New Phytologist

Research

Cheating in arbuscular mycorrhizal mutualism: a network and phylogenetic analysis of mycoheterotrophy

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Received: *2 September 2019*
Accepted: *20 January 2020*

New Phytologist (2020)
doi: 10.1111/nph.16474

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

Summary

- Although 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 vs noncheating autotrophic plants and their respective fungi, in a global arbuscular mycorrhizal network with> 25 000 interactions.
- We show that mycoheterotrophy evolved repeatedly 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 vs autotrophic plants provides a network and phylogenetic framework to assess the presence of constraints upon cheating emergences in mutualisms.

Introduction

Mutualistic interactions are ubiquitous in nature and largely help to generate and maintain biodiversity (Bronstein, 2015). Because 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 used extensively 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 asymmetrical specialization with partners that are themselves generalists?), and modularity (existence of distinct subnetworks; Bascompte & Jordano, 2013). 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 (Thébault & 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.* (2019) 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 (vs 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 ≥ 407 Myr old (Strullu-Derrien *et al.*, 2018) and concerns *c.* 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 (C) 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), because multiple fungi colonize most plants, whereas fungi are usually shared among surrounding plant species (Verbruggen *et al.*, 2012). Thus, fungi

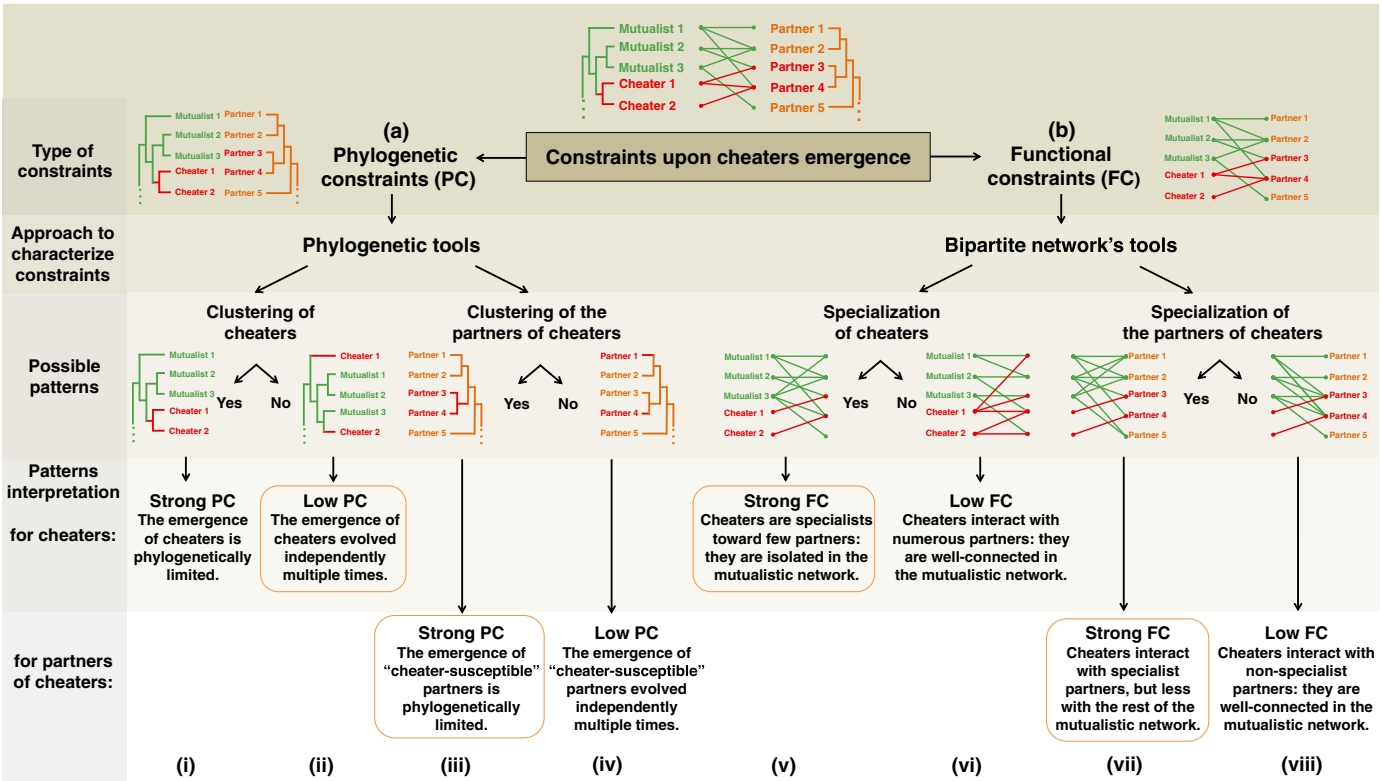

Fig. 1 Conceptual framework used in this study to evaluate the constraints upon the emergence of mycoheterotrophic cheater plants in arbuscular mycorrhizal symbiosis. (a) Strong phylogenetic constraints (PC) should affect the phylogenetic distributions of mycoheterotrophic cheater plants and/or their fungal partners. However, (b) functional constraints (FC; e.g. physiological or ecological constraints) should affect the network structure that is 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 weak 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 nestedness), whereas if they are specialists (vii – high constraints), reciprocal specialization on both sides drives the isolation of mycoheterotrophic plants into modules, thus decreasing nestedness. Mutualistic species are represented in green and their partners are in orange, whereas cheaters and their partners are represented in red. Mutualistic interactions are thus represented in green, whereas antagonistic interactions (cheating) are in red. The patterns and interpretations from the present study on mycoheterotrophic cheaters are shown in the orange frames.

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 C 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, whereas 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 C (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 C and are mostly cooperative, whereas mycoheterotrophs never supply photosynthetic C 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 C via surrounding autotrophic plants, therefore increasing nestedness (Fig. 1b-v,viii). On the other, if autotrophic plants are able to avoid costly interactions with fungi associated with mycoheterotrophs (physiological constraints), or if mycoheterotrophs are tolerated only in particular habitats (ecological constraints), we expect a reciprocal specialization between mycoheterotrophs and their fungi and thus an increase of modularity and a decrease of nestedness (Fig. 1b-v,vii). Establishment of an extreme reciprocal specialization between entirely mycoheterotrophs and fungi exclusively associated with such plants seems unlikely though, because an autotrophic C source is required.

