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Original article

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The manuscript contains 2 tables and 4 color figures, and 8 supplementary tables and 7 supplementary figures.

Abstract (159 words)

- While mutualistic interactions are widespread and essential in ecosystem functioning, the emergence of uncooperative cheaters threatens their stability, unless there are some physiological or ecological mechanisms limiting interactions with cheaters.
- In this framework, we investigated the patterns of specialization and phylogenetic distribution of mycoheterotrophic cheaters *versus* non-cheating autotrophic plants and their respective fungi in a global arbuscular mycorrhizal network with >25,000 interactions.
- We show that mycoheterotrophy repeatedly evolved among vascular plants, suggesting low phylogenetic constraints for plants. However, mycoheterotrophic plants are significantly more specialized than autotrophic plants, and they tend to be associated with specialized and closely related fungi. These results raise new hypotheses about the mechanisms (e.g. sanctions, or habitat filtering) that actually limit the interaction of mycoheterotrophic plants and their associated fungi with the rest of the autotrophic plants.
- Beyond mycorrhizal symbiosis, this unprecedented comparison of mycoheterotrophic *versus* autotrophic plants provides a network and phylogenetic framework to assess the presence of constraints upon cheating emergences in mutualisms.

Keywords: arbuscular mycorrhiza, mutualism, cheating, mycoheterotrophy, ecological networks, reciprocal specialization, phylogenetic constraint.

Introduction

Mutualistic interactions are ubiquitous in nature and largely help to generate and maintain biodiversity (Bronstein, 2015). Since benefits in mutualism often come at a cost for cooperators (Douglas, 2008), some species, referred to as *cheaters*, have evolved an adaptive uncooperative strategy by retrieving benefits from an interaction without paying the associated cost (Sachs *et al.*, 2010). Although cheating compromises the evolutionary stability of mutualistic interactions (Ferriere *et al.,* 2002), its evolutionary origin and persistence until present (hereafter referred to as cheating emergence) is often limited by factors securing the persistence of mutualism (Bronstein *et al.*, 2003; Frederickson, 2013; Jones *et al.*, 2015). For instance, species often favor the most cooperative partners (e.g. conditional investment; Roberts & Sherratt, 1998), stop interactions with cheaters (Pellmyr & Huth, 1994), or even sanction them (Kiers et al. 2003). Cheating emergence can thus be constrained through physiological or biochemical mechanisms of the interaction and its regulation. In addition, cheating can be restricted to particular habitats or to partners with specific niches. Therefore, cheaters might be constrained to specialize on susceptible partners and/or particular habitats. Moreover, these different constraints (hereafter referred to as *functional constraints*) can be evolutionarily conserved or not (Gómez *et al.* 2010). If they are conserved, there will be *phylogenetic constraints* on the emergence of cheaters, as some species will have evolutionarily conserved traits that make them more or less likely to cheat or to be cheated upon (Lallemand *et al.*, 2016).

The framework of bipartite interaction networks, combined with the phylogeny of partners, is useful for analyzing the patterns susceptible to arise from constraints limiting the emergence of cheaters in mutualisms (Fig. **1**). Analyses of bipartite networks have been extensively used to showcase the properties of mutualistic interactions (Bascompte *et al.*, 2003; Rezende *et al.*, 2007; Martos *et al.*, 2012), such as their level of specialization (number of partners), nestedness (do specialists establish asymmetric specialization with partners that are themselves generalists?), and modularity (existence of distinct sub-networks; Bascompte & Jordano 2014). These studies, most of them describing species interactions at a local scale, have shown that mutualistic networks are generally nested with specialists establishing asymmetric specialization with more generalist partners,

unlike antagonistic networks, which tend to be modular, with partners establishing reciprocal specialization (Thebault & Fontaine, 2010). However, few analyses of bipartite networks have focused on the specialization of cheaters and how they influence nestedness and modularity (Fontaine *et al.*, 2011). By assembling networks at a regional scale, Joffard *et al.* (2018) showed that specialization of orchids toward pollinators was higher in deceptive cheaters (both sexual and food deceits) than in cooperative nectar-producing species, and Genini *et al.* (2010) showed that a network dominated by cooperative pollinators was nested, whereas another network dominated by nectar thieving insects was more modular. If cheaters specialize and form modules, this would suggest the presence of functional constraints limiting the set of species that they can exploit (Fig. **1b**-v). Additionally, if cheaters emerged only once in a phylogeny (*versus* repeatedly), and/or if 'cheating-susceptible' partners are phylogenetically related (Merckx *et al.,* 2012), this would suggest that cheating involves some rare evolutionary innovations (Pellmyr *et al,.* 1996) and/or that cheating susceptibility is limited to few clades, meaning that cheating is phylogenetically constrained (Fig. **1a**-i).

Here we study cheating emergences in arbuscular mycorrhizal mutualism between plant roots and soil Glomeromycotina fungi (Selosse & Rousset, 2011; Jacquemyn & Merckx, 2019). This symbiosis is at least 407 Myr-old (Strullu-Derrien *et al.*, 2018) and concerns ca. 80% of extant land plants and several hundred fungal taxa (Davison *et al.,* 2015; van der Heijden *et al*., 2015). Arbuscular mycorrhizal fungi colonize plant roots and provide host plants with water and mineral nutrients, in return for organic carbon compounds (Rich *et al.*, 2017). Although obligate for both partners, this symbiosis is generally diffuse and not very specific (van der Heijden *et al.*, 2015), since multiple fungi colonize most plants, while fungi are usually shared among surrounding plant species (Verbruggen *et al.* 2012). Thus, fungi interconnect plant individuals of different species and allow resource movement between plants (Selosse *et al.*, 2006; Merckx, 2013). This allowed the emergence of achlorophyllous cheating plants, called *mycoheterotrophs*, which obtain carbon from their mycorrhizal fungi that are themselves fed by surrounding autotrophic plants (Merckx, 2013) – these plants are thus permanent cheaters, whatever the conditions or partners. Some of these plant species are *entirely mycoheterotrophic* over their lifecycle, while others are mycoheterotrophic only at early stages before turning

autotrophic (*initially mycoheterotrophic*), therefore shifting from being cheaters to becoming potentially cooperative partners (Merckx, 2013). Unlike other systems where cheaters are costly (they receive the benefits without paying the cost of the interaction) mostly for direct partners (e.g. in plant pollination), mycoheterotrophs are costly for both their direct fungal partners and the interconnected autotrophic plants, whose photosynthesis supplies the carbon (it represents a projected cost, transmitted through the network). Although uncooperative strategies between autotrophic plants and arbuscular mycorrhizal fungi may exist under certain conditions (Klironomos, 2003; Jacquemyn & Merckx, 2019; but discussed in Frederickson, 2017), autotrophs can supply photosynthetic carbon and are mostly cooperative, while mycoheterotrophs never supply photosynthetic carbon and are therefore necessarily uncooperative.

We evaluate the presence of functional constraints upon cheating by measuring specialization, nestedness and modularity in a composite plant-mycorrhizal fungal interaction network built from associations between species at multiple sites across the entire globe (Öpik *et al.*, 2010). Mycoheterotrophic plants are thought to be specialists interacting with few fungal species (Leake, 1994; Merckx, 2013), but whether or not these plant species are unusually specialized compared to autotrophic plants is still debated (Merckx *et al.*, 2012). Mycoheterotrophs could specialize on few fungal species if some functional constraints limit the set of fungi or habitats they can exploit, and if they have evolved particular strategies to obtain nutrients from their specific fungal partners (Blüthgen *et al.,* 2007). In terms of nestedness and modularity, arbuscular mycorrhizal networks are generally nested (Chagnon *et al.*, 2012; Sepp *et al.*, 2019); this pattern of asymmetrical specialization is generally thought to confer greater stability in relation to disturbance and resistance to species extinction (Thébault & Fontaine, 2010). How mycoheterotrophic plants affect nestedness has yet to be investigated. On the one hand, in the absence of functional constraints upon cheating, we would expect that mycoheterotrophs interact with generalist fungi to increase their indirect access to carbon *via* surrounding autotrophic plants, therefore increasing nestedness (Fig. **1b**–v,viii). On the other hand, if autotrophic plants are able to avoid costly interactions with fungi associated with mycoheterotrophs (physiological constraints), or if mycoheterotrophs are only tolerated in particular habitats (ecological constraints), we

153 expect a reciprocal specialization between mycoheterotrophs and their fungi and thus an
154 increase of modularity and a decrease of nestedness (Fig. **1b**–v,vii). Establishment of an
155 extreme reciprocal specialization between entirely mycoheterotrophs and fungi
156 exclusively associated with such plants seems unlikely though, since an autotrophic
157 carbon source is required.

158

159 With regards to phylogenetic constraints on mycoheterotrophy, we already know that
160 mycoheterotrophic strategies evolved multiple times (Merckx, 2013), generating
161 monophyletic groups of mycoheterotrophic plants, which suggests weak phylogenetic
162 constraints on the emergence of mycoheterotrophy in plants. However, the fungi
163 interacting with independent mycoheterotrophic lineages might be phylogenetically
164 closely related (Merckx *et al*., 2012), which would indicate phylogenetic constraints on
165 fungi (Fig. **1a**–iii). The presence of such phylogenetic constraints has yet to be confirmed
166 in a large phylogenetic context including the fungi of autotrophic plants. Moreover, if as
167 we expect, only a set of phylogenetically close fungi interact with all mycoheterotrophic
168 plant lineages, an important follow-up question is whether these fungi were acquired
169 independently by autotrophic ancestors, or whether they were acquired by symbiont shift
170 from other mycoheterotrophic plants.

