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1 Original article

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

5

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24

25

26 **Content:** Words in the main text: 6,501, including introduction (1,270), materials and
27 methods (1,958), results (1,045), and discussion (2,228).

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

30 **Abstract (159 words)**

31

32 • While mutualistic interactions are widespread and essential in ecosystem
33 functioning, the emergence of uncooperative cheaters threatens their stability,
34 unless there are some physiological or ecological mechanisms limiting interactions
35 with cheaters.

36 • In this framework, we investigated the patterns of specialization and phylogenetic
37 distribution of mycoheterotrophic cheaters *versus* non-cheating autotrophic
38 plants and their respective fungi in a global arbuscular mycorrhizal network with
39 >25,000 interactions.

40 • We show that mycoheterotrophy repeatedly evolved among vascular plants,
41 suggesting low phylogenetic constraints for plants. However, mycoheterotrophic
42 plants are significantly more specialized than autotrophic plants, and they tend to
43 be associated with specialized and closely related fungi. These results raise new
44 hypotheses about the mechanisms (e.g. sanctions, or habitat filtering) that actually
45 limit the interaction of mycoheterotrophic plants and their associated fungi with
46 the rest of the autotrophic plants.

47 • Beyond mycorrhizal symbiosis, this unprecedented comparison of
48 mycoheterotrophic *versus* autotrophic plants provides a network and
49 phylogenetic framework to assess the presence of constraints upon cheating
50 emergences in mutualisms.

51

52

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

56 **Introduction**

57

58 Mutualistic interactions are ubiquitous in nature and largely help to generate and
59 maintain biodiversity (Bronstein, 2015). Since benefits in mutualism often come at a cost
60 for cooperators (Douglas, 2008), some species, referred to as *cheaters*, have evolved an
61 adaptive uncooperative strategy by retrieving benefits from an interaction without
62 paying the associated cost (Sachs *et al.*, 2010). Although cheating compromises the
63 evolutionary stability of mutualistic interactions (Ferriere *et al.*, 2002), its evolutionary
64 origin and persistence until present (hereafter referred to as cheating emergence) is often
65 limited by factors securing the persistence of mutualism (Bronstein *et al.*, 2003;
66 Frederickson, 2013; Jones *et al.*, 2015). For instance, species often favor the most
67 cooperative partners (e.g. conditional investment; Roberts & Sherratt, 1998), stop
68 interactions with cheaters (Pellmyr & Huth, 1994), or even sanction them (Kiers *et al.*
69 2003). Cheating emergence can thus be constrained through physiological or biochemical
70 mechanisms of the interaction and its regulation. In addition, cheating can be restricted
71 to particular habitats or to partners with specific niches. Therefore, cheaters might be
72 constrained to specialize on susceptible partners and/or particular habitats. Moreover,
73 these different constraints (hereafter referred to as *functional constraints*) can be
74 evolutionarily conserved or not (Gómez *et al.* 2010). If they are conserved, there will be
75 *phylogenetic constraints* on the emergence of cheaters, as some species will have
76 evolutionarily conserved traits that make them more or less likely to cheat or to be
77 cheated upon (Lallemand *et al.*, 2016).

78

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

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

104

105 Here we study cheating emergences in arbuscular mycorrhizal mutualism between plant
106 roots and soil Glomeromycotina fungi (Selosse & Rousset, 2011; Jacquemyn & Merckx,
107 2019). This symbiosis is at least 407 Myr-old (Strullu-Derrien *et al.*, 2018) and concerns
108 ca. 80% of extant land plants and several hundred fungal taxa (Davison *et al.*, 2015; van
109 der Heijden *et al.*, 2015). Arbuscular mycorrhizal fungi colonize plant roots and provide
110 host plants with water and mineral nutrients, in return for organic carbon compounds
111 (Rich *et al.*, 2017). Although obligate for both partners, this symbiosis is generally diffuse
112 and not very specific (van der Heijden *et al.*, 2015), since multiple fungi colonize most
113 plants, while fungi are usually shared among surrounding plant species (Verbruggen *et al.*
114 2012). Thus, fungi interconnect plant individuals of different species and allow resource
115 movement between plants (Selosse *et al.*, 2006; Merckx, 2013). This allowed the
116 emergence of achlorophyllous cheating plants, called *mycoheterotrophs*, which obtain
117 carbon from their mycorrhizal fungi that are themselves fed by surrounding autotrophic
118 plants (Merckx, 2013) – these plants are thus permanent cheaters, whatever the
119 conditions or partners. Some of these plant species are *entirely mycoheterotrophic* over
120 their lifecycle, while others are mycoheterotrophic only at early stages before turning