With regards to phylogenetic constraints on mycoheterotrophy, we already know that mycoheterotrophic strategies evolved multiple times (Merckx, 2013), generating monophyletic groups of mycoheterotrophic plants, which suggests weak phylogenetic constraints on the emergence of mycoheterotrophy in plants. However, the fungi interacting with independent mycoheterotrophic lineages might be phylogenetically closely related (Merckx *et al.*, 2012), which would indicate phylogenetic constraints on fungi (Fig. 1a-iii). The presence of such phylogenetic constraints has yet to be confirmed in a large phylogenetic context including the fungi of autotrophic plants. Moreover, if as we expect, only a set of phylogenetically close fungi interact with all mycoheterotrophic plant lineages, an important follow-up question is whether these fungi were acquired independently by autotrophic ancestors, or whether they were acquired by symbiont shift 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.*, 2019). 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): (1) amplification of the 18S rRNA gene; (2) fungus identified from plant roots (i.e. excluding soil samples); (3) interaction in a natural ecosystem (i.e. excluding anthropogenic or highly disturbed ecosystems); (4) host plant identified at the species level; and (5) a virtual taxon (VT) assignation available in MaarjAM. The VTs are a classification (= species proxy) of arbuscular mycorrhizal fungi designed by applying a $\geq 97\%$ 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 world-wide ecosystems (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 (in R/VEGAN, based on Chao index; Oksanen *et al.*, 2016). 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 (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, Methods S1). We obtained the phylogenetic relationships between the 490 host plants by pruning the time-calibrated supertree from Zanne *et al.* (2014) using PHYLOMATIC (http://phylodiversity.net/phylomatic/). We also used the Open Tree of Life website (http://opentreeoflife.org) and the R/ROTL package (Michonneau *et al.*, 2016; R Core Team, 2017) for grafting of 41 plant taxa missing from the pruned supertree (as polytomies at the lowest taxonomy level possible; Methods S1). We set tree root calibrations at 505 Myr ago (Ma) for the fungi (Davison *et al.*, 2015) and 440 Ma 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 (C) nutrition mode thanks to an online database (http://mhp.myspecies.info/) and individual publications (Boullard, 1979; Winther & Friedman, 2008; Field *et al.*, 2015): autotrophic ($n = 434$, 88.6%), entirely mycoheterotrophic ($n = 41$, 8.4%), or initially mycoheterotrophic ($n = 15$, 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 mycoheterotrophic plant ($n = 54$, 15.4%), 'associated with initially mycoheterotrophs' if the fungus interacts with at least one initially mycoheterotrophic plant ($n = 23$, 6.6%), or 'associated with mycoheterotroph' if the fungus interacts with at least one entirely or initially mycoheterotrophic plants ($n = 71$, 20.2%; Table S2). Only five fungi are associated with both entirely and initially mycoheterotrophic plants. Our dataset included mycoheterotrophs from 18 publications. Although only 41 entirely mycoheterotrophic species were included out of 267 described

