CHEATING BELOWGROUND INTERACTIONS

- Diversity, ecology and distribution of mycoheterotrophy -

Sofia I.F. Gomes

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Cheating belowground interactions

Diversity, ecology and distribution of mycoheterotrophy

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To my father

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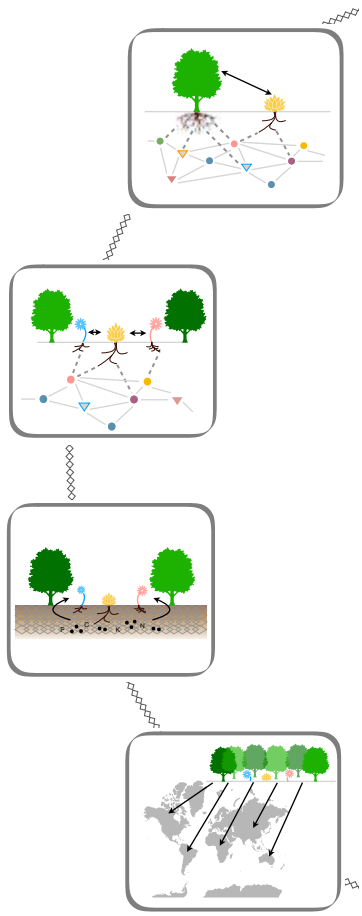


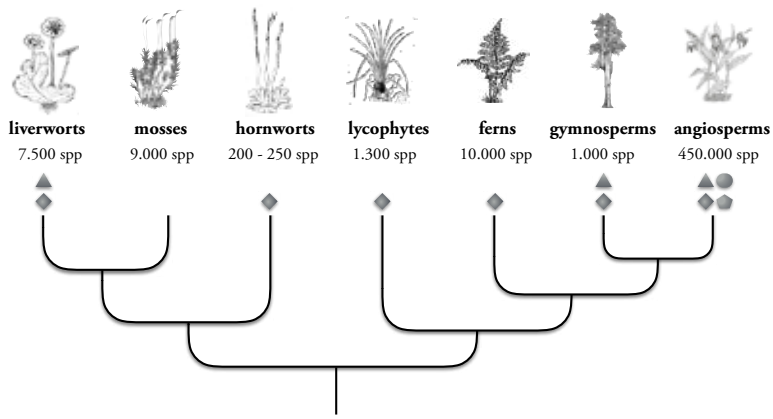
CHAPTER 1

Introduction to mycoheterotrophy

Sofia I.F. Gomes

Background

Mycorrhizal fungi are allies of more than 90% of the half a million land plant species on Earth (Wang & Qiu 2006; Corlett 2016), establishing one of the most widespread and ecologically important mutualisms. The mycorrhizal symbiosis consists of associations between plant roots and specialized soil fungi, where plants transfer photosynthetically fixed carbon to their fungal partners, which in turn facilitate the uptake of limiting soil nutrients, mainly phosphorus and nitrogen (Smith & Read 2008). All major lineages of land plants, with the exception of mosses (Pressel *et al.* 2010), form associations with fungi belonging to the phyla Mucoromycota, Basidiomycota or Ascomycota (van der Heijden *et al.* 2015; Spatafora *et al.* 2016). The classification of mycorrhizal types depends on the morphological features and the identity of the partners within the interaction (van der Heijden *et al.* 2015). There are four main types: arbuscular mycorrhiza (AM), ectomycorrhiza (EM), ericoid mycorrhiza (ErM) and orchid mycorrhiza (OrM). Figure 1 provides a summary of the phylogenetic distribution of these mycorrhizal types and the number of plant and fungal taxa involved in the mycorrhizal symbiosis.



Mycorrhizal type	Plant species[1]	Fungal species[2]
orchid mycorrhiza	28.000	25.000
ericoid mycorrhiza	4.000	> 150
ectomycorrhiza	> 6.000	20.000
arbuscular mycorrhiza	210.000	300 - 1.600

Figure 1 | Representation of the best supported hypothesis of the relationships between the major lineages of land plants (Puttick et al. 2018). Symbols represent the different mycorrhizal types. References for the estimated number of species per lineage are the following: angiosperms (Pimm & Joppa 2015), gymnosperms (Christenhusz et al. 2011), ferns (Ranker & Sundue 2015), lycophytes and mosses (Magill 2010), hornworts (Villarreal et al. 2010), and liverworts (Von Konrat et al. 2014). Total number of plant[1] (after Brundrett 2017) and fungal[2] species (after van der Heijden et al. 2015) estimated as taking part in the mycorrhizal symbiosis are depicted in the table.