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

132

133 We evaluate the presence of functional constraints upon cheating by measuring
134 specialization, nestedness and modularity in a composite plant-mycorrhizal fungal
135 interaction network built from associations between species at multiple sites across the
136 entire globe (Öpik *et al.*, 2010). Mycoheterotrophic plants are thought to be specialists
137 interacting with few fungal species (Leake, 1994; Merckx, 2013), but whether or not these
138 plant species are unusually specialized compared to autotrophic plants is still debated
139 (Merckx *et al.*, 2012). Mycoheterotrophs could specialize on few fungal species if some
140 functional constraints limit the set of fungi or habitats they can exploit, and if they have
141 evolved particular strategies to obtain nutrients from their specific fungal partners
142 (Blüthgen *et al.*, 2007). In terms of nestedness and modularity, arbuscular mycorrhizal
143 networks are generally nested (Chagnon *et al.*, 2012; Sepp *et al.*, 2019); this pattern of
144 asymmetrical specialization is generally thought to confer greater stability in relation to
145 disturbance and resistance to species extinction (Thébault & Fontaine, 2010). How
146 mycoheterotrophic plants affect nestedness has yet to be investigated. On the one hand,
147 in the absence of functional constraints upon cheating, we would expect that
148 mycoheterotrophs interact with generalist fungi to increase their indirect access to
149 carbon *via* surrounding autotrophic plants, therefore increasing nestedness (Fig. **1b**–
150 v,viii). On the other hand, if autotrophic plants are able to avoid costly interactions with
151 fungi associated with mycoheterotrophs (physiological constraints), or if
152 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.

171 **Materials and Methods**

172

173 ***MaarjAM database and interaction matrix***

174 The MaarjAM database is a web-based database (<http://maarjam.botany.ut.ee>; accessed
175 in June 2019 after a very recent update) of publicly available sequences of
176 Glomeromycotina fungi, with information on the host plants, geographical location and
177 biomes for the recorded interactions (Öpik *et al.*, 2010). We used an approach with a
178 compiled network, where all locally described physical mycelial interactions between
179 species are merged and studied at larger scales (as in Joffard *et al.*, 2018). Although such
180 a compiled network can be sensitive to several biases (see Discussion), it offers unique
181 opportunities to study the emergence of mycoheterotrophy in a large evolutionary and
182 ecological perspective (e.g. Werner *et al.*, 2018). Among the 41,989 interactions between
183 plants and Glomeromycotina, we filtered out the data from MaarjAM for the fungi to
184 satisfy the following criteria (Supporting Information Table **S1a**): (i) amplification of the
185 18S rRNA gene, (ii) fungus identified from plant roots (i.e. excluding soil samples), (iii)
186 interaction in a natural ecosystem (i.e. excluding anthropogenic or highly disturbed
187 ecosystems), (iv) host plant identified at the species level, and (v) a virtual taxon (VT)
188 assignment available in MaarjAM. The VTs are a classification (=species proxy) of
189 arbuscular mycorrhizal fungi designed by applying a $\geq 97\%$ sequence similarity threshold
190 to the 18S rRNA gene sequences, and by running phylogenetic analysis to ensure VT
191 monophyly (Öpik *et al.*, 2013, 2014). In the following, we assumed that we have a full
192 representation of all fungal partners associated with each plant species in the dataset. The
193 filtered dataset yielded a binary interaction matrix of 490 plant species (hereafter
194 'plants'), 351 VTs (hereafter 'fungi'), and 26,350 interactions (Fig. 2), resulting from the
195 compilation of 112 publications from worldwide ecosystems (Supporting Information
196 Fig. **S1**; Table **S1b**). In order to estimate the sampling fraction of Glomeromycotina fungi
197 in our dataset, we plotted rarefaction curves of the number of fungal species as a function
198 of the sampling fraction (for the observed number of interactions or for the number of
199 sampled plant species) and we estimated the total number of species using the 'specpool'
200 function ('vegan' R-package, based on Chao index; Oksanen *et al.*, 2019). We separately
201 performed rarefaction analyses for mycoheterotrophic species only. Moreover, in order
202 to check the robustness of our results, we repeated all the analyses on a subsampled

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

205

206 ***Phylogenetic reconstructions***

207 We aligned consensus sequences of the 351 fungi with MUSCLE (Edgar, 2004) and ran a
208 Bayesian analysis using BEAST2 to reconstruct the fungal phylogeny (Bouckaert *et al.*
209 2014, Supporting Information Methods **S1**). We obtained the phylogenetic relationships
210 between the 490 host plants by pruning the time-calibrated supertree from Zanne *et al.*
211 (2013) using Phylomatic (<http://phylodiversity.net/phyloomatic/>). We also used the Open
212 Tree of Life website (<http://opentreeoflife.org>) and the 'rotl' R-package (Michonneau *et al.*
213 2016; R Core Team, 2019) for grafting of 41 plant taxa missing from the pruned
214 supertree (as polytomies at the lowest taxonomy level possible; Supporting Information
215 Methods **S1**). We set tree root calibrations at 505 million years (Myr) for the fungi
216 (Davison *et al.*, 2015) and 440 Myr for the plants (Zanne *et al.*, 2014).