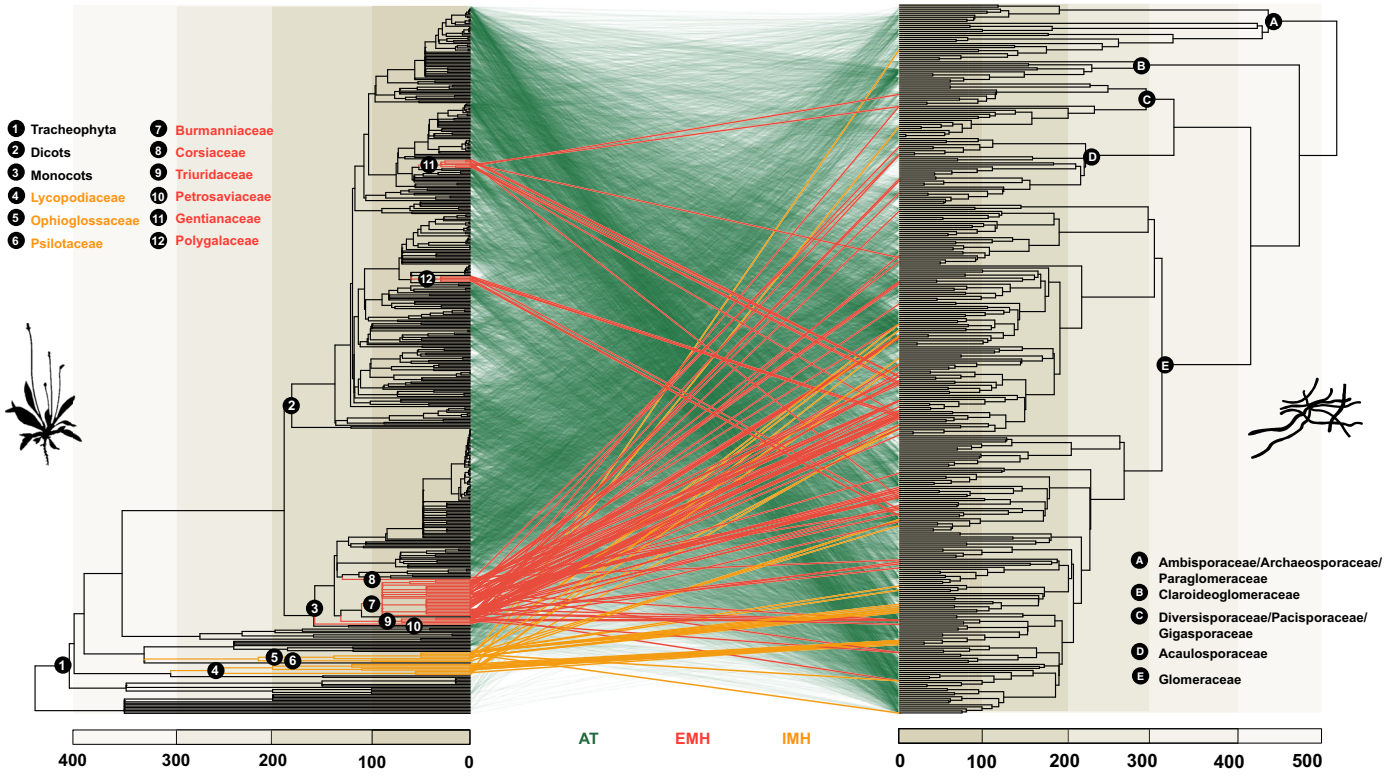

Fig. 2 Phylogenetic distribution of mycoheterotrophy in global arbuscular mycorrhizal mutualism. (Categories are defined according to the plant carbon nutrition modes: AT, autotrophic; EMH, entirely mycoheterotrophic throughout the life cycle of the individual plant; and IMH, initially mycoheterotrophic 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 six monophyletic families (Burmanniaceae (25 spp.), Gentianaceae (six spp.), Triuridaceae (four spp.), Polygalaceae (four spp.), Corsiaceae (one sp.), and Petrosaviaceae (one sp.)), and 15 initially mycoheterotrophic species in three families (Ophioglossaceae (ferns; five spp.), Psilotaceae (ferns; two spp.), and Lycopodiaceae (clubmoss; eight spp.)). Scales of the phylogenetic trees are in Myr.

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). Likewise, 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: the overall network (490 plants, 351 fungi and 26 350 interactions), the network restricted to autotrophic plants (434, 344 and 26 087), and the network restricted to entirely and initially mycoheterotrophic plants (56, 71 and 263), using the function 'NODF2' in R/BIPARTITE (Dormann *et al.*, 2008)(NODF, nestedness metric based on overlap and decreasing fill). We tested the significance of NODF values (Methods S2 – List of abbreviations) by using two types of null models ($N = 100$ for each type): the first model (*'r2dtable'* from R/STATS– *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 R/BIPARTITE– *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 null-models values divided by their standard deviation (*Z*-scores > 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 ($N = 1000$) using the function 'permatfull' in R/VEGAN *(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. > 50% exclusive partners). We used 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 R/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, that is 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 the Results section).