The AM fungi colonize the roots of about 71% plant species including most families of vascular plants (Brundrett & Tedersoo 2018). These fungi penetrate the root cells and form arbuscules from inter-cellular hyphae or from neighbouring cells. The AM fungi belong to the phylum Mucoromycota, in which most mycorrhizal taxa are included in the sub-phylum Glomeromycotina (former phylum Glomeromycota; Spatafora *et al.* 2016). The EM fungi usually form a Hartig net with a differential hyphal mantle that surrounds the roots cells, without intracellular colonization. The EM type occurs in 2% of plant species, within few families of gymnosperms and angiosperms. The fungi involved belong to distantly-related lineages in the phyla Ascomycota and Basidiomycota. The ErM fungi colonize the plants by penetrating the root cells forming hyphal coils with each individual cell being colonized from the root surface. The ErM type is restricted to members of the Ericaceae family and the fungal partners are mostly Ascomycota. Finally, the OrM fungi form hyphal pelotons within the root cells and colonization occurs from root surface mycelia or from neighbouring cells. This mycorrhizal type occurs exclusively within members of the Orchidaceae, and involves mostly Basidiomycota fungi (Brundrett & Tedersoo 2018).

The mycorrhizal fungi have the potential to link plants from different species creating extensive underground networks. These underground mycorrhizal networks are complex to understand, yet play crucial roles in the ecosystem. The mycorrhizal symbiosis has great impact at the ecosystem level since plants allocate between 10 to 30% of their fixed carbon to their fungal partners, and receive from them up to 90% of their nutritional demands (Drigo *et al.* 2010; van der Heijden *et al.* 2015). Furthermore, AM networks are estimated to retain up to 20% carbon that plants fixate through photosynthesis (Parniske 2008), while EM networks are estimated to retain 30-70% (Leake *et al.* 2004; Clemmensen *et al.* 2013). Besides, it has been shown that within the EM symbiosis plants transfer carbon to each other from mother trees to tree seedlings (Simard & Perry 1997), but also between trees of different species, representing up to 4% of the forest net primary productivity. Thus, mycorrhizal networks are, most likely, responsible for tree to tree carbon transfer (Klein *et al.* 2016).

Evolution and stability of the mycorrhizal symbiosis

The majority of land plants associate with AM fungi and this symbiosis is believed to have been instrumental in the colonization of land by plants (Read *et al.* 2000; Bonfante & Selosse 2010). The AM symbiosis has evolved since the colonisation of land by plants,

representing a great example of evolutionary stability (Selosse & Le Tacon 1998), which has been extensively studied during the past decades. Fossil evidence shows the presence of arbuscule-like structures resembling the current morphological features formed by arbuscular mycorrhizal fungi for at least 407 Mya (Field *et al.* 2015; Strullu-Derrien *et al.* 2018). Moreover, molecular evolutionary analysis revealed the presence of conserved genes required for the establishment of mycorrhizal symbiosis in the common ancestor of land plants (Wang *et al.* 2010). With the colonization of land by plants, environmental conditions drastically changed due to the substantial reduction of carbon dioxide and increase of oxygen towards the atmosphere composition of the present day (Selosse *et al.* 2015). Thus, the dawn of mycorrhizal symbiosis represents a key event that allowed the evolution of life on Earth as we know today.

The evolutionary persistence of the mycorrhizal mutualism demonstrates the stability of cooperation between species, despite the selfish interest of individuals (Kiers & van der Heijden 2006). The maintenance of a stable interaction is expected by the reciprocal reward between partners (Kiers *et al.* 2011; Fellbaum *et al.* 2014). Mycorrhizal fungi, particularly AM fungi, are strictly dependent on the carbon provided by the plant for their growth. Kiers *et al.* (2011) has demonstrated that the exchange of nutrients for carbon is reciprocally regulated and the most beneficial partner is rewarded with the most resources in return. This mutualistic association is thus maintained due to the reciprocal exchange of goods between partners where both benefit, suggesting that a biological market dynamics have been critical for the stability of the interaction over the course of evolution.