217

218 ***Nature of the interaction***

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

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

239

240 ***Network nestedness, modularity, and specialization of cheaters***

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

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

266 values feature a reciprocal specialization (Fig. **1b-v,vii**). We tested whether k and P_{sp} were
267 statistically different among autotrophic, entirely mycoheterotrophic and initially
268 mycoheterotrophic plants using non-parametric Kruskal-Wallis tests and pairwise Mann-
269 Whitney U tests. To assess the significance of k and P_{sp} values, we built null-model
270 networks ($N=1,000$) using the function 'permatfull' in the 'vegan' R-package (*null model*
271 *1*), keeping the connectance constant but allowing different marginal sums. Then, in order
272 to detect specialization at the clade scale toward partners, for any given clade of every
273 node in the plant or fungus phylogenies, we calculated the partner fidelity (F_x) as the ratio
274 of partners exclusively interacting with this particular clade divided by the total number
275 of partners interacting with it. We consider the clade as 'faithful' and the corresponding
276 set of partners as 'clade-specific' when $F_x > 0.5$ (i.e., more than 50% exclusive partners).
277 We used analysis of covariance (ANCOVA) to test the effect of the nature of the interaction
278 on partner fidelity F_x accounting for clade size, which corrects the bias of having high
279 partner fidelity F_x in older clades including many plants. To confirm that the patterns of
280 specialization at the global scale held at a more local scale, we reproduced the analyses of
281 specialization (k and P_{sp}) in two continental networks in South America and Africa, which
282 represented a high number of interactions and mycoheterotrophic species.

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

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

294

295 ***Phylogenetic distribution of cheating***

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

298 phylogenetic distribution of mycoheterotrophy, i.e. if mycoheterotrophic plants and their
299 fungal partners were more or less phylogenetically related than expected by chance
300 (patterns of clustering *versus* overdispersion). We computed the net relatedness index
301 (NRI) and the nearest taxon index (NTI) using the 'PICANTE' R-package (Kembel *et al.*,
302 2010). NRI quantifies the phylogenetic structure of a species set based on the mean
303 pairwise distances, whereas NTI quantifies the terminal structure of the species set by
304 computing the mean phylogenetic distance to the nearest taxon of every species (Gotelli
305 & Rohde, 2002). To standardize the indices, we generated 999 null models with the option
306 '*taxa.labels*' (shuffles the taxa labels). Significant positive (resp. negative) NRI and NTI
307 values indicate phylogenetic clustering (resp. overdispersion). We computed these
308 indices (*i*) on the plant phylogeny to evaluate the phylogenetic structure of entirely
309 mycoheterotrophic and initially mycoheterotrophic plant distribution, and (*ii*) on the
310 fungal phylogeny to investigate if fungi associated with mycoheterotrophs were
311 phylogenetically structured (we successively tested the distribution of the fungi
312 associated with mycoheterotrophs, entirely mycoheterotrophs, or initially
313 mycoheterotrophs, and then of the fungi associated with each specific mycoheterotrophic
314 family). Similarly, for each plant, we computed the partners' mean phylogenetic pairwise
315 distance (*MPD*), that is the average phylogenetic distance across pairs of fungal partners
316 (Kembel *et al.*, 2010): a low value of *MPD* indicates that the set of partners is constituted
317 of closely related species. The effect of mycoheterotrophy on *MPD* values and its
318 significance were evaluated as for *k* and *Psp* values above.

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

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

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

340

341 **Results**

342 ***Completeness of the dataset***

343 We estimated a total number of 373 \pm 9 fungal species (Chao index), which indicated that
344 the 351 fungi in the dataset included most of the arbuscular mycorrhizal fungal diversity
345 (94% \pm 2%; Supporting Information Fig. **S3**). Concerning mycoheterotrophic species, we
346 estimated a total of 117 \pm 19 fungi associated with all mycoheterotrophs, 110 \pm 27 fungi
347 associated with entirely mycoheterotrophs, and 54 \pm 24 fungi associated with initially
348 mycoheterotrophs. Our dataset thus encompassed sampling fractions of 60% \pm 10% for
349 fungi associated with mycoheterotrophs, 49% \pm 10% for fungi associated with entirely
350 mycoheterotrophs, and 40% \pm 28% for fungi associated with initially mycoheterotrophs.
351 Although our dataset did not include all the fungi associated with mycoheterotrophic
352 species, the following results were not sensitive to the sampling fractions of
353 mycoheterotrophs and their fungal partners (Supporting Information Fig. **S2**).