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, that is if mycoheterotrophic plants and their fungal partners were more or less phylogenetically related than expected by chance (patterns of clustering vs overdispersion). We computed the net relatedness index (NRI) and the nearest taxon index (NTI) using R/PICANTE (Kembel *et al.*, 2010). Whereas NRI quantifies the phylogenetic structure of a species set based on the mean pairwise distances, 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 on the plant phylogeny to evaluate the phylogenetic structure of entirely mycoheterotrophic and initially mycoheterotrophic plant distribution, and 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). Likewise, 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 (1) every pair of plant species of this family, (2) every pair comprising one plant of this family and one plant of the most closely related autotrophic family (see later Table 2), (3) every pair composed of one plant of this family and one plant belonging to other mycoheterotrophic families, and (4) 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/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 ($94\% \pm 2\%$; Fig. S3). Concerning mycoheterotrophic species, we estimated a total of 117 ± 19 fungi associated with all mycoheterotrophs, 110 ± 27 fungi associated with entirely mycoheterotrophic plants, and 54 ± 24 fungi associated with initially mycoheterotrophic plants. Our dataset thus encompassed sampling fractions of $60\% \pm 10\%$ for fungi associated with mycoheterotrophs, $49\% \pm 10\%$ for fungi associated entirely with mycoheterotrophs, and $40\% \pm 28\%$ for fungi associated initially with mycoheterotrophs. Although our dataset did not include all of the fungi associated with mycoheterotrophic species, the following results were not sensitive to the sampling fractions of mycoheterotrophs and their fungal partners (Fig. S2).