According to the biological market theory (Noë & Hammerstein 1995) applied to the mycorrhizal symbiosis, the trading partners – plants and mycorrhizal fungi – have the ability to regulate resource supply by discriminating between different mutualistic partners and, thus, allocating resources preferentially towards more beneficial partners (Kiers & van der Heijden 2006; Bever *et al.* 2009; Kiers *et al.* 2011). Besides the preferential allocation of resources rewarding most beneficial partners, this mechanism implies the application of sanctions to the least cooperative ones (Kiers & Denison 2008). This theory leads to the expectation of low specificity among partners, as is observed globally in the AM symbiosis, supporting partner choice as an evident component in maintaining the stability of the mycorrhizal mutualism (Kiers & van der Heijden 2006; Kiers & Denison 2008; Kiers *et al.* 2011; Walder & van der Heijden 2015). However, the processes of recognition and discrimination of the best partners from both plants and fungi still remain unclear.

Cheaters

Mycorrhizal symbiosis contains relationships that likely span a mutualism-parasitism continuum of plant-fungus interactions. The mutual reciprocal interactions between plants and fungi during the mycorrhizal symbiosis can be regarded as the midpoint, while the exploitation from each of the partners represent the endpoints of the mutualism-parasitism continuum (Bronstein 1994; Egger & Hibbett 2004). Additionally, mutualisms are predicted to persist when the costs to obtain resources are lower than the benefits gained from it (Foster & Wenseleers 2006). The costs to obtain resources within the mycorrhizal symbiosis show temporal fluctuation, such as seasonal or circadian cycles, and vary according to environmental conditions that determine resource availability (García & Mendoza 2008; Hernandez & Allen 2013; Klabi *et al.* 2014). Hence, resource acquisition can become costly, and cheating may arise. Antagonistic interactions are, thus, predicted to occur on both sides of the interaction.

Plant growth reduction in response to AM fungi inoculation has been recorded in several experiments (see, for example, Johnson *et al.* 1997; Graham & Abbott 2000). Despite the negative impact in the host plants, high colonisation of AM fungi in the roots was detected in some cases (Veiga *et al.* 2011, amongst others), suggesting a parasitic interaction of the fungi towards the host plants. However, due to the interlink between partner performance and environmental influence, which has high dependency on plant and fungi identity (Grman 2012; Walder & van der Heijden 2015), it is challenging to find substantial evidence of exclusive fungal parasitism in natural mycorrhizal systems. Despite the context-dependent outcome of mycorrhizal interactions, several mechanisms have been put forth to explain growth depression scenarios in plants influenced by AM fungi (Jin *et al.* 2017). Overall, the effectiveness of AM fungal performance between the mutualistic and the parasitic outcome has been attributed to light availability (Reinhard *et al.* 1993), which in turn is dependent on host identity (Stonor *et al.* 2014), and in combination with the relative availability of nutrients in the soil (Johnson 2010). Possible natural scenarios of plant growth depression related to AM performance include the negative effect of AM fungi in the growth of seedlings established near non-conspecific adult plants (Burke 2012); the low performance of AM partners which can be masked by the presence of more effective partners within the total fungal community (Hart *et al.* 2013); and stress conditions where AM fungi increase their carbon demands due to preferentially allocation of resources to vesicles (Johnson 1993). In addition, high fertility environments combined with a luxurious supply of

phosphorus have been hypothesized to benefit the rise of parasitic associations within the AM symbiosis. In this situation, fungal growth is only limited by carbon leading to an increase of carbon demand to a point that may depress plant growth (Thrall *et al.* 2007; Johnson 2010).