Materials and Methods

MaarjAM database and interaction matrix

The MaarjAM database is a web-based database (http://maarjam.botany.ut.ee; accessed in June 2019 after a very recent update) of publicly available sequences of Glomeromycotina fungi, with information on the host plants, geographical location and biomes for the recorded interactions (Öpik *et al.*, 2010). We used an approach with a compiled network, where all locally described physical mycelial interactions between species are merged and studied at larger scales (as in Joffard *et al.*, 2018). Although such a compiled network can be sensitive to several biases (see Discussion), it offers unique opportunities to study the emergence of mycoheterotrophy in a large evolutionary and ecological perspective (e.g. Werner *et al.*, 2018). Among the 41,989 interactions between plants and Glomeromycotina, we filtered out the data from MaarjAM for the fungi to satisfy the following criteria (Supporting Information Table **S1a**): *(i)* amplification of the 18S rRNA gene, *(ii)* fungus identified from plant roots (i.e. excluding soil samples), *(iii)* interaction in a natural ecosystem (i.e. excluding anthropogenic or highly disturbed ecosystems), *(iv)* host plant identified at the species level, and *(v)* a virtual taxon (VT) assignation available in MaarjAM. The VTs are a classification (=species proxy) of arbuscular mycorrhizal fungi designed by applying a ≥97% sequence similarity threshold to the 18S rRNA gene sequences, and by running phylogenetic analysis to ensure VT monophyly (Öpik *et al.*, 2013, 2014). In the following, we assumed that we have a full representation of all fungal partners associated with each plant species in the dataset. The filtered dataset yielded a binary interaction matrix of 490 plant species (hereafter 'plants'), 351 VTs (hereafter 'fungi'), and 26,350 interactions (Fig. **2**), resulting from the compilation of 112 publications from worldwide ecosystems (Supporting Information Fig. **S1**; Table **S1b**). In order to estimate the sampling fraction of Glomeromycotina fungi in our dataset, we plotted rarefaction curves of the number of fungal species as a function of the sampling fraction (for the observed number of interactions or for the number of sampled plant species) and we estimated the total number of species using the '*specpool*' function ('*vegan*' R-package, based on Chao index; Oksanen *et al.*, 2019). We separately performed rarefaction analyses for mycoheterotrophic species only. Moreover, in order to check the robustness of our results, we repeated all the analyses on a subsampled

version of the MaarjAM database accessed in October 2017 (Supporting Information Fig. **S2**).

Phylogenetic reconstructions

We aligned consensus sequences of the 351 fungi with MUSCLE (Edgar, 2004) and ran a Bayesian analysis using BEAST2 to reconstruct the fungal phylogeny (Bouckaert *et al.* 2014, Supporting Information Methods **S1**). We obtained the phylogenetic relationships between the 490 host plants by pruning the time-calibrated supertree from Zanne *et al.* (2013) using Phylomatic (http://phylodiversity.net/phylomatic/). We also used the Open Tree of Life website (http://opentreeoflife.org) and the '*rotl*' R-package (Michonneau et al. 2016; R Core Team, 2019) for grafting of 41 plant taxa missing from the pruned supertree (as polytomies at the lowest taxonomy level possible; Supporting Information Methods **S1**). We set tree root calibrations at 505 million years (Myr) for the fungi (Davison *et al.*, 2015) and 440 Myr for the plants (Zanne *et al.*, 2014).

Nature of the interaction

We assigned to each plant its 'nature of the interaction' with fungi according to its carbon nutrition mode according to an on-line database (http://mhp.myspecies.info/category/myco-heterotrophic-plants/) and individual publications (Boullard, 1979; Winther & Friedman, 2008; Field et al. 2015): autotroph (n=434, 88.6%), entirely mycoheterotroph (n=41, 8.4%), or initially mycoheterotroph (n=15, 3.1%). We assigned each fungus to three categories: 'associated with autotrophs' if the fungus interacts with autotrophic plants only (n=280, 79.8%), 'associated with entirely mycoheterotrophs' if the fungus interacts with at least one entirely mycoheterotroph (n=54, 15.4%), 'associated with initially mycoheterotrophs' if the fungus interacts with at least one initially mycoheterotroph (n=23, 6.6%), or 'associated with mycoheterotroph' if the fungus interacts with at least one entirely or initially mycoheterotroph (n=71, 20.2%; Supporting Information Table **S2**). Only five fungi are associated with both entirely and initially mycoheterotrophic plants. Our dataset included mycoheterotrophs from 18 publications. While only 41 entirely mycoheterotrophic species were included out of 267 described species (Jacquemyn & Merckx, 2019), all known entirely mycoheterotrophic families were represented by at least one plant

species, except the families Aneuraceae (liverwort, one mycoheterotrophic species), Iridaceae (monocotyledons, three species), and Podocarpaceae (gymnosperm, one controversial species). Similarly, our dataset missed only a few initially mycoheterotrophic families, such as Schizaeaceae (Boullard, 1979).

Network nestedness, modularity, and specialization of cheaters

In order to assess the functional constraints upon cheating, we tested the effect of mycoheterotrophy on network structure (Fig. **1b**). First, we measured nestedness in: *(i)* the overall network (490 plants, 351 fungi, and 26,350 interactions), *(ii)* the network restricted to autotrophic plants (434, 344, and 26,087) and *(iii)* the network restricted to entirely and initially mycoheterotrophic plants (56, 71, and 263), using the function '*NODF2*' in the R-package '*bipartite*' (Dormann et al. 2008). We tested the significance of NODF values (nestedness metric based on overlap and decreasing fill; Supporting Information Methods **S2** - *List of abbreviations*) by using two types of null models (N=100 for each type): the first model (*'r2dtable'* from *'stats'* R-package - *null model 3*) maintains the marginal sums of the network (the sums of each row and each column), whereas the less stringent second model (*'vaznull'* from '*bipartite'* R-package - *null model 2*) produces slightly different marginal sums (interactions are randomized with species marginal sums as weights, and each species must have at least one interaction), while maintaining the connectance (proportion of observed interactions). We calculated the Z-score, which is the difference between the observed value and the mean of the of null-models values divided by their standard deviation (Z-scores greater than 1.96 validate a significant nestedness with an alpha-risk of 5%). Positive z-scored NODF values indicate nested networks.

Second, to further evaluate the specialization of mycoheterotrophic plants, we computed several network indices for each plant. The degree (k) is the number of partners with which a given plant or fungus interacts in the bipartite network. The degree is high (*vice versa* low) when the species is generalist (*vice versa* specialist). The partner specialization (*Psp*) is the mean degree (k) averaged for all the fungal partners for a given plant species (Taudiere *et al.*, 2015): a high (*vice versa* low) *Psp* characterizes a species interacting mainly with generalist (*vice versa* specialist) partners. Simultaneously low k and *Psp*

values feature a reciprocal specialization (Fig. **1b**-v,vii). We tested whether *k* and *Psp* were statistically different among autotrophic, entirely mycoheterotrophic and initially mycoheterotrophic plants using non-parametric Kruskal-Wallis tests and pairwise Mann-Whitney U tests. To assess the significance of *k* and *Psp* values, we built null-model networks (*N*=1,000) using the function '*permatfull'* in the '*vegan'* R-package *(null model 1)*, keeping the connectance constant but allowing different marginal sums. Then, in order to detect specialization at the clade scale toward partners, for any given clade of every node in the plant or fungus phylogenies, we calculated the partner fidelity (*Fx*) as the ratio of partners exclusively interacting with this particular clade divided by the total number of partners interacting with it. We consider the clade as 'faithful' and the corresponding set of partners as 'clade-specific' when *Fx*>0.5 (i.e., more than 50% exclusive partners). We used analysis of covariance (ANCOVA) to test the effect of the nature of the interaction on partner fidelity *Fx* accounting for clade size, which corrects the bias of having high partner fidelity *Fx* in older clades including many plants. To confirm that the patterns of specialization at the global scale held at a more local scale, we reproduced the analyses of specialization (*k* and *Psp*) in two continental networks in South America and Africa, which represented a high number of interactions and mycoheterotrophic species.

Third, we investigated signatures of reciprocal specialization in the overall network structure. We used the DIRTLPAwb+ algorithm (Beckett, 2016) to infer modules and assess their significance (a module is significant if it encompasses a subset of species interacting more with each other than with the rest of the species) and used the function *components* of the R-package igraph (Csardi & Nepusz, 2006) to detect cases of extreme reciprocal specializations leading to independent modules (two species belong to two distinct independent modules if there is no path in the network going from one to the other, *i.e.* an independent module is the smallest subset of species exclusively interacting with each other).

We replicated these statistical tests without the initially mycoheterotrophic Lycopodiaceae forming different network patterns (see Results).

Phylogenetic distribution of cheating

In order to assess phylogenetic constraints, we explored the phylogenetic distribution of mycoheterotrophic plants and their associated fungi (Fig. **1a**). First, we investigated the

phylogenetic distribution of mycoheterotrophy, i.e. if mycoheterotrophic plants and their fungal partners were more or less phylogenetically related than expected by chance (patterns of clustering *versus* overdispersion). We computed the net relatedness index (NRI) and the nearest taxon index (NTI) using the '*PICANTE*' R-package (Kembel *et al.*, 2010). NRI quantifies the phylogenetic structure of a species set based on the mean pairwise distances, whereas NTI quantifies the terminal structure of the species set by computing the mean phylogenetic distance to the nearest taxon of every species (Gotelli & Rohde, 2002). To standardize the indices, we generated 999 null models with the option '*taxa.labels*' (shuffles the taxa labels). Significant positive (resp. negative) NRI and NTI values indicate phylogenetic clustering (resp. overdispersion). We computed these indices *(i)* on the plant phylogeny to evaluate the phylogenetic structure of entirely mycoheterotrophic and initially mycoheterotrophic plant distribution, and *(ii)* on the fungal phylogeny to investigate if fungi associated with mycoheterotrophs were phylogenetically structured (we successively tested the distribution of the fungi associated with mycoheterotrophs, entirely mycoheterotrophs, or initially mycoheterotrophs, and then of the fungi associated with each specific mycoheterotrophic family). Similarly, for each plant, we computed the partners' mean phylogenetic pairwise distance (*MPD*), that is the average phylogenetic distance across pairs of fungal partners (Kembel *et al.*, 2010): a low value of *MPD* indicates that the set of partners is constituted of closely related species. The effect of mycoheterotrophy on *MPD* values and its significance were evaluated as for *k* and *Psp* values above.