Network nestedness, modularity and specialization of mycoheterotrophs

The overall network had a significant positive nestedness value ($Z = 9.2$, $P = 1.10^{-20}$, Table S3). Nestedness increased when only autotrophic plants were considered ($Z = 16.6$, $P = 8.10^{-62}$), whereas it was not significant in the network of only mycoheterotrophs ($Z = 1.44$, $P = 0.075$): 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 intermodule than intramodule interactions), suggesting that the overall structure was not modular, but detected few significant small independent modules (Table S4). In addition to a main module encompassing most species (481 of 490 plants and 346 of 351 fungi), we found three small independent modules: six initially mycoheterotrophic Lycopodiaceae plants and three exclusive fungi (*Glomus* VT127, VT158, VT394); two autotrophic plants from salt marshes (*Salicornia europaea* and *Limonium vulgare*) with one *Glomus* (VT296); and 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-fold fewer fungi (Kruskal–Wallis $H = 87.2$; $P = 1.2.10^{-19}$; Fig. 3a; Table 1). The *Psp* values 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 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 (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 *Fx* values 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 (Fig. S5). Thus, not only did these six 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 Ma from the other *Glomus* fungi.

Phylogenetic distribution of cheating

The partners' mean phylogenetic pairwise distance (*MPD*) indicated that fungi associated with entirely or initially mycoheterotrophic plants (or even with all mycoheterotrophs) were phylogenetically more closely related than fungi associated with

