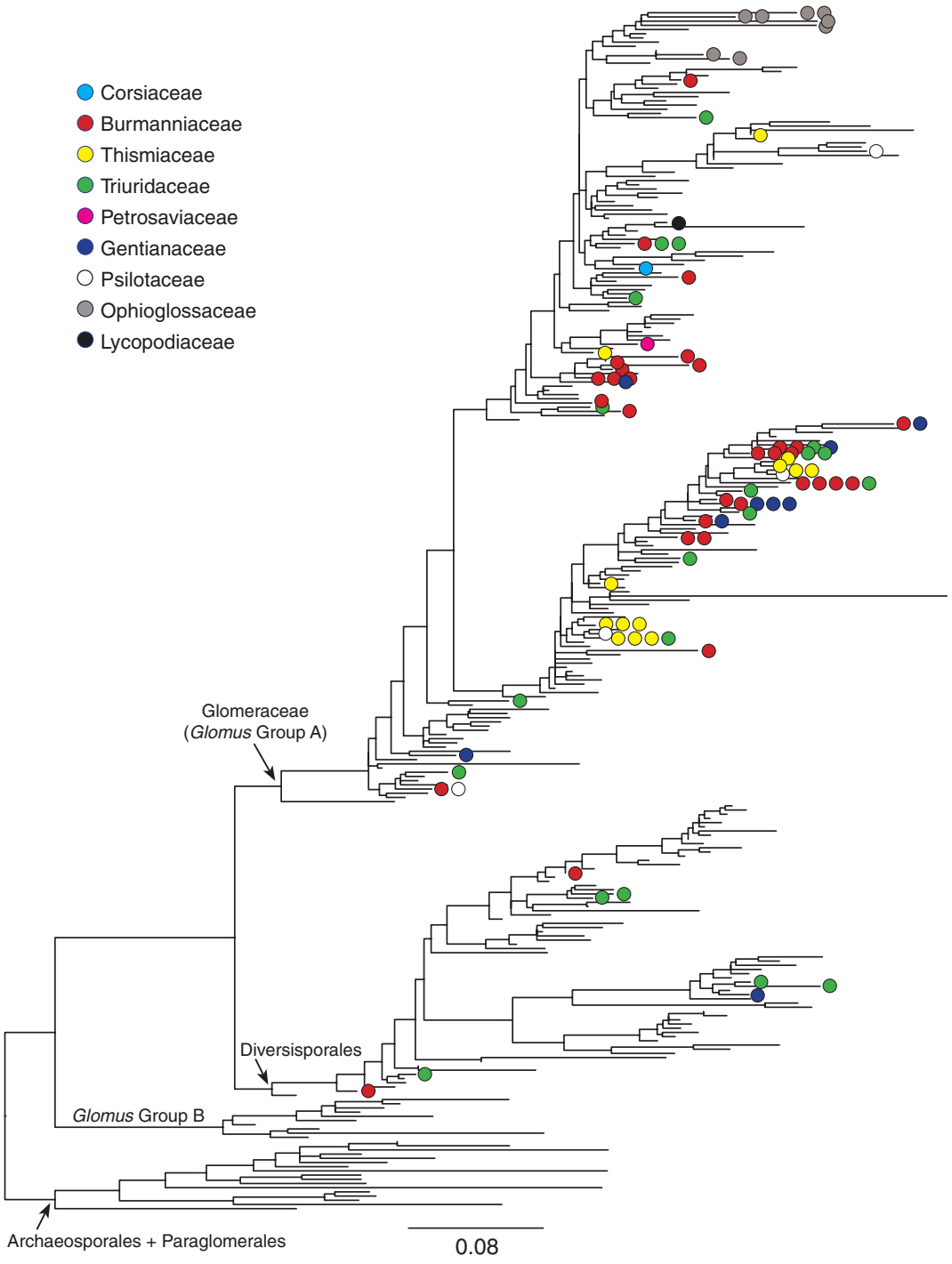


**Fig. 2** Maximum clade credibility tree of partial SSU rDNA sequences of 286 virtual taxa of arbuscular mycorrhizal fungi, inferred with Maximum Likelihood phylogenetic analysis. Coloured dots represent mycoheterotrophic species in which a particular virtual taxon has been detected. Bar shows number of substitutions per site.