Second, in order to assess whether fungal partners of a given mycoheterotrophic family were derived from fungal partners of autotrophic ancestors or were secondarily acquired from other mycoheterotrophic lineages, we compared in an evolutionary framework the sets of fungi associated with plants with different natures of the interaction. To do so, we computed the unweighted UniFrac distance (Lozupone & Knight, 2005) between sets of fungi interacting with each pair of plants in the network. For each of the seven mycoheterotrophic families, we compared the UniFrac distances across *(i)* every pair of plant species of this family, *(ii)* every pair comprising one plant of this family and one plant of the most closely related autotrophic family (see Table **2**), *(iii)* every pair composed of one plant of this family and one plant belonging to other mycoheterotrophic families, and *(iv)* every pair comprising one plant of this family and one more distant autotrophic plant (i.e. all autotrophic plants except those of the most closely related

autotrophic family). This analysis was not performed on mycoheterotrophic Petrosaviaceae, which were represented by only one species and were too divergent to define a reliable autotrophic sister clade.

We tested differences between groups of distances using Mann-Whitney U tests. We also performed a principal coordinates analysis (PCoA) from all the UniFrac dissimilarities of sets of fungal partners, and tested the effect of the nature of the interaction on the two principal coordinates, using Kruskal-Wallis tests. Finally, to examine the extent to which the nature of the interaction affects fungal partners, we used permutational analysis of variance (PerMANOVA, '*adonis'* function in R-package '*vegan'*), with 10,000 permutations.

Results

Completeness of the dataset

We estimated a total number of 373+/-9 fungal species (Chao index), which indicated that the 351 fungi in the dataset included most of the arbuscular mycorrhizal fungal diversity (94%+/-2%; Supporting Information Fig. **S3**). Concerning mycoheterotrophic species, we estimated a total of 117+/-19 fungi associated with all mycoheterotrophs, 110+/-27 fungi associated with entirely mycoheterotrophs, and 54+/-24 fungi associated with initially mycoheterotrophs. Our dataset thus encompassed sampling fractions of 60%+/-10% for fungi associated with mycoheterotrophs, 49%+/-10% for fungi associated with entirely mycoheterotrophs, and 40%+/-28% for fungi associated with initially mycoheterotrophs. Although our dataset did not include all the fungi associated with mycoheterotrophic species, the following results were not sensitive to the sampling fractions of mycoheterotrophs and their fungal partners (Supporting Information Fig. **S2**).

Network nestedness, modularity, and specialization of mycoheterotrophs

The overall network had a significant positive nestedness value (Z-score=9.2, $P=1.10^{-20}$, Supporting Information Table **S3**). Nestedness increased when only autotrophic plants were considered (Z-score=16.6, $P=8.10^{-62}$), whereas it was not significant in the network of only mycoheterotrophs (Z-score=1.44, P=0.075): mycoheterotrophic plants reduced nestedness, signifying that they displayed higher reciprocal specializations.

Reciprocal specializations were confirmed by the analyses of modularity, which found no significant large modules (i.e. the inferred large modules presented more inter-modules than intra-module interactions), suggesting that the overall structure was not modular, but detected few significant small independent modules (Supporting Information Table **S4**). In addition to a main module encompassing most species (481 out of 490 plants and 346 out of 351 fungi), we found three small independent modules: *(i)* 6 initially mycoheterotrophic Lycopodiaceae plants and three exclusive fungi (*Glomus* VT127, VT158, VT394); *(ii)* two autotrophic plants from salt marshes (*Salicornia europaea* and *Limonium vulgare*) with one *Glomus* (VT296); and *(iii)* the entirely mycoheterotrophic *Kupea martinetugei* with a unique *Glomus* (VT204).

From the degrees (*k*), we found that entirely and initially mycoheterotrophic plants were significantly more specialized than autotrophic plants and interacted with on average more than five times fewer fungi (Kruskal-Wallis H=87.2; P=$1.2.10^{-19}$; Fig. **3a**; Table **1**). Partner specializations (*Psp*) indicated that mycoheterotrophs interacted with more specialized fungi (fungi associated with mycoheterotrophs interact on average with two times fewer plants; Kruskal-Wallis H=47.2; P=$5.6.10^{-11}$; Fig. **3a**). We found similar evidence for mycoheterotrophic reciprocal specializations by reanalyzing the network excluding the family Lycopodiaceae (Table **1**; significance assessments using null models are shown in Supporting Information Table **S5**). This pattern of reciprocal specialization of mycoheterotrophic plants and their associated fungi held at a smaller geographical scale in the African and South American networks (Supporting Information Fig. **S4**; Table **S6**; yet the difference was not significant for *Psp* in the South American network, probably due to the small number of species and the low power of the statistical tests).

The partner fidelity index (*Fx*) showed that very few plant and fungi clades interacted with 'clade-specific' partners (i.e. *Fx*>0.5), and most fungi were shared between different plant clades (Fig. **3c**). Among exceptions, however, the clade of initially mycoheterotrophic Lycopodiaceae was characterized by a high partner fidelity index (*Fx*>0.8), reflecting a strong association with a clade of three Lycopodiaceae-associated fungi (Supporting Information Fig. **S5**). Thus, not only did these 6 Lycopodiaceae species and their fungal partners form an independent module, but the Lycopodiaceae-associated fungi also formed a monophyletic clade within Glomeromycotina. The estimated clade age was 250 Myr for the Lycopodiaceae and 49 Myr for the Lycopodiaceae-associated fungi (Fig. **3d**), which diverged 78 Myr ago from the other *Glomus* fungi.

Phylogenetic distribution of cheating

The partners' mean phylogenetic pairwise distance (*MPD*) indicated that fungi associated with entirely or initially mycoheterotrophs (or even with all mycoheterotrophs) were phylogenetically more closely related than fungi associated with autotrophs (Kruskal-Wallis H=18.0; P=$1.2.10^{-4}$; Table **1**; Fig. **3b**). NRI and NTI values (Supporting Information Table **S7**) also confirmed significant clustering on the fungal phylogeny on fungi associated with mycoheterotrophs, entirely mycoheterotrophs, or initially mycoheterotrophs; this clustering held at the family level for fungi associated with each

of four main mycoheterotrophic families (namely Burmanniaceae, Triuridaceae, Polygalaceae, and Ophioglossaceae). In terms of the plants, only the entirely mycoheterotrophs were significantly clustered, mainly because they all were angiosperms and mostly monocotyledons, but this did not apply to mycoheterotrophs in general, nor to initially mycoheterotrophs (Supporting Information Table **S7**). These phylogenetic clusters were visually noticeable on fungal and plant phylogenetic trees (Supporting Information Fig. **S6; S7**). This suggests that although mycoheterotrophy evolved several times independently in plants, mycoheterotrophic plants interact mainly with closely related fungi (see also Fig. **2**).

Looking specifically at the fungi shared among mycoheterotrophic plants highlighted differences between entirely and initially mycoheterotrophs (Table **2**). While the initially mycoheterotrophic Lycopodiaceae family formed an independent module with three specific *Glomus* VTs, another initially mycoheterotrophic family Ophioglossaceae also had 2 exclusive fungi (*Glomus* VT134 and VT173) among a total of 15 fungi. When comparing the fungi shared between mycoheterotrophic families (Table **2**), mainly two closely related families, Burmanniaceae and Triuridaceae, tended to share some fungi with other mycoheterotrophic families.

The decomposition of UniFrac dissimilarities between sets of fungal partners using a PCoA, showed a clear pattern of clustering of mycoheterotrophic species, indicating that the set of fungal partners associated with mycoheterotrophs were more similar than expected by chance ($P<1.10^{-16}$ for PCoA1; $P=9.10^{-3}$ for PCoA2; Fig. **4a**). Similarly, the PerMANOVA analysis indicated that the nature of the interaction (initially mycoheterotrophic, entirely mycoheterotrophic, or autotrophic) predicted 6.5% of the variance (P=0.0001). By comparing the UniFrac dissimilarities between sets of fungal partners according to the nature of the interaction and plant family relatedness, we observed that all mycoheterotrophic families had fungal partners more similar to each other than those of other autotrophic families (Fig. **4b**; Supporting Information Table **S8**). Some families (Burmanniaceae, Polygalaceae, Triuridaceae, Lycopodiaceae, and Ophioglossaceae) had fungal partners significantly more similar to partners interacting with their closest autotrophic relatives (P>0.05) than to partners interacting with other autotrophic families ($P<10^{-16}$). This suggests phylogenetic conservatism of fungal partners during the evolution of mycoheterotrophic nutrition in these families. For other

435 mycoheterotrophic families (Corsiaceae, Gentianaceae, and Psilotaceae), fungal partners
436 were significantly more similar to partners interacting with other mycoheterotrophic
437 families than to partners interacting with their closest autotrophic relatives, the latter
438 being as distant as other autotrophic families (Supporting Information Table **S8**). This
439 points to a shift to new fungal partners correlated with the evolution of
440 mycoheterotrophic nutrition in these three families.

Discussion

By combining network and phylogenetic analyses, we assessed constraints upon the emergence of mycoheterotrophic cheating in arbuscular mycorrhizal mutualism. Although the network was nested, we found evidence for reciprocal specialization in the case of mycoheterotrophic plants (specialists) and their fungal partners (also specialists). We even observed unexpected, extreme reciprocal specialization for some initially mycoheterotrophic lineages associating with fungi exclusively interacting with these plant lineages. Finally, we found that independently emerged mycoheterotrophic plant lineages share many closely related fungi, and that in some of these lineages fungal partners were likely acquired from autotrophic ancestors, while in others they were likely acquired by symbiont shift, suggesting different evolutionary pathways leading to mycoheterotrophy.