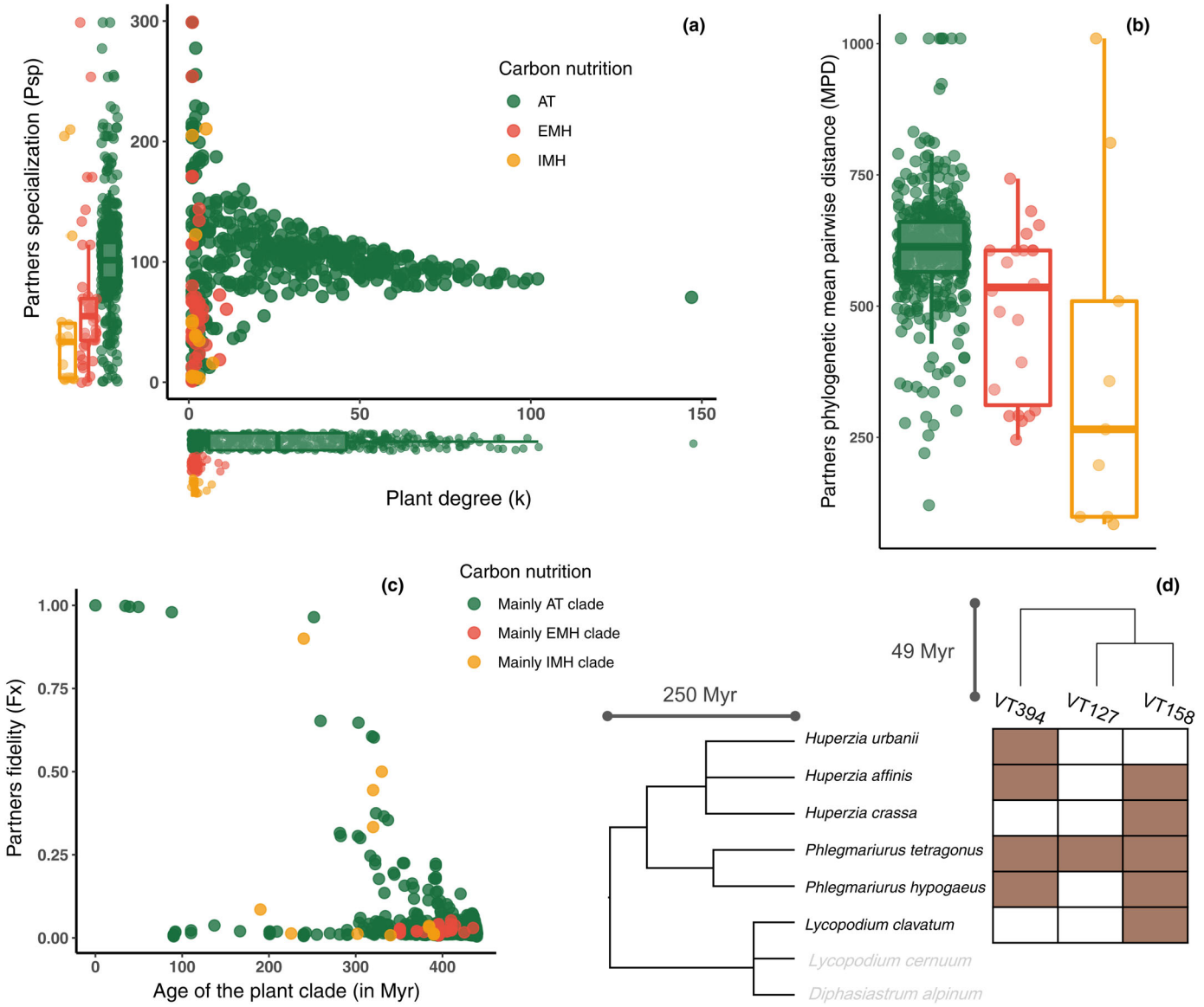

Fig. 3 Effect of the nature of the interaction on specialization (plant degree *k* and fungal partner specialization *Psp*), the partner's mean phylogenetic distance (*MPD*) and partner fidelity (*Fx* – Supporting Information Methods S2):(Categories are defined according to the plant carbon nutrition modes: AT, autotrophic; EMH, entirely mycoheterotrophic over development; and IMH, initially mycoheterotrophic in development). (a) *k* against *Psp* (i.e. the average degree of fungal partners); dots in the bottom left corner indicate reciprocal specialization. For each axis, boxplots represent the 1D projection of *k* and *Psp*. (b) Mean phylogenetic pairwise distance (*MPD*) of the sets of fungal partners. 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. (c) Fidelity (*Fx*) toward fungal partners in relation to the age of the plant clade. Clades are defined according to their main carbon nutrition mode of their plants (> 50%). The yellow dots departing from other mycoheterotrophic clades (high *Fx* values) correspond to clades of Lycopodiaceae. (d) Independent network between the clubmoss family Lycopodiaceae (rows) and their three arbuscular mycorrhizal fungi (columns), with their respective phylogenetic relationships.

autotrophs (Kruskal–Wallis $H = 18.0$; $P = 1.2.10^{-4}$; Table 1; Fig. 3b). NRI and NTI values (Table S7) also confirmed significant clustering on the fungal phylogeny on fungi associated with mycoheterotrophs, entirely mycoheterotrophic, or initially mycoheterotrophic plants; 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 mycoheterotrophic plants were significantly clustered, mainly because they all were angiosperms and mostly monocotyledons, but this did not apply to mycoheterotrophs in general, nor to initially mycoheterotrophic plants (Table S7). These phylogenetic clusters were visually apparent on fungal and plant phylogenetic trees (Figs 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 mycoheterotrophic plants (Table 2). Although the initially mycoheterotrophic Lycopodiaceae family formed an

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

		Mann–Whitney *U*-tests		
Index	Kruskal–Wallis test	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