354

355 ***Network nestedness, modularity, and specialization of mycoheterotrophs***

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

361 Reciprocal specializations were confirmed by the analyses of modularity, which found no
362 significant large modules (i.e. the inferred large modules presented more inter-modules
363 than intra-module interactions), suggesting that the overall structure was not modular,
364 but detected few significant small independent modules (Supporting Information Table
365 **S4**). In addition to a main module encompassing most species (481 out of 490 plants and
366 346 out of 351 fungi), we found three small independent modules: (i) 6 initially
367 mycoheterotrophic Lycopodiaceae plants and three exclusive fungi (*Glomus* VT127,
368 VT158, VT394); (ii) two autotrophic plants from salt marshes (*Salicornia europaea* and
369 *Limonium vulgare*) with one *Glomus* (VT296); and (iii) the entirely mycoheterotrophic
370 *Kupea martinetegei* with a unique *Glomus* (VT204).

371 From the degrees (k), we found that entirely and initially mycoheterotrophic plants were
372 significantly more specialized than autotrophic plants and interacted with on average
373 more than five times fewer fungi (Kruskal-Wallis $H=87.2$; $P=1.2 \cdot 10^{-19}$; Fig. **3a**; Table **1**).
374 Partner specializations (Psp) indicated that mycoheterotrophs interacted with more
375 specialized fungi (fungi associated with mycoheterotrophs interact on average with two
376 times fewer plants; Kruskal-Wallis $H=47.2$; $P=5.6 \cdot 10^{-11}$; Fig. **3a**). We found similar
377 evidence for mycoheterotrophic reciprocal specializations by reanalyzing the network
378 excluding the family Lycopodiaceae (Table **1**; significance assessments using null models
379 are shown in Supporting Information Table **S5**). This pattern of reciprocal specialization
380 of mycoheterotrophic plants and their associated fungi held at a smaller geographical
381 scale in the African and South American networks (Supporting Information Fig. **S4**; Table
382 **S6**; yet the difference was not significant for Psp in the South American network, probably
383 due to the small number of species and the low power of the statistical tests).

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

394

395 ***Phylogenetic distribution of cheating***

396 The partners' mean phylogenetic pairwise distance (MPD) indicated that fungi associated
397 with entirely or initially mycoheterotrophs (or even with all mycoheterotrophs) were
398 phylogenetically more closely related than fungi associated with autotrophs (Kruskal-
399 Wallis $H=18.0$; $P=1.2 \cdot 10^{-4}$; Table **1**; Fig. **3b**). NRI and NTI values (Supporting Information
400 Table **S7**) also confirmed significant clustering on the fungal phylogeny on fungi
401 associated with mycoheterotrophs, entirely mycoheterotrophs, or initially
402 mycoheterotrophs; this clustering held at the family level for fungi associated with each

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

412 Looking specifically at the fungi shared among mycoheterotrophic plants highlighted
413 differences between entirely and initially mycoheterotrophs (Table **2**). While the initially
414 mycoheterotrophic Lycopodiaceae family formed an independent module with three
415 specific *Glomus* VTs, another initially mycoheterotrophic family Ophioglossaceae also had
416 2 exclusive fungi (*Glomus* VT134 and VT173) among a total of 15 fungi. When comparing
417 the fungi shared between mycoheterotrophic families (Table **2**), mainly two closely
418 related families, Burmanniaceae and Triuridaceae, tended to share some fungi with other
419 mycoheterotrophic families.

420 The decomposition of UniFrac dissimilarities between sets of fungal partners using a
421 PCoA, showed a clear pattern of clustering of mycoheterotrophic species, indicating that
422 the set of fungal partners associated with mycoheterotrophs were more similar than
423 expected by chance ($P < 1.10 \cdot 10^{-16}$ for PCoA1; $P = 9.10 \cdot 10^{-3}$ for PCoA2; Fig. **4a**). Similarly, the
424 PerMANOVA analysis indicated that the nature of the interaction (initially
425 mycoheterotrophic, entirely mycoheterotrophic, or autotrophic) predicted 6.5% of the
426 variance ($P = 0.0001$). By comparing the UniFrac dissimilarities between sets of fungal
427 partners according to the nature of the interaction and plant family relatedness, we
428 observed that all mycoheterotrophic families had fungal partners more similar to each
429 other than those of other autotrophic families (Fig. **4b**; Supporting Information Table **S8**).
430 Some families (Burmanniaceae, Polygalaceae, Triuridaceae, Lycopodiaceae, and
431 Ophioglossaceae) had fungal partners significantly more similar to partners interacting
432 with their closest autotrophic relatives ($P > 0.05$) than to partners interacting with other
433 autotrophic families ($P < 10^{-16}$). This suggests phylogenetic conservatism of fungal
434 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.