Besides the parasitism of AM fungi towards host plants, plants can also exploit their mycorrhizal partners – a phenomenon in which the plants are described as mycoheterotrophic plants (Leake 1994). Although it is difficult to measure the reduction of fitness of fungi, mycoheterotrophic plants cannot perform photosynthesis and, subsequently, there is no exchange of carbon for nutrients; thus, these plants are considered cheaters of the mycorrhizal symbiosis (Bidartondo 2005b). It remains unclear whether mycoheterotrophic plants provide benefits other than carbon to their mycorrhizal partners, such as vitamins or protection, engaging still in a mutualistic interaction, but no evidence for this has been presented so far (Selosse & Rousset 2011). Conversely, evidence points to a biotrophic parasitic nutritional mode of mycoheterotrophic plants due to the apparent digestion of the AM fungi colonising their roots (Imhof 1999). Yet, the mechanism underpinning the persistence of mycoheterotrophy through which plants subvert the carbon flux from their fungal partners to themselves still remains unknown.

Mycoheterotrophy

Leake (1994) coined the term mycoheterotrophy to define plants that rely on their fungal partners for the uptake of carbon (Bidartondo *et al.* 2002). Stable isotope natural abundance analyses have revealed that mycoheterotrophic plants are enriched in the heavy isotopes of carbon ^{13}C and nitrogen ^{15}N compared to the surrounding autotrophic species, pointing out for a carbon source coming from the mycorrhizal network, with numerous examples of partial mycoheterotrophy where plants combine both autotrophic and mycoheterotrophic strategies (for example, Bidartondo *et al.* 2004; Hynson *et al.* 2009; Gebauer *et al.* 2016; Schiebold *et al.* 2018). Both AM (Merckx *et al.* 2010) and EM (Hynson *et al.* 2009) fungi can participate in mycoheterotrophic interactions.

Some plants exhibit mycoheterotrophic mode only at initial stages of their life, called initial mycoheterotrophy (Merckx 2013). This is the case for the gametophytes of some ferns and lycopophytes which rely on mycorrhizal fungi for carbon (Leake 1994; Pressel *et al.* 2016). A possible explanation for the fungi to engage in mycoheterotrophic

relationships at this stage has been suggested as a 'take now, pay later' strategy in which the fungi invest carbon in supporting the mycoheterotrophic gametophytes and subterranean sporophytes (Cameron *et al.* 2008), leading to the fungi to be repaid once these plants have established as autotrophs. This has been shown for the fern *Ophioglossum vulgatum* (Field *et al.* 2015). Such strategy has also been extensively suggested for orchids, which, by having dust seeds with no endosperm, rely on carbon from mycorrhizal fungi in their early life stages. Later in their development, most orchids develop leaves gaining the ability to perform photosynthesis. However, for many orchid species it is not clear whether adult plants become totally autotrophic, since isotopic analysis of several lineages have recently suggested a partial mycoheterotrophic mode for a wider number of orchids than previously expected (Gebauer *et al.* 2016). In this

Figure 2 | Examples of mycoheterotrophic plants: (a) *Voyria spruceana*, Colombia; (b) *Sciaphila* sp., Malaysia; (c) *Apteria aphylla*, French Guiana; (d) *Gastrodia cunninghamii*, New Zealand; (e) *Afrothismia hydra*, Cameroon; (f) *Burmannia hexaptera*, Cameroon; (g) *Epirixanthes pallida*, Malaysia; (h) *Voyria* sp. nov., French Guiana; (i) *Thismia clavarioides*, Australia; (j) *Corsia* cf. *brassii*, Papua New Guinea; (k) *Monotropa hypopitys*, Netherlands; (l) *Petrosavia stellaris*, Malaysia; (m) *Pterospora andromedea*, USA. Photo credits: a, c, h, k by Sofia Gomes; b, d, e, f, g, i, l, m by Vincent Merckx; j by Stephanie Lyon.

case, orchids are able to shift the dependence on their mycorrhizal partners depending on their nutritional demands and light availability in the environment, and can vary throughout their life cycle.

Fully mycoheterotrophic plants (see Figure 2) remain on the extreme end of the spectrum of the mycorrhizal mutualistim-parasitism continuum throughout their development. These plants have completely lost their ability to photosynthesize and depend entirely on their fungi to meet their carbon demands, which ultimately rely on the surrounding autotrophic plants as carbon sources (epiparasitic mycoheterotrophs), or in rare cases obtain carbon from saprotrophic activity (Hynson *et al.* 2013). The focus of this thesis is on the epiparasitic mycoheterotrophs.