(Categories are defined according to the plant carbon nutrition modes: 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 Mann–Whitney *U*-tests (pairwise tests) for the overall network including the Lycopodiaceae. *P*-values lower than 5% (significance level) are shown in bold.

independent module with three specific *Glomus* VTs, another initially mycoheterotrophic family Ophioglossaceae also had two 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). Likewise, 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; 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 ($P < 1.10^{-16}$). This suggests phylogenetic conservatism of fungal partners during the evolution of mycoheterotrophic nutrition in these families. For other mycoheterotrophic families (Corsiaceae, Gentianaceae and Psilotaceae), fungal partners were significantly more similar to partners interacting with other mycoheterotrophic families than to partners interacting with their closest autotrophic relatives, the latter being as distant as other autotrophic families (Table S8). This points to a shift to new fungal partners correlated with the evolution of 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, whereas 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 Independent networks and parental nurture in initially mycoheterotrophs), because 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 also has 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 (C) 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 C 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

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

	Most closely related autotrophic sister-clade in our dataset (and divergence time, Ma (Myr ago))	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 Ma)	25	38		0%	13%	3%	8%	16%	2%	16%	0%	16	0
Corsiaceae	Melanthiaceae Liliaceae Smilacaceae (129 Ma)	1	1	0		0%	0%		0%	0%			0	0
Gentianaceae	Apocynaceae (52 Ma)	6	8	5	0		0%	9%	8%	0%			6	0
Petrosaviaceae		1	2	1	0	0		0%	5%	0%			1	0
Polygalaceae	Polygalaceae (60 Ma)	4	4	3	0	1	0						3	1
Triuridaceae	Dioscoreaceae (131 Ma)	4	19	8	0	2	1	2		8%	3%		11	1
IMH														
Lycopodiaceae	Selaginellaceae (303 Ma)	8	7	1	0	0	0	0	2				3	3
Ophioglossaceae	Aspleniaceae Dryopteridaceae Gleicheniaceae Lygodiaceae	5	15	3	0	0	0	0	1	0		6%	4	2
Psilotaceae	Osmundaceae Pteridaceae (330 Ma)	2	2	0	0	0	0	0	0	0	1		1	0

Number (lower part of the matrix) and percentage (upper part) of fungi shared between family pairs. The last two columns represent the total number of fungi shared with other entirely or initially mycoheterotrophic families, and 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).