441 **Discussion**

442

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

454

455 ***Cheaters are isolated by reciprocal specialization***

456 We confirmed that mycoheterotrophic plants are more specialized toward few
457 mycorrhizal fungal partners than autotrophic plants (Merckx *et al.*, 2012) and showed for
458 the first time that their fungal partners are overall more specialized than fungi associated
459 with autotrophic plants. This reciprocal specialization is not strict (with the exception of
460 Lycopodiaceae, see below), since mycoheterotrophs and their fungal partners need some
461 connection to autotrophic plants, yet sufficient to lower nestedness in the arbuscular
462 mycorrhizal network. The observed trend toward reciprocal specialization and reduced
463 nestedness suggests that mycoheterotrophic cheating is an unstable ecological and
464 evolutionary strategy, which could explain the relatively recent origin of
465 mycoheterotrophic clades (Fig. 2). Indeed, reciprocal specialization confers high
466 extinction risks for both interacting partners, which is one of the main hypotheses
467 explaining why mutualistic networks tend to be nested, with asymmetrical
468 specialization (i.e. specialists interact with generalist partners; Thébault & Fontaine,
469 2010). Whatever its origin, the reciprocal specialization of cheaters and their partners has
470 also been suggested in other mutualisms (Genini *et al.*, 2010). A parasitic nature of
471 entirely mycoheterotrophic plants has often been mooted (Bidartondo, 2005; Merckx,
472 2013), albeit without direct support, in the absence of data on fitness of fungal partners

473 and autotrophic plants providing carbon to mycoheterotrophs (van der Heijden *et al.*,
474 2015). Our analysis *a posteriori* supports the view of entirely mycoheterotrophic plants
475 as parasitic cheaters. However, we cannot exclude the possibility that mycoheterotrophs
476 might provide some advantages to their mycorrhizal fungi (e.g. shelter or vitamins;
477 Brundrett, 2002; Selosse & Rousset, 2011), making them useful partners for some specific
478 fungal species, despite their carbon cost. Further empirical evidence is needed to clarify
479 this.

480

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

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

515

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

529

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

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

541

542 In this study, we used a simple dichotomy of plants considered either as mutualistic
543 autotrophs or as (either entirely or initially) mycoheterotrophic cheaters. However,
544 mycoheterotrophy is not the only uncooperative strategy in this symbiosis: mycorrhizal
545 interactions rather represent a continuum between mutualism and parasitism, both in
546 terms of plants (Jacquemyn & Merckx, 2019) and fungi (Johnson *et al.*, 1997; Klironomos,
547 2003). Physiological constraints are thus thought to constitutively maintain the stability
548 of the mycorrhizal symbiosis (Kiers *et al.*, 2003, 2011) against many forms of cheating,
549 including the specific case of mycoheterotrophy. Moreover, we did not consider context
550 dependency, which has a non-negligible impact on the functioning of mycorrhizal
551 interactions (Chaudhary *et al.*, 2016). Although the mutualism-parasitism continuum or
552 the context dependency could have hidden the observed patterns, the fact that we
553 observed significant differences in the specialization between autotrophic and
554 mycoheterotrophic plants and high similarities between sets of fungal partners
555 associated with different mycoheterotrophic plant lineages suggests that the observed
556 patterns are likely robust to our simplifications.

557

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

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

568

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

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

582

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

599

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

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

612

613 ***Independent networks and parental nurture in initially mycoheterotrophs***

614 Our results serendipitously revealed that two initially mycoheterotrophic families,
615 Ophioglossaceae and Lycopodiaceae, seem to have exclusive mycorrhizal associations, as
616 they interacted with fungi that did not interact with any other plant family. In these
617 families, the fungi are present during both mycoheterotrophic underground spore
618 germination and in the roots of adult autotrophic individuals (Winther & Friedman, 2007,
619 2008). Autotrophic adults likely act as the carbon source (Field *et al.*, 2015), part of which
620 is dedicated to the offspring. This further supports the hypothesis by Leake *et al.* (2008)
621 proposing parental nurture where germinating spores would be indirectly nourished by
622 surrounding conspecific sporophytes. Parental nurture is not universal to all initially
623 mycoheterotrophic families though; in the initially mycoheterotrophic Psilotaceae, for
624 example, fungal partners are shared with surrounding autotrophic plants (Winther &
625 Friedman, 2009). In initially mycoheterotrophic independent networks, the overall
626 outcome for the fungus over the plant lifespan may actually be positive: fungi invest in
627 mycoheterotrophic germinations that represent future carbon sources (Field *et al.*, 2015).
628 In other words, initially mycoheterotrophic plants do not cheat their exclusive fungi, but
629 postpone the reward. We note, however, that the existence of independent networks for
630 these families should be confirmed in studies of local communities.