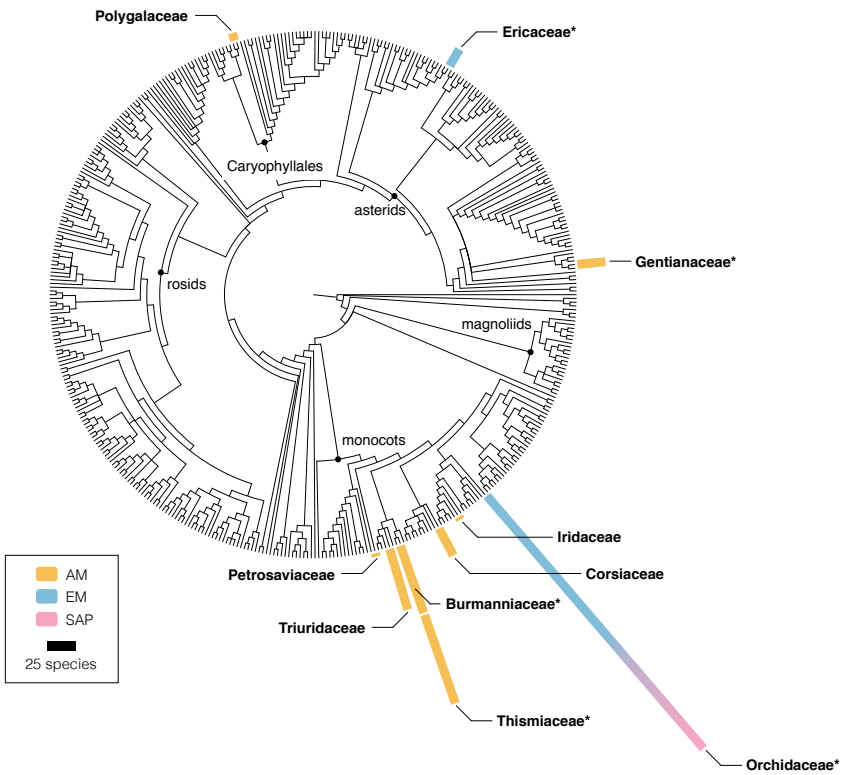


Figure 3 | Phylogenetic relationships of families within the flowering plants based on Gastauer & Meira Neto 2017, including the APG IV relationships of orders (Chase et al. 2016). Number of species of fully mycoheterotrophic plants described to date are represented by the coloured bars according to their mycorrhizal associations (yellow, with AM fungi; blue, with EM fungi; pink, with saprotrophic fungi). Asterisks indicates families in which there are multiple independent evolutionary origins of full mycoheterotrophy.

Diversity of mycoheterotrophic plant species

Considering the half a million land plant species, the emergence and establishment of full mycoheterotrophy is a rare event in evolution (Corlett 2016b; Merckx 2013). Yet, it evolved multiple times independently harbouring a wide variety of taxa, and it is present in almost every major group of land plants with a major presence within the flowering plants (Fig. 3). The conspicuous habit of mycoheterotrophic plants does not pass unnoticed, however their reduced size and unpredictable flowering intervals hinder their discovery even in frequently visited places, thus new species to science are discovered every year mainly in tropical rainforests. Within the flowering plants, the first complete overviews of mycoheterotrophic species estimated ca. 400 species (Furman & Trappe 1971; Leake 1994). This number has increased to ca. 515 species (Merckx 2013), and currently there are 579 mycoheterotrophic species described (Merckx 2013; Tsukaya *et al.* 2016; WCSP 2016). Of these, 530 are in 7 families of monocots, and 49 in 3 families of eudicots.

Fungal diversity

The ecology and evolution of mycoheterotrophic plants is tightly linked to their associated fungi. The fungi involved in mycoheterotrophic interactions were first investigated with molecular methods to belong to EM fungi in temperate regions (Cullings *et al.* 1996; Taylor & Bruns 1997; McKendrick *et al.* 2000; Bidartondo & Bruns 2001; Bidartondo & Bruns 2002). A decade later, Bidartondo *et al.* (2002), investigated for the first time using molecular methods the nature of fungal associations of non-ectomycorrhizal mycoheterotrophs and confirmed the involvement of AM fungi in these cheating interactions, as suggested by morphological observations (see, for example, Janse 1897; Imhof 1999).