Cheaters are isolated by reciprocal specialization

We confirmed that mycoheterotrophic plants are more specialized toward few mycorrhizal fungal partners than autotrophic plants (Merckx *et al.*, 2012) and showed for the first time that their fungal partners are overall more specialized than fungi associated with autotrophic plants. This reciprocal specialization is not strict (with the exception of Lycopodiaceae, see below), since mycoheterotrophs and their fungal partners need some connection to autotrophic plants, yet sufficient to lower nestedness in the arbuscular mycorrhizal network. The observed trend toward reciprocal specialization and reduced nestedness suggests that mycoheterotrophic cheating is an unstable ecological and evolutionary strategy, which could explain the relatively recent origin of mycoheterotrophic clades (Fig. **2**). Indeed, reciprocal specialization confers high extinction risks for both interacting partners, which is one of the main hypotheses explaining why mutualistic networks tend to be nested, with asymmetrical specialization (i.e. specialists interact with generalist partners; Thébault & Fontaine, 2010). Whatever its origin, the reciprocal specialization of cheaters and their partners has also been suggested in other mutualisms (Genini *et al.*, 2010). A parasitic nature of entirely mycoheterotrophic plants has often been mooted (Bidartondo, 2005; Merckx, 2013), albeit without direct support, in the absence of data on fitness of fungal partners

and autotrophic plants providing carbon to mycoheterotrophs (van der Heijden *et al.*, 2015). Our analysis *a posteriori* supports the view of entirely mycoheterotrophic plants as parasitic cheaters. However, we cannot exclude the possibility that mycoheterotrophs might provide some advantages to their mycorrhizal fungi (e.g. shelter or vitamins; Brundrett, 2002; Selosse & Rousset, 2011), making them useful partners for some specific fungal species, despite their carbon cost. Further empirical evidence is needed to clarify this.

There are several not mutually exclusive explanations for this reciprocal mycoheterotrophic specialization. First, physiological constraints may act if conditional investment and partner choice occur in the mycorrhizal symbiosis (Kiers *et al.,* 2011), meaning that each partner would preferentially interact with the most mutualistic of the many partners they encounter in soil. Mycoheterotrophic cheaters might have been able to successfully avoid these constraints by specifically targeting a few specific fungi susceptible to mycoheterotrophy, with which they now interact in specialized parasitism (Selosse & Rousset, 2011). Regarding the fungi, we can speculate that 'cheated' fungi that provide mycoheterotrophs with carbon entail a greater carbon cost for autotrophic plants than other fungi, and that autotrophic plants therefore tend to avoid interactions with these fungi. This would result in a trend to reciprocal specialization, and the partial isolation of mycoheterotrophic cheaters and their fungal partners from the mutualistic network. Second, the pattern of reciprocal specialization could result from physiological traits of the fungal species, as yet unknown to us, which make them more likely to be avoided by autotrophic plants and to associate with mycoheterotrophic plants. Third, such a pattern of reciprocal specializations could also come from ecological constraints limiting the niches and habitats of mycoheterotrophic plants. Indeed, mycoheterotrophic plants often tend to occur specifically in patches of low soil fertility (Gomes *et al.*, 2019). It is important to acknowledge that although the global pattern of reciprocal specialization observed in the present work is likely to be linked to cheating, it might also be influenced by the specific local environmental conditions where cheating is promoted. For instance, because mycoheterotrophs primarily persist in these low fertility habitats where access to essential mineral nutrients for autotrophic plants is limiting, we can speculate that it might still be advantageous for autotrophic plants to interact with poorly cooperative fungal partners associated with mycoheterotrophs, which provide less

mineral nutrient in relation to their carbon cost. Additionally, low nutrient availability in the environments of mycoheterotrophs might also limit the available pool of mycorrhizal fungi: the relative specialization of mycoheterotrophic plants could be the consequence of low availability of fungal partners in these specific habitats. Yet, there is ample evidence that mycoheterotrophic species are specialized on one or few fungi in various environments from all over the world, where several to many suitable fungi should also be available. For instance, in a similar symbiosis, mycoheterotrophic orchids specialize on few saprotrophic fungi in tropical forests where many saprotrophic fungi occur (Martos *et al.*, 2009).

An in-depth sampling of mycorrhizal networks (particularly weighted networks) in various local communities containing mycoheterotrophs would be required to test whether reciprocal specialization occurs at the local scale and will shed more light on the mechanisms regulating the interaction. Indeed, we observed a trend to reciprocal specialization in a large-scale interaction network compiled from mycorrhizal interactions described in different ecosystems around the world, not in locally described physical mycelial networks. This allowed us to analyze a global ecological pattern, representing the complete evolutionary history of the partners, and is justified by the very low endemism of arbuscular mycorrhizal fungi and thus the absence of strong geographic structure (Davison *et al.*, 2015; Savary *et al.*, 2018). It is noteworthy that similar patterns of specialization were found in the African and South American networks (Supporting Information Fig. **S4**). On the other hand, a species may appear to be relatively more specialized in a global network than it actually is in local communities.

Our rarefaction analyses indicated that including more mycoheterotrophic species in this dataset should reveal more fungal species associated with mycoheterotrophs. Yet, given that our dataset covers almost all mycoheterotrophic families and that our results are robust to the sampling fraction of mycoheterotrophs and their associated fungi (Supporting Information Fig. **S2**), we expect the unsampled fungi associated with unsampled mycoheterotrophs to be phylogenetically related and specialists to the same degree as the sampled fungi associated with sampled mycoheterotrophs. A low sampling fraction of fungi associated with mycoheterotrophic plants is even expected given the trend of reciprocal specialization: as mycoheterotrophic species tend to be specialists

interacting with specialist fungi, we would need to sample most of the mycoheterotrophic species to obtain most of their specialist associated fungi.

In this study, we used a simple dichotomy of plants considered either as mutualistic autotrophs or as (either entirely or initially) mycoheterotrophic cheaters. However, mycoheterotrophy is not the only uncooperative strategy in this symbiosis: mycorrhizal interactions rather represent a continuum between mutualism and parasitism, both in terms of plants (Jacquemyn & Merckx, 2019) and fungi (Johnson *et al.*, 1997; Klironomos, 2003). Physiological constraints are thus thought to constitutively maintain the stability of the mycorrhizal symbiosis (Kiers *et al.*, 2003, 2011) against many forms of cheating, including the specific case of mycoheterotrophy. Moreover, we did not consider context dependency, which has a non-negligible impact on the functioning of mycorrhizal interactions (Chaudhary *et al.*, 2016). Although the mutualism-parasitism continuum or the context dependency could have hidden the observed patterns, the fact that we observed significant differences in the specialization between autotrophic and mycoheterotrophic plants and high similarities between sets of fungal partners associated with different mycoheterotrophic plant lineages suggests that the observed patterns are likely robust to our simplifications.

Independent emergences of entirely mycoheterotrophic cheating converge on closely related susceptible fungi

Mycoheterotrophic cheating emerged multiple times in different clades of the phylogeny of vascular land plants, indicating weak phylogenetic constraints. This likely results from the low specificity in arbuscular mycorrhizal symbiosis, which allows convergent interactions (Bittleston *et al.*, 2016) in different plant clades. Such convergences would have happened during the evolution of mycoheterotrophic plants with similar fungi susceptible to cheating. Thus, physiological or ecological constraints leading to reciprocal specialization appear to be the main barrier to the emergence of cheating in arbuscular mycorrhizal mutualism.

There are, however, phylogenetic constraints on the fungal side. We found few fungal clades that interacted with independent mycoheterotrophic plant lineages, and these clades were phylogenetically related, as already reported by Merckx *et al.* (2012);

accordingly, fungal partners associated with mycoheterotrophs seem to be less phylogenetically diverse than those associated with autotrophic plants. The physiological traits that underlie variation in susceptibility of fungi to mycoheterotrophy remain unclear (Chagnon et al. 2013; van der Heijden & Scheublin 2007) and obtaining more information on fungal functional traits would greatly improve our understanding of mycoheterotrophic systems, the habitat distribution of mycoheterotrophs and their associated fungi, and what make fungi susceptible to mycoheterotrophy or not. Studying the functional traits of susceptible fungi, which are exceptions to the widespread avoidance of non-cooperative partners (Selosse & Rousset, 2011), will be particularly useful for understanding how fungi avoid cheating.

The acquisition of susceptible fungi depends on the mycoheterotrophic plant lineage. In some mycoheterotrophic lineages, such as Burmanniaceae, fungal partners were closely related to the fungal partners of autotrophic relatives, suggesting that the fungi associated with mycoheterotrophs are derived from the fungal partners of cooperative autotrophic ancestors. In other mycoheterotrophic lineages, such as Gentianaceae or Corsiaceae, fungal partners were more closely related to fungal partners of other mycoheterotrophic lineages than to autotrophic relatives, suggesting that the fungi associated with mycoheterotrophs were acquired secondarily rather than derived from the partners of autotrophic ancestors. A few mycoheterotrophic plant lineages lacked closest autotrophic relatives in our analysis (e.g. mycoheterotrophic Gentianaceae should be compared to autotrophic Gentianaceae, not represented in the MaarjAM database), which may bias our analyses towards supporting secondary transfer from other mycoheterotrophic plants rather than acquisition from autotrophic ancestors. Still, similar fungi were found in mycoheterotrophic Burmanniaceae and their closest autotrophic relative after a 110-Myr-old divergence, while mycoheterotrophic Gentianaceae and their closest autotrophic relative have distinct fungal partners after a divergence of only 52 Myr.