631

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

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

642

643 **Conclusions**

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

657

658

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669

670 **Author contributions**

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

674

675 **Data Availability Statement**

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

678

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- 865

866 **Tables**

867

868

869 **Table 1: Effect of the nature of the interaction (i.e. plant carbon nutrition modes)**
870 **on indices of network structure and phylogenetic distributions.**871 (Categories are defined according to the plant carbon nutrition modes, i.e. AT:
872 autotrophic; EMH: entirely mycoheterotrophic over development; and IMH: initially
873 mycoheterotrophic in development).874 The second column corresponds to *P*-values of Kruskal-Wallis tests for the overall
875 network with or without (in brackets) the Lycopodiaceae.876 The last three columns correspond to *P*-values of Whitney U tests (pairwise tests) for the
877 overall network including the Lycopodiaceae.878 *P*-values lower than 5% (significance level) are shown in bold.

879

880

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

881

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

884 Number (lower part of the matrix) and percentage (upper part) of fungi shared between
885 family pairs. The last two columns represent (*i*) the total number of fungi shared with
886 other entirely or initially mycoheterotrophic families, and (*ii*) the number of fungi
887 exclusive to this family (i.e. not shared with any other mycoheterotrophic or autotrophic
888 family). The second column indicates the most closely related autotrophic sister clade of
889 each family; it can be one family, a higher clade, the family itself if autotrophic species
890 were compiled in the MaarjAM database (e.g. Polygalaceae), or none in the case of
891 Petrosaviaceae (which forms a too divergent distinct branch).
892 Boxes are shaded according to the number of shared fungi (white: no shared fungus,
893 black: many shared fungi).

894

		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
<i>EMH</i>	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
<i>IMH</i>	Lycopodiaceae	Selaginellaceae (303 Myr)	8	7	1	0	0	0	0	2	/	0%	0%	3	3
	Ophioglossaceae	Aspleniaceae Dryopteridaceae Gleicheniaceae Lygodiaceae	5	15	3	0	0	0	0	1	0	/	6%	4	2
	Psilotaceae	Osmundaceae Pteridaceae (330 Myr)	2	2	0	0	0	0	0	0	0	1	/	1	0

895

897 **Figure legends**

898

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

902

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

913 Interpreting the observed patterns of phylogenetic clustering and network structure
914 directly indicates the strength of the constraints. For instance, strong phylogenetic
915 clustering of the cheaters and their partners (i-iii) suggests that the emergence of cheaters
916 and their susceptible partners is rare and limited, whereas low phylogenetic clustering
917 (ii-iv) suggests that cheating evolved multiple times. Regarding functional constraints,
918 generalist cheaters (vi) might indicate that their partners do not have any mechanisms
919 preventing uncooperative interactions (low constraints). Conversely, specialist cheaters
920 (v) might indicate that cheaters cannot interact with most partners (high constraints).
921 Moreover, if the partners of cheaters are generalists (viii - low constraints), asymmetrical
922 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.

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

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

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

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

946 **Figure 3:** Effect of the nature of the interaction on specialization (k and P_{sp}), the partner's
947 mean phylogenetic distance (MPD), and partner fidelity (F_x - 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 (P_{sp}) (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 P_{sp} .
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 (F_x) 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 F_x 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.

965

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

968 **(a):** Principal coordinates analysis (PCoA) from UniFrac dissimilarities of sets of fungal
969 partners. Every dot corresponds to a plant species and is colored according to its
970 autotrophic (green), entirely mycoheterotrophic (red), or initially mycoheterotrophic
971 (orange) nature. Only the first two principal axes explaining, respectively, 15.6% and
972 3.8% of the variation were kept.

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

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

985 **Supporting Information**

986

987 **Supplementary methods:**

988

989 **Methods S1:** Phylogenetic reconstructions

990

991 **Methods S2:** List of abbreviations

992

993 **Supporting Information Tables:**

994

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

996

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

999

1000 **Table S3:** Effect of mycoheterotrophy on the network nestedness.

1001

1002 **Table S4:** Independent sub-networks (modules) in the overall arbuscular mycorrhizal
1003 network and their respective numbers of partners.

1004

1005 **Table S5:** Null model to assess the significance of the effect of the nature of the interaction
1006 on indices of network structure and phylogenetic distributions.

1007

1008 **Table S6:** Effect of the nature of the interaction (i.e. plant carbon nutrition modes) on
1009 indices of the network structure in South America and Africa.

1010

1011 **Table S7:** Measure of the phylogenetic distributions of mycoheterotrophy: measure of
1012 NRI (net relatedness index) and NTI (nearest taxon index).

1013

1014 **Table S8:** Pairwise comparisons of UniFrac dissimilarities between sets of fungal
1015 partners associated with different plant families.

1016

1017

1018 **Supporting Information Figures:**

1019

1020 **Fig. S1:** The global geographic distribution of sampling sites used in our analysis.