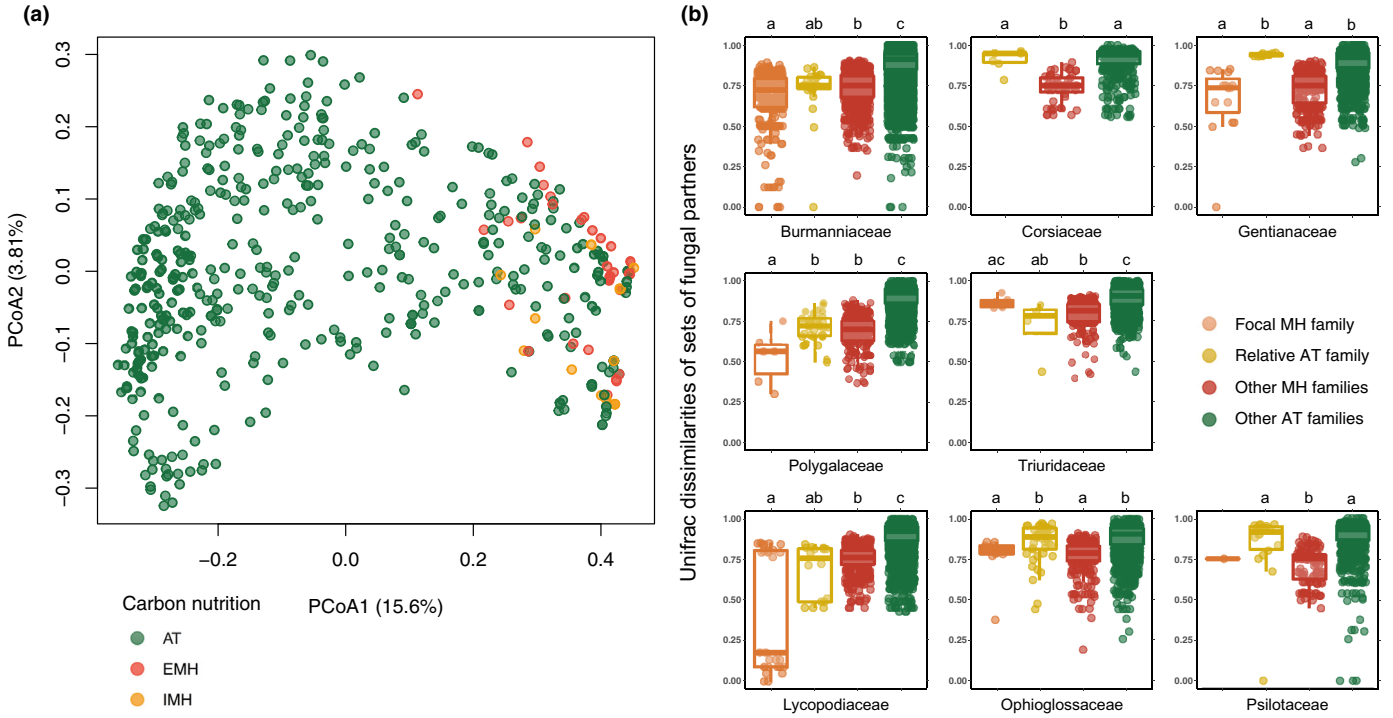

Fig. 4 Dissimilarities between sets of fungal partners associated according to the nature of the interaction. (a) Principal coordinates analysis (PCoA) from UniFrac dissimilarities of sets of fungal partners. Every dot corresponds to a plant species and is colored according to its autotrophic (green), 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 interquartile range.

that each partner preferentially would 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 C entail a greater C 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 also might be influenced by the specific local environmental conditions where cheating is promoted. For instance, because mycoheterotrophs persist primarily 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 C cost. Additionally, low nutrient availability in the environments of mycoheterotrophs also might 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 also should 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 geographical 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 (Fig. S4). However, 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 (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 (van der Heijden & Scheublin, 2007; Chagnon *et al.*, 2013) 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 noncooperative 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 divergence 110 Myr ago (Ma), whereas mycoheterotrophic Gentianaceae and their closest autotrophic relative have distinct fungal partners after a divergence at only 52 Ma.

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 (*c.* 50–60 Myr old; Fig. 2). The oldest mycoheterotrophic families show conservatism for fungal partners, whereas 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 C 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 C 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 Ma from all other *Glomus* (Rimington *et al.*, 2018). Thus, this highly specific interaction results from a secondary acquisition: some species of Lycopodiaceae initially may have 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).

Data availability

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

Acknowledgements

The authors thank members of the INEVEF team at ISYEB and the BIODIV team at IBENS for helpful comments on the article. They also thank Colin Fontaine, Marianne Elias and Damien de Vienne for helpful discussions, David Marsh for English editing, the Editor Björn Lindahl, and four anonymous reviewers for improvements of earlier versions of this manuscript. This work was supported by a doctoral fellowship from the École Normale Supérieure de Paris attributed to BPL and the École Doctorale FIRE – Programme Bettencourt. MÖ was supported by the European Regional Development Fund (Centre of Excellence EcolChange) and the Estonian Research Council (IUT20-28). HM acknowledges support from the European Research Council (grant CoG-PANDA).

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.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The global geographical 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 *Fx* of plant partners 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.

Methods S1 Phylogenetic reconstructions.

Methods S2 List of abbreviations.

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 subnetworks (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 C 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 and NTI.

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

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