Interestingly, all entirely mycoheterotrophic families are evolutionarily relatively recent: the oldest monocotyledonous entirely mycoheterotrophic families, such as Burmanniaceae and Triuridaceae, are only 110-130 Myr old, and the dicotyledonous entirely mycoheterotrophic families Gentianaceae and Polygalaceae are even more recent (around 50-60 Myr; Fig. **2**). The oldest mycoheterotrophic families show conservatism for

fungal partners, while the most recently evolved ones display secondary acquisition. We can speculate that mycoheterotrophy initially emerged in the monocotyledons thanks to suitable cheating-susceptible fungal partners; more recently evolved entirely mycoheterotrophic lineages (especially in dicotyledons) then convergently reutilized these fungal partners. Complementary analyses including more sampling of the mycoheterotrophic families and their closest autotrophic relatives would be needed to test this speculation.

Independent networks and parental nurture in initially mycoheterotrophs

Our results serendipitously revealed that two initially mycoheterotrophic families, Ophioglossaceae and Lycopodiaceae, seem to have exclusive mycorrhizal associations, as they interacted with fungi that did not interact with any other plant family. In these families, the fungi are present during both mycoheterotrophic underground spore germination and in the roots of adult autotrophic individuals (Winther & Friedman, 2007, 2008). Autotrophic adults likely act as the carbon source (Field *et al.*, 2015), part of which is dedicated to the offspring. This further supports the hypothesis by Leake *et al.* (2008) proposing parental nurture where germinating spores would be indirectly nourished by surrounding conspecific sporophytes. Parental nurture is not universal to all initially mycoheterotrophic families though; in the initially mycoheterotrophic Psilotaceae, for example, fungal partners are shared with surrounding autotrophic plants (Winther & Friedman, 2009). In initially mycoheterotrophic independent networks, the overall outcome for the fungus over the plant lifespan may actually be positive: fungi invest in mycoheterotrophic germinations that represent future carbon sources (Field *et al.*, 2015). In other words, initially mycoheterotrophic plants do not cheat their exclusive fungi, but postpone the reward. We note, however, that the existence of independent networks for these families should be confirmed in studies of local communities.

We found an extreme reciprocal specialization between Lycopodiaceae and a single *Glomus* clade. More studies are required to confirm that this pattern does not result from undersampling of the fungi interacting with these Lycopodiaceae species. Unlike other early-diverging plant clades that tend to interact with early-diverging fungal clades, the Lycopodiaceae (250-Myr-old) associate with a 49-Myr-old clade that diverged 78 Myr ago from all other *Glomus* (Rimington *et al.,* 2018). Thus, this highly specific interaction results

from a secondary acquisition: some species of Lycopodiaceae may have initially developed mycoheterotrophic interactions with a wider set of fungi, and later evolved into a specific mutualistic parental nurture with their exclusive fungi, raising the possibility of co-evolution between both clades.

Conclusions

Our analysis of mycoheterotrophy in arbuscular mycorrhizal symbiosis illustrates a globally mutualistic system where cheaters tend to be limited by reciprocal specialization. Such reciprocal specialization between mycoheterotrophic cheaters and their 'cheating-susceptible' partners, potentially due to partner choice, sanctions, and/or habitat restrictions, reduces nestedness in the network. Phylogenetic constraints occur on the fungal but not the plant side, as independently emerged mycoheterotrophic families convergently interact with closely related fungi. In addition, our results challenge the general cheater status of mycoheterotrophy, highlighting a dichotomy between true mycoheterotrophic cheaters and possibly cooperative, initially mycoheterotrophic systems with parental nurture. Beyond mycorrhizal symbiosis, we invite the use of our combination of network and phylogenetic approaches to evaluate the nature of constraints upon cheating in other multiple-partner mutualisms (e.g. pollination or seed dispersal).

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Author contributions

BPL, MAS, MÖ, HM, and FM designed the study. MÖ gathered the data, BPL performed the analyses and wrote the first draft of the manuscript, and all authors contributed substantially to the writing and revisions.

Data Availability Statement

All the data used in this work are available in the MaarjAM database (https://maarjam.botany.ut.ee; Öpik *et al.*, 2010).

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Tables

Table 1: Effect of the nature of the interaction (i.e. plant carbon nutrition modes) on indices of network structure and phylogenetic distributions.

(Categories are defined according to the plant carbon nutrition modes, i.e. AT: autotrophic; EMH: entirely mycoheterotrophic over development; and IMH: initially mycoheterotrophic in development).

The second column corresponds to *P*-values of Kruskal-Wallis tests for the overall network with or without (in brackets) the Lycopodiaceae.

The last three columns correspond to *P*-values of Whitney U tests (pairwise tests) for the overall network including the Lycopodiaceae.

P-values lower than 5% (significance level) are shown in bold.

Index	Kruskal-Wallis test	Whitney U tests		
		AT vs. EMH	AT vs. IMH	IMH vs. EMH
Plant degree (*k*)	**1.2e-19 (1.4e-17)**	**5.3e-16**	**4.2e-7**	0.97
Fungal partner specialization (*Psp*)	**5.6e-11 (1.3e-8)**	**1.1e-4**	**4.5e-9**	0.054
Mean phylogenetic pairwise distance of fungal partners (*MPD*)	**1.2e-4 (2.0e-3)**	**8.0e-4**	**6.8e-3**	0.11

Table 2: Fungal sharing between nine entirely (EMH) or initially (IMH) mycoheterotrophic plant families.

Number (lower part of the matrix) and percentage (upper part) of fungi shared between family pairs. The last two columns represent *(i)* the total number of fungi shared with other entirely or initially mycoheterotrophic families, and *(ii)* the number of fungi exclusive to this family (i.e. not shared with any other mycoheterotrophic or autotrophic family). The second column indicates the most closely related autotrophic sister clade of each family; it can be one family, a higher clade, the family itself if autotrophic species were compiled in the MaarjAM database (e.g. Polygalaceae), or none in the case of Petrosaviaceae (which forms a too divergent distinct branch).

Boxes are shaded according to the number of shared fungi (white: no shared fungus, black: many shared fungi).

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		Most closely related autotrophic sister-clade in our dataset (and divergence time in million years)	Number of plant species	Number of fungal partners	Burmanniaceae	Corsiaceae	Gentianaceae	Petrosaviaceae	Polygalaceae	Triuridaceae	Lycopodiaceae	Ophioglossaceae	Psilotaceae	Total number of shared fungi	Total number of exclusive fungi
EMH	**Burmanniaceae**	Dioscoreaceae **(110 Myr)**	25	38		0%	13%	3%	8%	16%	2%	6%	0%	16	0
	Corsiaceae	Melanthiaceae Liliaceae Smilacaceae **(129 Myr)**	1	1	0		0%	0%	0%	0%	0%	0%	0%	0	0
	Gentianaceae	Apocynaceae **(52 Myr)**	6	8	5	0		0%	9%	8%	0%	0%	0%	6	0
	Petrosaviaceae		1	2	1	0	0		0%	5%	0%	0%	0%	1	0
	Polygalaceae	Polygalaceae **(60 Myr)**	4	4	3	0	1	0		10%	0%	0%	0%	3	1
	Triuridaceae	Dioscoreaceae **(131 Myr)**	4	19	8	0	2	1	2		8%	3%	0%	11	1
IMH	**Lycopodiaceae**	Selaginellaceae **(303 Myr)**	8	7	1	0	0	0	0	2		0%	0%	3	3
	Ophioglossaceae	Aspleniaceae Dryopteridaceae Gleicheniaceae Lygodiaceae Osmundaceae Pteridaceae **(330 Myr)**	5	15	3	0	0	0	0	1	0		6%	4	2
	Psilotaceae		2	2	0	0	0	0	0	0	0	1		1	0

895

Figure legends

Figure 1: Conceptual framework used in this study to evaluate the constraints upon the emergence of mycoheterotrophic cheater plants in arbuscular mycorrhizal symbiosis.

(a) Strong phylogenetic constraints (PC) should affect the phylogenetic distributions of mycoheterotrophic cheater plants and/or their fungal partners; whereas *(b)* functional constraints (FC; e.g. physiological or ecological constraints) should affect the network structure i.e. level of specialization of mycoheterotrophic cheater plants and/or their partners. Therefore, by investigating specialization and phylogenetic clustering of mycoheterotrophic cheaters and of their fungal partners, we evaluated functional and phylogenetic constraints. This can be done by using and interpreting bipartite network tools (a – e.g. computation of nestedness, measures of partner degree, and partner specialization) or phylogenetic tools (b – e.g. measure of phylogenetic dispersion), respectively.

Interpreting the observed patterns of phylogenetic clustering and network structure directly indicates the strength of the constraints. For instance, strong phylogenetic clustering of the cheaters and their partners (i-iii) suggests that the emergence of cheaters and their susceptible partners is rare and limited, whereas low phylogenetic clustering (ii-iv) suggests that cheating evolved multiple times. Regarding functional constraints, generalist cheaters (vi) might indicate that their partners do not have any mechanisms preventing uncooperative interactions (low constraints). Conversely, specialist cheaters (v) might indicate that cheaters cannot interact with most partners (high constraints). Moreover, if the partners of cheaters are generalists (viii - low constraints), asymmetrical specialization ensures that cheaters are well connected in the interaction network (high

923 nestedness), whereas if they are specialists (vii - high constraints), reciprocal
924 specialization on both sides drives the isolation of mycoheterotrophic plants into
925 modules, thus decreasing nestedness.
926 Mutualistic species are represented in green and their partners are in orange, whereas
927 cheaters and their partners are represented in red. Mutualistic interactions are thus
928 represented in green, whereas antagonistic interactions (cheating) are in red.
929 The patterns and interpretations from the present study on mycoheterotrophic cheaters
930 are shown in the orange frames.

Figure 2: Phylogenetic distribution of mycoheterotrophy in global arbuscular mycorrhizal mutualism.

(Categories are defined according to the plant carbon nutrition modes, i.e. AT: autotrophic; EMH: entirely mycoheterotrophic throughout the life cycle of the individual plant; and IMH: initially mycoheterotrophic in the life cycle).