1021

1022 **Fig. S2:** Analysis on a smaller network (accessed in the MaarjAM database in October
1023 2017)

1024

1025 **Fig. S3:** Rarefaction curves representing the number of fungal taxa as a function of the
1026 sampling fraction.

1027

1028 **Fig. S4:** Effect of the nature of the interaction on specializations in the South American
1029 network and the African network.

1030

1031 **Fig. S5:** Fidelity of plant partners (F_x) according to the age of the fungal clade.

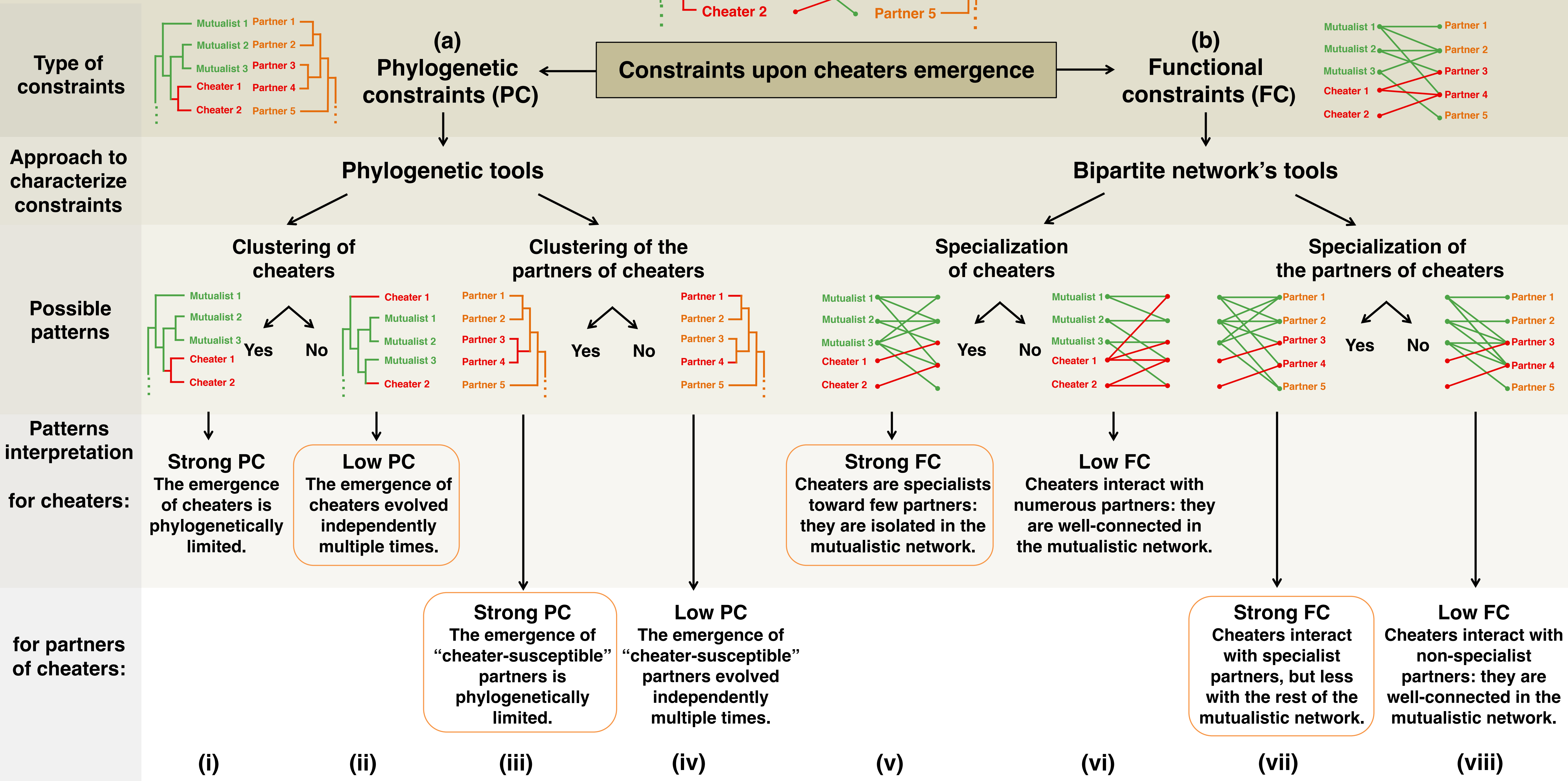
1032

1033 **Fig. S6:** Calibrated phylogenetic tree of the 490 plant species.