phylogeny. NTI and NRI values show that these VT are phylogenetically clustered. In addition, VT that are associated with Burmanniaceae and Thismiaceae also show phylogenetic clustering using both NTI and NRI values (Table 1). However, we expect that NRI values, which measure clustering across the whole phylogeny including deep-level relationships, may be influenced by the fact that Archaeosporales, Paraglomerales and *Glomus* Group B fungi have not been detected in mycoheterotrophic plants. Indeed, when these clades

**Table 1** Results of the phylogenetic community analyses. Measurements of nearest taxon index (NTI) and net relatedness index (NRI) are shown for virtual taxa (VT) sets found in different plant groups

| | Number of VT | Glomeromycota | | Glomeraceae + Diversisporales | |
|---|---|---|---|---|---|
| | | NTI | NRI | NTI | NRI |
| All MHP's | 58 | **3.74** | **2.17** | **2.15** | 0.37 |
| | | **3.57** | **1.96** | **2.14** | 0.29 |
| Burmanniaceae | 22 | **2.95** | **1.88** | **2.29** | 0.97 |
| | | **3.05** | **1.90** | **2.32** | 0.92 |
| Thismiaceae | 7 | **2.45** | **2.34** | **2.25** | **2.53** |
| | | **2.36** | **2.39** | **2.24** | **2.33** |
| Triuridaceae | 19 | 1.07 | **2.27** | 0.03 | 1.49 |
| | | 1.09 | **2.07** | 0.02 | 1.52 |
| Gentianaceae | 7 | 0.75 | 0.61 | 0.21 | 0.05 |
| | | 0.78 | 0.65 | 0.22 | 0.02 |
| Psilotaceae | 4 | 0.69 | 0.33 | 0.28 | 0.21 |
| | | 0.66 | 0.33 | 0.35 | 0.21 |
| Ophioglossaceae | 6 | **2.23** | 1.35 | **2.07** | 1.04 |
| | | **2.23** | 1.25 | **1.94** | 1.03 |

Results are shown for calculations on the complete Glomeromycota phylogeny and for calculations on the Glomeraceae-Diversisporales clade only. Results in the first row were calculated using the taxa.labels null model, results in the second row were calculated using the phylogeny.pool null model. Significant NTI and NRI values are shown in bold. NTI and NRI are not applicable for VT of Corsiaceae, Petrosaviaceae and Lycopodiaceae because in these families only a single VT has been detected.

are excluded from the Glomeromycota phylogeny, all but the Thismiaceae VT taxon set lost their significant NRI values. In contrast, NTI values remained more or less similar (Table 1), again indicating that within Glomeromycota, most major clades contain taxa that are involved in mycoheterotrophic interactions yet within these clades these taxa are more phylogenetically clustered than for randomly generated phylogenies. Our results indicate that at the ordinal level, mycoheterotrophic interactions through AMF are generally restricted to Diversisporales and particularly Glomeraceae. Within these two clades, VT that are involved in mycoheterotrophic interactions are phylogenetically clustered. However, it is to be expected that sampling of additional specimens, species and families of mycoheterotrophic plants from different or the same geographical areas will increase the total number of fungal lineages involved in mycoheterotrophic interactions, and thus the phylogenetic breadth of mycoheterotrophic interactions.

### *Fungal overlap*

At the virtual taxon level, there is considerable overlap in the AMF hosts between different mycoheterotrophic plant species and families. Nine VT were found in at least two different mycoheterotrophic plant families. Fungal overlap between Burmanniaceae, Gentianaceae Thismiaceae and Triuridaceae is most pronounced. This is possibly influenced by their similar distribution patterns. Burmanniaceae, Gentianaceae, Thismiaceae and Triuridaceae have overlapping pantropical distributions and were often sampled at sites where species from two or three of these families occurred. It is likely that these species have potential access to the same fungi. Glomeromycota VT have recently been found to occupy restricted distribution ranges at biogeographical scales reflecting climatic zones and historical and current continental limits (Öpik *et al.* 2010). However, we do not find strong indications for continental differences in AMF of mycoheterotrophic plants. For example, the West African species *S. ledermannii* and *C. congestus* grow with many VT that are also found in the roots of Neotropical Burmanniaceae species. And VTX00106 has been found in *Gymnosiphon divaricatus* (Neotropics), *S. ledermannii* (tropical West Africa) and *S. tosaensis* (Japan). It must be noted that for most species analyzed here, the current data are based on a very limited sampling—in a context of both number of individuals and geographical coverage. Therefore, the diversity of associated mycorrhizal fungi per species may be considerably underestimated, as well as the extent of fungal overlap between different mycoheterotrophic plant species.