Phylogenetic trees of 390 plants (left side) and 351 fungi (right side) forming 26,350 interactions (links) in the MaarjAM database. Links are colored according to the autotrophic (green), entirely mycoheterotrophic (red), or initially mycoheterotrophic (orange) nature of the plant. Major plant and fungal clades are named. Mycoheterotrophy encompasses 41 entirely mycoheterotrophic species in 6 monophyletic families [Burmanniaceae (25 spp.), Gentianaceae (6 spp.), Triuridaceae (4 spp.), Polygalaceae (4 spp.), Corsiaceae (1 sp.), and Petrosaviaceae (1 sp.)], and 15 initially mycoheterotrophic species in 3 families [Ophioglossaceae (ferns; 5 spp.), Psilotaceae (ferns; 2 spp.), and Lycopodiaceae (clubmoss; 8 spp.)].

Scales of the phylogenetic trees are in million years (Myr).

946 **Figure 3:** Effect of the nature of the interaction on specialization (*k* and *Psp*), the partner's
947 mean phylogenetic distance (*MPD*), and partner fidelity (*Fx* - Supporting Information
948 Methods S2 - *List of abbreviations*):
949 (Categories are defined according to the plant carbon nutrition modes, i.e. AT:
950 autotrophic; EMH: entirely mycoheterotrophic over development; and IMH: initially
951 mycoheterotrophic in development).
952 **(a):** Plant degree (*k*) against fungal partner specialization (*Psp*) (i.e. the average degree of
953 fungal partners); dots in the bottom left corner indicate reciprocal specialization. For each
954 axis, boxplots represent the one-dimensional projection of *k* and *Psp*.
955 **(b):** Mean phylogenetic pairwise distance (*MPD*) of the sets of fungal partners. Boxplots
956 present the median surrounded by the first and third quartiles, and whiskers extend to
957 the extreme values but no further than 1.5 of the inter-quartile range.
958 **(c):** Fidelity (*Fx*) toward fungal partners in relation to the age of the plant clade. Clades
959 are defined according to their main carbon nutrition mode of their plants (over 50%). The
960 yellow dots departing from other mycoheterotrophic clades (high *Fx* values) correspond
961 to clades of Lycopodiaceae.
962 **(d):** Independent network between the clubmoss family Lycopodiaceae (rows) and their
963 three arbuscular mycorrhizal fungi (columns), with their respective phylogenetic
964 relationships.

Figure 4: Dissimilarities between sets of fungal partners associated according to the nature of the interaction.

(a): Principal coordinates analysis (PCoA) from UniFrac dissimilarities of sets of fungal partners. Every dot corresponds to a plant species and is colored according to its autotrophic (green), entirely mycoheterotrophic (red), or initially mycoheterotrophic (orange) nature. Only the first two principal axes explaining, respectively, 15.6% and 3.8% of the variation were kept.

(b): Dissimilarities between sets of fungal partners associated with different mycoheterotrophic plant families. For each mycoheterotrophic family, UniFrac dissimilarities of sets of fungal partners are calculated between one particular mycoheterotrophic species belonging to the focal mycoheterotrophic family and another plant species (from the same family, from the closest related autotrophic family, from other mycoheterotrophic families, or from other autotrophic plant families). All the groups cannot be calculated for every mycoheterotrophic family, due to the low number of species within families Corsiaceae and Psilotaceae.

Lowercase letters above each panel represent significant differences between categories (Mann-Whitney U tests). Boxplots present the median surrounded by the first and third quartiles, and whiskers extend to the extreme values but no further than 1.5 of the inter-quartile range.

Supporting Information

Supplementary methods:

Methods S1: Phylogenetic reconstructions

Methods S2: List of abbreviations

Supporting Information Tables:

Table S1: Data selection from the MaarjAM database: description of the filters used.

Table S2: Number and percentage of mycoheterotrophic plants and their associated fungi.

Table S3: Effect of mycoheterotrophy on the network nestedness.

Table S4: Independent sub-networks (modules) in the overall arbuscular mycorrhizal network and their respective numbers of partners.

Table S5: Null model to assess the significance of the effect of the nature of the interaction on indices of network structure and phylogenetic distributions.

Table S6: Effect of the nature of the interaction (i.e. plant carbon nutrition modes) on indices of the network structure in South America and Africa.

Table S7: Measure of the phylogenetic distributions of mycoheterotrophy: measure of NRI (net relatedness index) and NTI (nearest taxon index).

Table S8: Pairwise comparisons of UniFrac dissimilarities between sets of fungal partners associated with different plant families.

Supporting Information Figures:

Fig. S1: The global geographic distribution of sampling sites used in our analysis.

Fig. S2: Analysis on a smaller network (accessed in the MaarjAM database in October 2017)

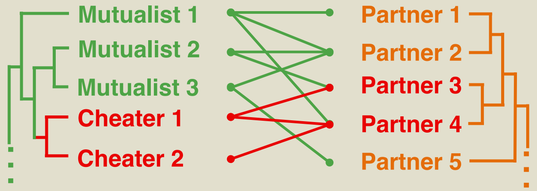
Fig. S3: Rarefaction curves representing the number of fungal taxa as a function of the sampling fraction.

Fig. S4: Effect of the nature of the interaction on specializations in the South American network and the African network.

Fig. S5: Fidelity of plant partners (*Fx*) according to the age of the fungal clade.

Fig. S6: Calibrated phylogenetic tree of the 490 plant species.

Fig. S7: Calibrated phylogenetic tree of the 351 arbuscular mycorrhizal fungi.



Constraints upon cheaters emergence

Type of constraints

(a) Phylogenetic constraints (PC)

(b) Functional constraints (FC)

Approach to characterize constraints

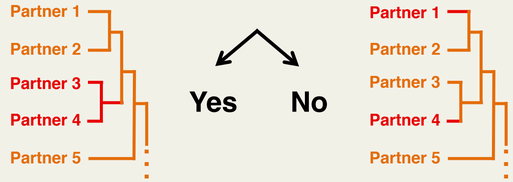
Phylogenetic tools

Bipartite network's tools

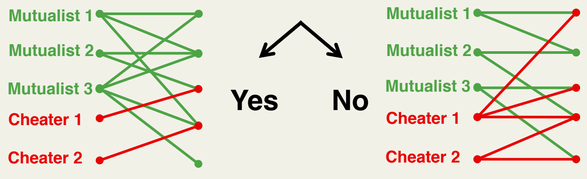
Possible patterns

Clustering of cheaters

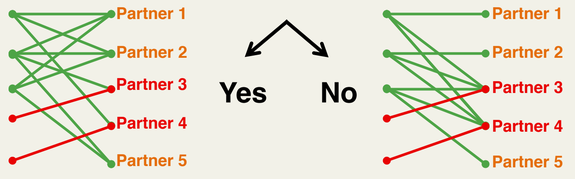
Clustering of the partners of cheaters



Specialization of cheaters



Specialization of the partners of cheaters



Patterns interpretation for cheaters:

Strong PC
The emergence of cheaters is phylogenetically limited.

Low PC
The emergence of cheaters evolved independently multiple times.

Strong FC
Cheaters are specialists toward few partners: they are isolated in the mutualistic network.

Low FC
Cheaters interact with numerous partners: they are well-connected in the mutualistic network.

for partners of cheaters:

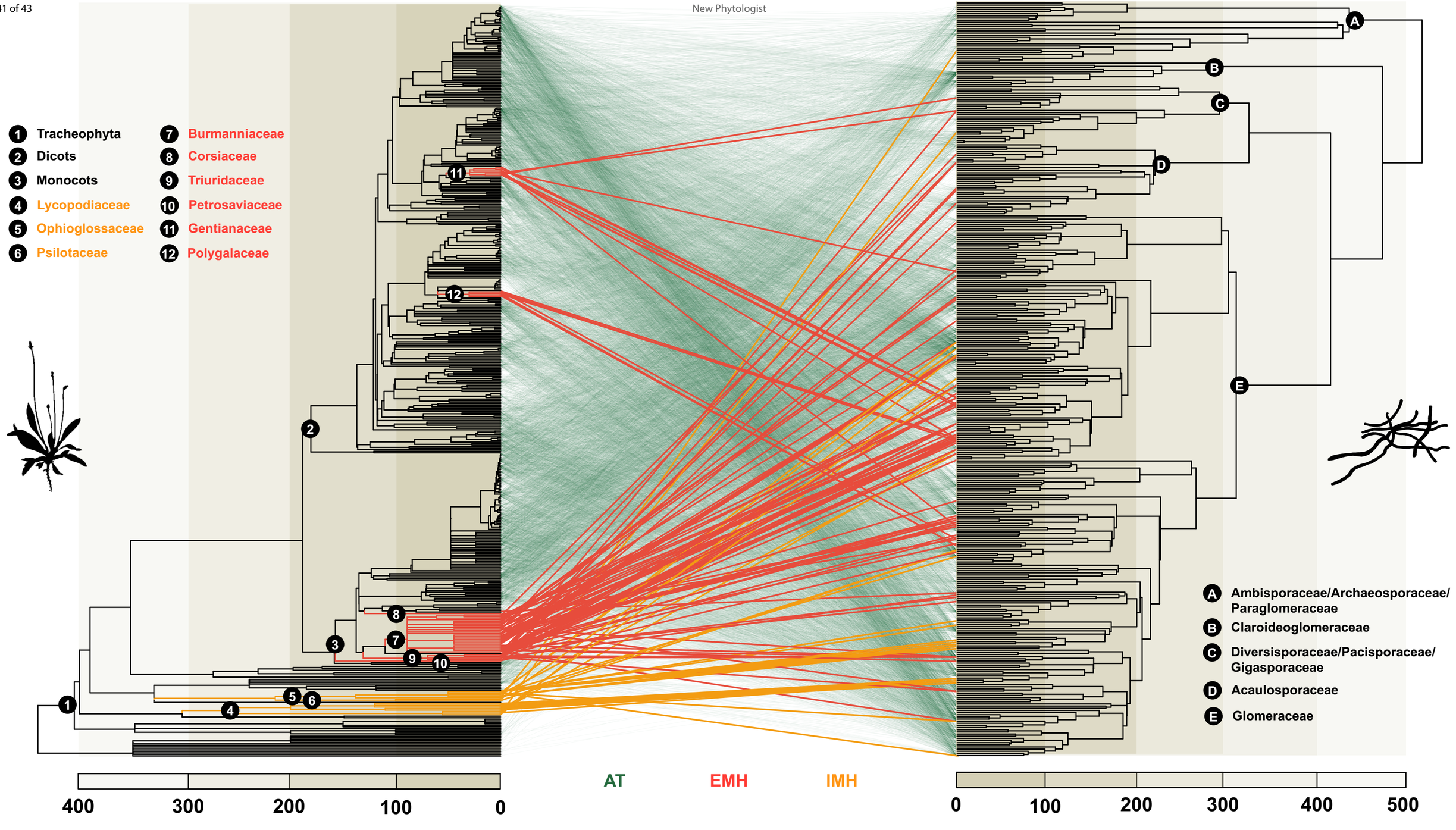
Strong PC
The emergence of "cheater-susceptible" partners is phylogenetically limited.