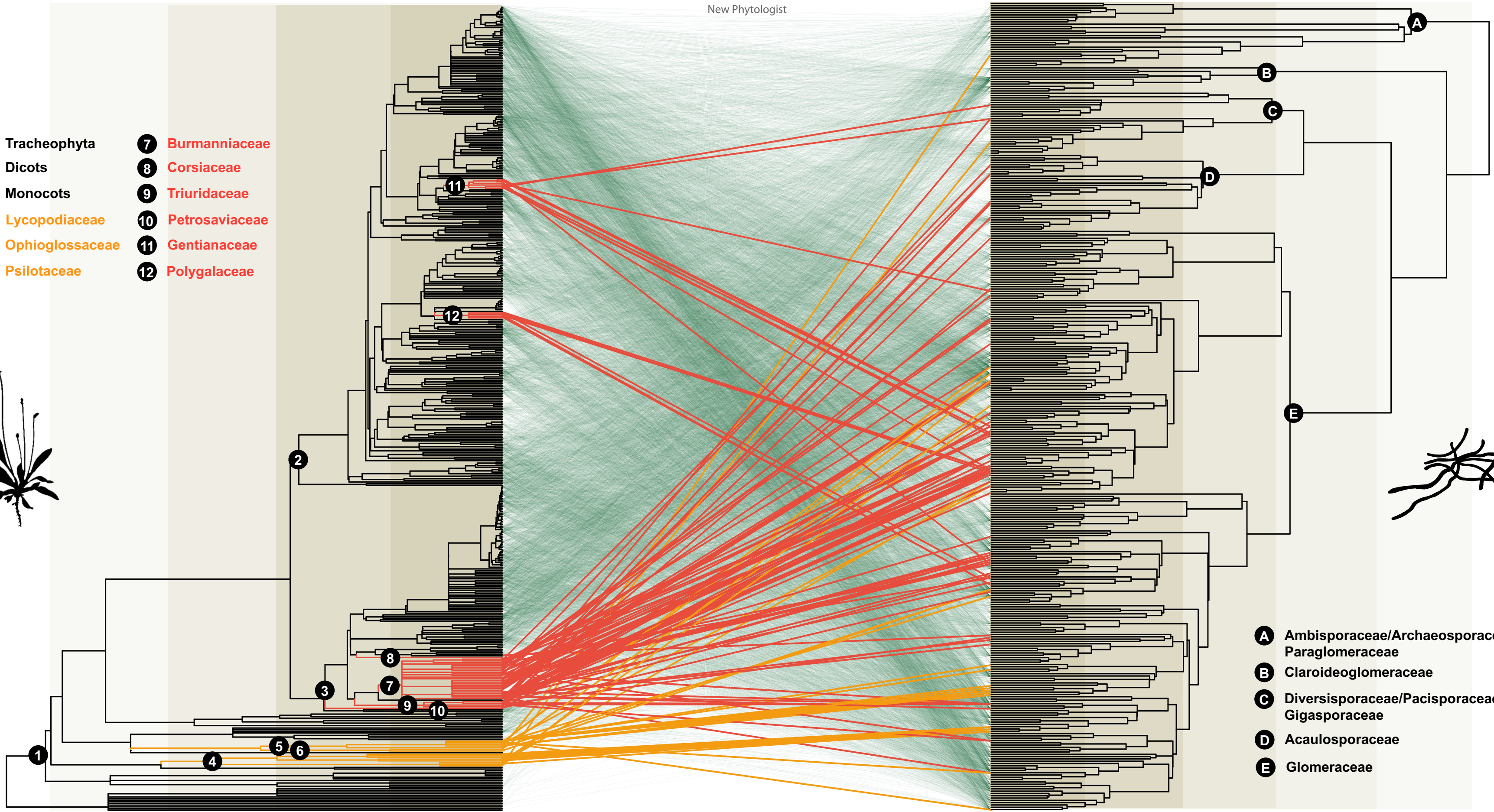
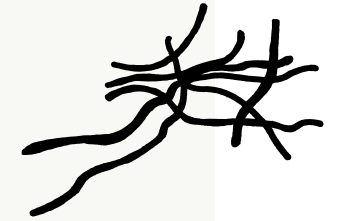
1034

1035 **Fig. S7:** Calibrated phylogenetic tree of the 351 arbuscular mycorrhizal fungi.

1036



- 1** Tracheophyta
- 2** Dicots
- 3** Monocots
- 4** Lycopodiaceae
- 5** Ophioglossaceae
- 6** Psilotaceae
- 7** Burmanniaceae
- 8** Corsiaceae
- 9** Triuridaceae
- 10** Petrosaviaceae
- 11** Gentianaceae
- 12** Polygalaceae



400 300 200 100 0

AT EMH IMH

0 100 200 300 400 500

- A** Ambisporaceae/Archaeosporaceae/
Paraglomeraceae
- B** Claroideoglomeraceae
- C** Diversisporaceae/Pacisporaceae/
Gigasporaceae
- D** Acaulosporaceae
- E** Glomeraceae