### *Specificity*

Some mycoheterotrophic plant species have the ability to associate with a wide range of AMF lineages.

Examples are *S. ledermannii*, which was found growing with 13 different fungal VT including Acaulosporaceae, Gigasporaceae, and Glomeraceae, and *Campylosiphon congestus*, which is able to associate with fungi from at least eight fungal VT in Acaulosporaceae and Glomeraceae. Again, it is likely that additional sampling will reveal a much broader fungal host range for many species in our data set. In contrast to these 'generalist' plant species, some mycoheterotrophs show very narrow host ranges, which suggest that these species are highly specialized in their fungal associations. Examples are *Arachnitis, Petrosavia* and most Thismiaceae species. Thus, extreme host specificity occurs in arbuscular mycorrhizal mycoheterotrophs, but it is certainly not a requirement for mycoheterotrophy, and it may be less common than previously assumed. The observation that so many different AMF lineages are being used by mycoheterotrophs shows that carbon transfer between plants through common arbuscular mycorrhizal networks does not require a narrow range of particular AMF. Similar observations have been made for mycoheterotrophic interactions through Basidiomycota fungi in species of Ericaceae and Orchidaceae. While many mycoheterotrophic species in Ericaceae and Orchidaceae exhibit high mycorrhizal specificity, a lack of mycorrhizal specificity has been observed for *Pyrola aphylla* (Ericaceae) (Hynson & Bruns 2009), *Wullschlaegelia aphylla* and species of *Aphyllorchis* (Orchidaceae) (Martos *et al.* 2009; Roy *et al.* 2009).

The arbuscular mycorrhizal symbiosis is generally characterized by low specificity between plants and fungi (Smith & Read 2008). Thus, mycoheterotrophs with high mycorrhizal specificity likely evolved from ancestors with more generalist fungal associations. It remains to be determined whether specialized mycoheterotrophs are growing with a subset of AMF that associate with their autotrophic relatives, or whether they have switched to different AMF lineages. Partially mycoheterotrophic plants may provide the key in unravelling this specialization process. Unfortunately, partial mycoheterotrophy through AMF has only been reported in *Obolaria* and *Bartonia* (Gentianaceae), and the degree of specificity of their associations with AMF remains unknown (Cameron & Bolin 2010). Many putative partially mycoheterotrophic plants, particularly photosynthetic plants closely related to nonphotosynthetic arbuscular mycorrhizal plants, remain to be examined for partial mycoheterotrophy (e.g. Polygalaceae, Dioscoreales and Iridaceae).

### *Limitations and future directions*

Assigning AMF sequences to VT is an elegant method to investigate the phylogenetic host range of mycoheterotrophic plant species. However, there is currently no evidence that fungal VT are functional entities for mycoheterotrophic plants or that VT based on partial 18S rDNA sequences are close to 'real' AMF species. Specificity may act well below the level of the current used VT. This is probably the case in *Afrothismia*, for which we detected overlap in VT between different species in this study. On a finer phylogenetic scale, this overlap disappears (Merckx & Bidartondo 2008). Thus, future studies may benefit from the inclusion of additional data from faster evolving DNA regions, such as ITS (e.g. Courty *et al.* 2011). *In vitro* propagation experiments of arbuscular mycorrhizal mycoheterotrophs may provide further insights into this issue. In addition, the plants analyzed in our study, and those from which data from the literature are included, were sampled during flowering stage; the possibility of differences in associated fungi and level of mycorrhizal specificity in different life stages or seasons cannot be excluded. Also, it remains to be determined which fungi are actually involved in the transfer of carbon to the plant when mycoheterotrophs are found growing with multiple fungal VT.

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## Data accessibility

DNA sequences: GenBank accessions JQ246029–JQ246072.

## Supporting information

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

**Appendix S1.** Final DNA sequence alignment.

**Table S1** List of myco-heterotrophic plants from which 18S rDNA sequences of their symbiotic fungi were included in the phylogenetic analyses.

**Table S2** GenBank accessions of sequences used to represent the virtual taxa.

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