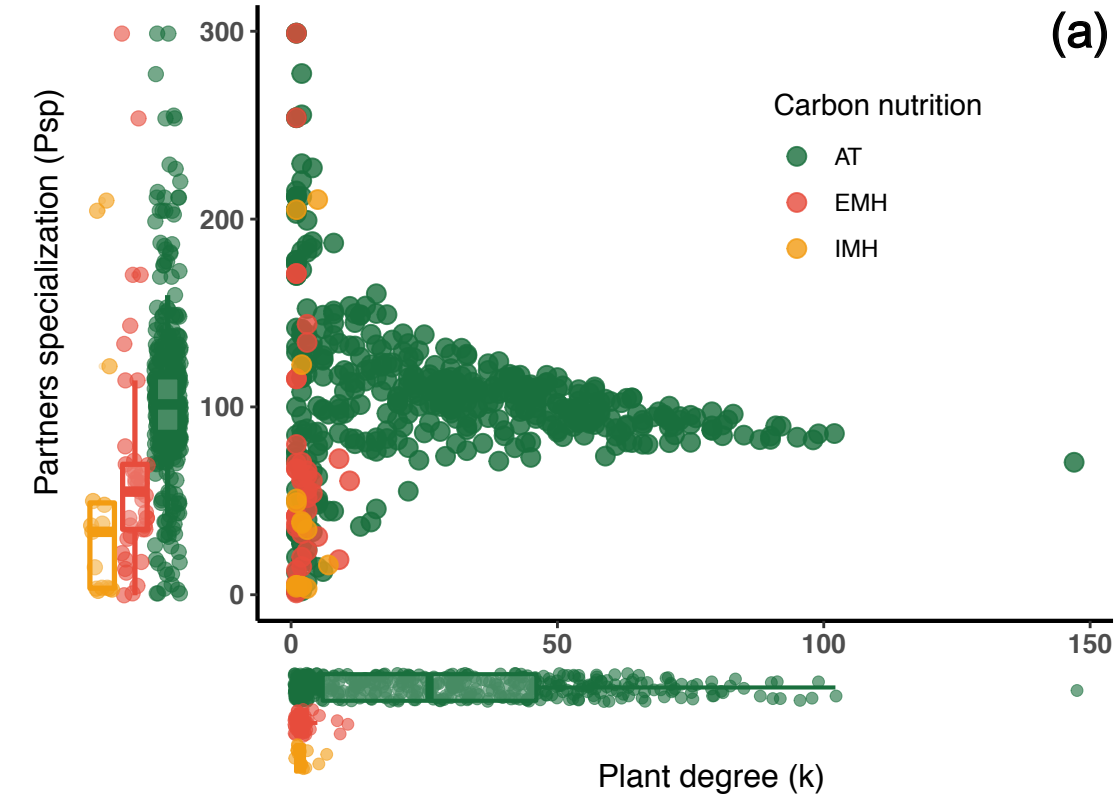
Low PC
The emergence of "cheater-susceptible" partners evolved independently multiple times.

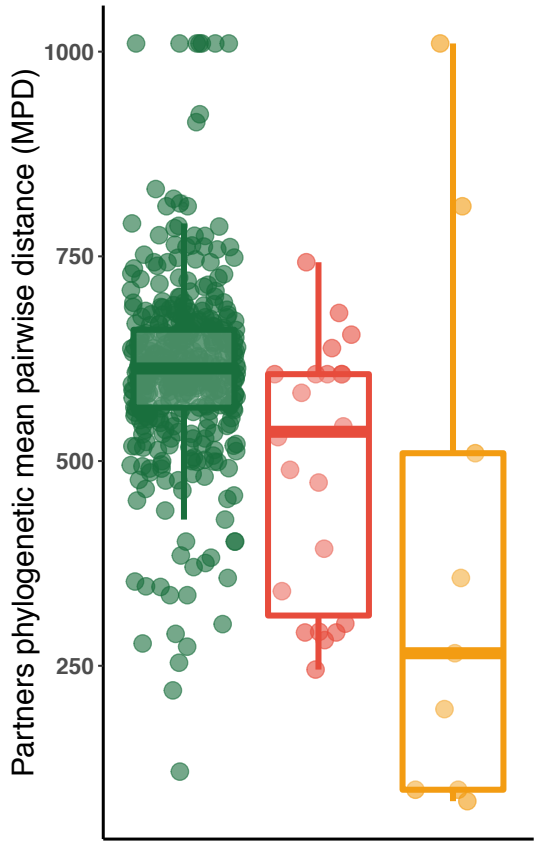
Strong FC
Cheaters interact with specialist partners, but less with the rest of the mutualistic network.

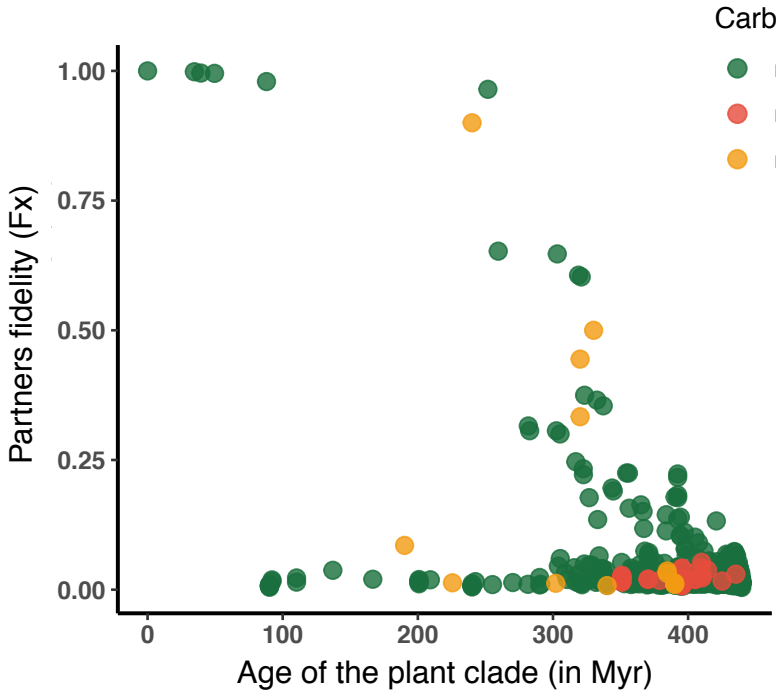
Low FC
Cheaters interact with non-specialist partners: they are well-connected in the mutualistic network.

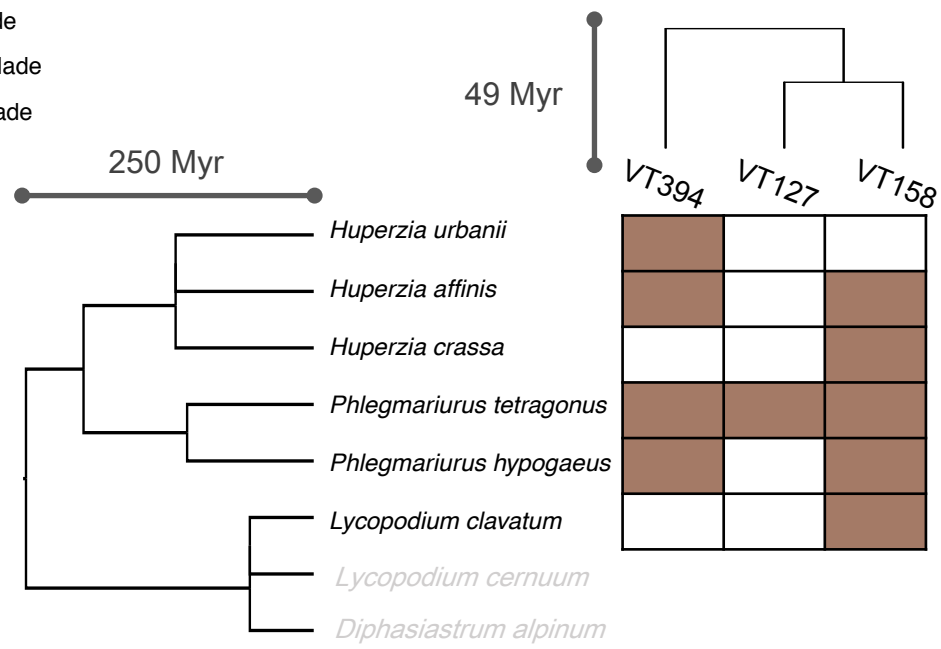
(i) **(ii)** **(iii)** **(iv)** **(v)** **(vi)** **(vii)** **(viii)**

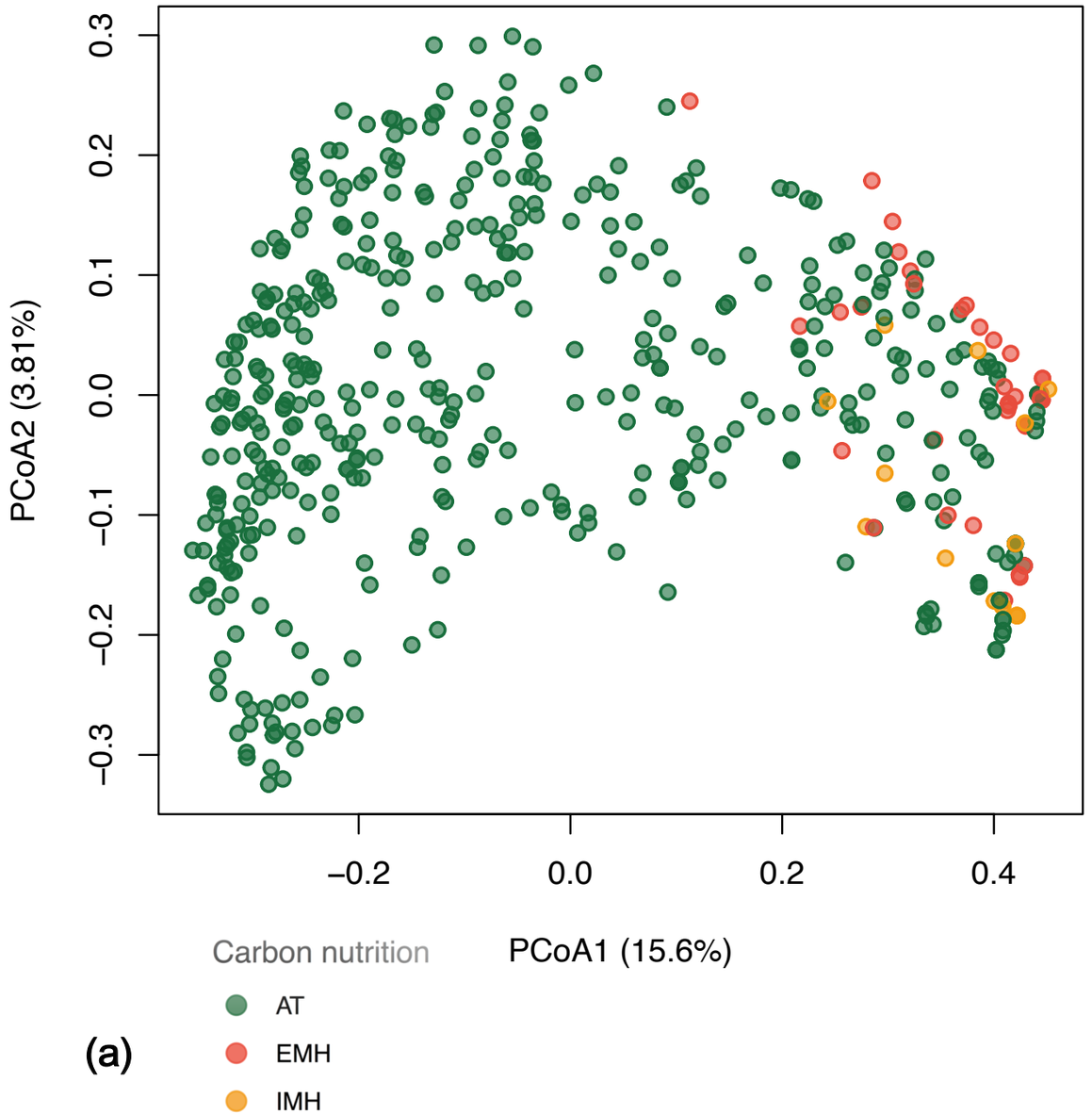

1 Tracheophyta
2 Dicots
3 Monocots
4 Lycopodiaceae
5 Ophioglossaceae
6 Psilotaceae
7 Burmanniaceae
8 Corsiaceae
9 Triuridaceae
10 Petrosaviaceae
11 Gentianaceae
12 Polygalaceae
A Ambisporaceae/Archaeosporaceae/ Paraglomeraceae
B Claroideoglomeraceae
C Diversisporaceae/Pacisporaceae/ Gigasporaceae
D Acaulosporaceae
E Glomeraceae
AT
EMH
IMH
400
300
200
100
0
0
100
200
300
400
500

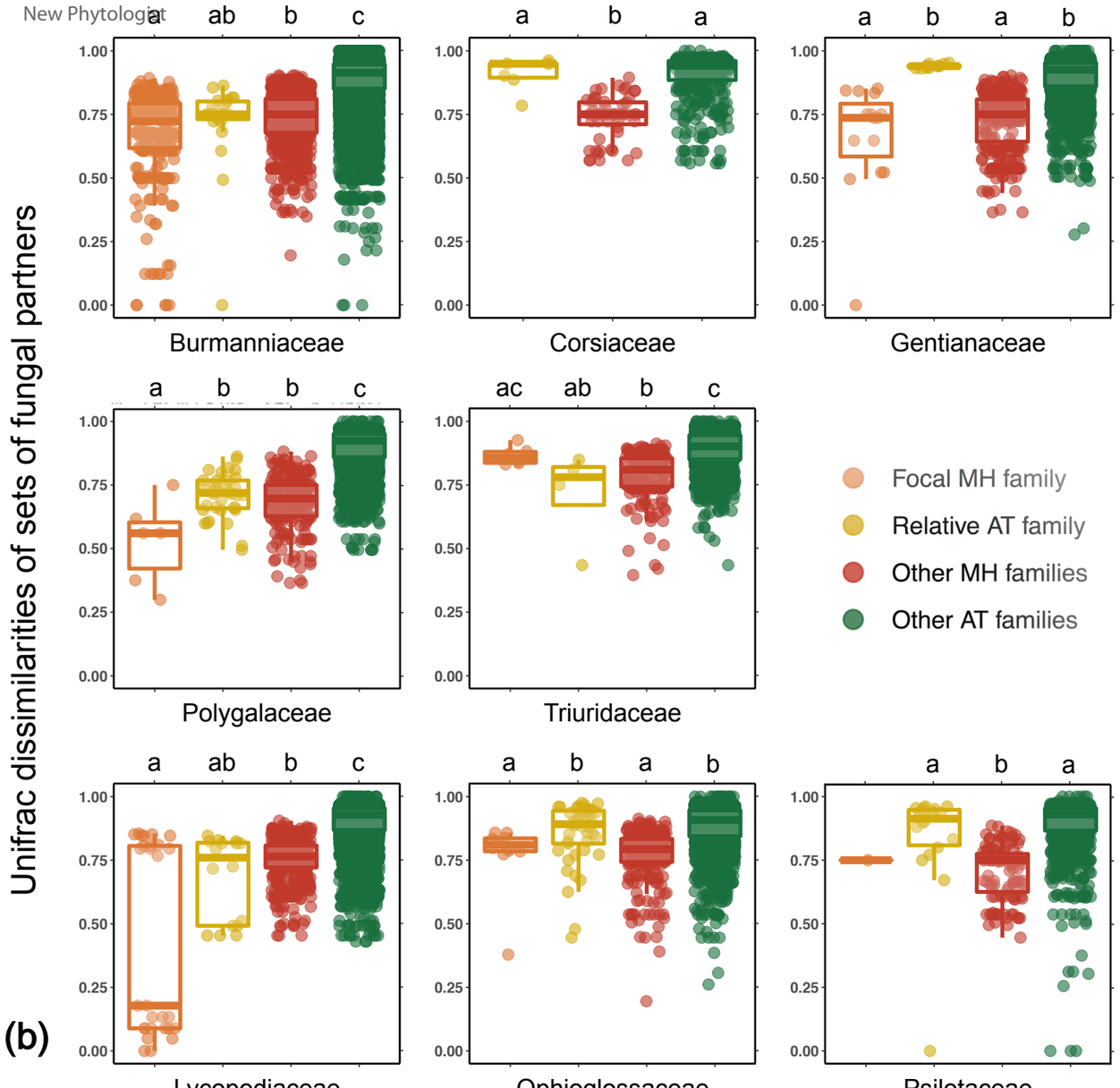

(a)
Partners specialization (Psp)
300
200
100
0
0
50
100
150
Plant degree (k)
Carbon nutrition
AT
EMH
IMH


(b)
Partners phylogenetic mean pairwise distance (MPD)
1000
750
500
250


Carbon nutrition
mainly AT clade
mainly EMH clade
mainly IMH clade
(c)
Partners fidelity (Fx)
1.00
0.75
0.50
0.25
0.00
0
100
200
300
400
Age of the plant clade (in Myr)


(d)
49 Myr
250 Myr
VT394
VT127
VT158
Huperzia urbanii
Huperzia affinis
Huperzia crassa
Phlegmariurus tetragonus
Phlegmariurus hypogaeus
Lycopodium clavatum
Lycopodium cernuum
Diphasiastrum alpinum


PCoA2 (3.81%)
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
0.0
0.2
0.4
PCoA1 (15.6%)
Carbon nutrition
AT
EMH
IMH
(a)


Unifrac dissimilarities of sets of fungal partners
a
ab
b
c
Burmanniaceae
a
b
a
Corsiaceae
a
b
a
b
Gentianaceae
a
b
b
c
Polygalaceae
ac
ab
b
c
Triuridaceae
Focal MH family
Relative AT family
Other MH families
Other AT families
a
ab
b
c
Lycopodiaceae
a
b
a
b
Ophioglossaceae
a
b
a
Psilotaceae
1.00
0.75
0.50
0.25
0.00
(b)