Probably because the mycorrhizal symbiosis has evolved in the three main phyla within the fungal kingdom – Mucoromycota, Ascomycota and Basidiomycota – cheating the mycorrhizal symbiosis occurred in all these lineages. Actually, among the mycorrhizal lineages, only within the sub-phylum Mucoromycotina, two orders within the sub-phylum Glomeromycotina, and one class of Ascomycota have not been yet found being cheated by mycoheterotrophic plants (Hynson & Bruns 2010). Due to the wide range of mycoheterotrophic interactions fungi engage in, it does not appear that specific fungal lineages are particularly more prone to cheating than others (Leake

& Cameron 2010), suggesting that mycoheterotrophic plants do not necessarily target particular clades of 'naïve' fungi (Merckx 2013).

All mycoheterotrophic plant lineages evolved from mutualistic mycorrhizal ancestors. In most cases, these ancestors were likely to have mutualistic interactions with mycorrhizal fungi of the same type than the extant mycoheterotrophic descendants (Merckx *et al.* 2013). However, in some plant clades, the evolutionary transition from autotrophy to mycoheterotrophy has been described to follow evident shifts in the identity of the associated mycorrhizal fungi (Motomura *et al.* 2010; Ogura-Tsujita *et al.* 2012; Yagame *et al.* 2016). Within Orchidaceae, some mycoheterotrophic species recruited non-mycorrhizal fungal lineages including wood-rotting and parasitic fungi (Brundrett 2002; Ogura-Tsujita *et al.* 2009). Mycoheterotrophic orchids exclusively dependent on saprotrophic fungi are quite widespread (Martos *et al.* 2009), including tropical rainforests, and also temperate forests (Hynson *et al.* 2013). So far, only orchids have been observed to be able to associate with fungi other than mycorrhizal ones, however there obviously remain mycoheterotrophic species to be studied, especially in the tropics.

Plant-fungus specificity

In general, host-parasite relationships tend to present a high degree of specificity (Page 2003). As a parasitic interaction, mycoheterotrophy is generally expected to exhibit greater host specificity with decreasing nestedness of their mycorrhizal networks compared to the mutualistic autotrophic plants (Bidartondo & Bruns 2005; van der Heijden *et al.* 2015; Põlme *et al.* 2018). Although this frequent high specificity is not fully understood, there are exceptions that show lack of specificity (Hynson & Bruns 2009; Roy *et al.* 2009). Also, even in partial mycoheterotrophy, high fungal specificity and fidelity are frequently observed throughout the entire life cycle of plants, probably due to selection for net overall fitness benefits for both partners over their lifetimes (Field, Leake, *et al.* 2015). Such an interaction for the fungi makes them vulnerable to plants not rewarding them back at any time of their life, favouring cheating to arise. In the case of fully mycoheterotrophs, where plants never pay back their fungi during their entire lifetime, plants may benefit from fine-tuning their physiological responses to match interactions with specific hosts, thus fostering broad host shifts unlikely (Hynson & Bruns 2010). Other mechanism such as partner filtering may occur instead, restricting these plants to cheat particular fungal lineages (Egger & Hibbett 2004).

There is evidence that mycoheterotrophic plants often present a high degree of host-fungus specificity at least within the EM symbiosis (Taylor *et al.* 2002; Bidartondo 2005a). The first indication of extreme fungal specificity has been shown in Monotropoideae where fidelity is displayed at species level among closely related species within the broad geographic distribution of the plant taxa, suggesting a complex geographical mosaic of specificity (Bidartondo & Bruns 2001). In many mycoheterotrophic species of Orchidaceae, sequencing data shows high specificity patterns (Ogura-Tsujita & Yukawa 2008; Ogura-Tsujita *et al.* 2009), and even some species e.g. *Corallorhiza*, *Gastrodia* and *Galeola* may only associate with a single fungal genus (Brundrett 2002; Taylor *et al.* 2004; Barrett *et al.* 2010). Mycoheterotrophic orchids exclusively dependent on saprotrophic fungi are still highly specific in their fungal partners (Ogura-Tsujita & Yukawa 2008; Ogura-Tsujita *et al.* 2009),

Testing specificity in AM mycoheterotrophic interactions remains poorly studied compared to EM mycoheterotrophs. On one hand, germination of seeds together with the AM partners represents a serious challenge since the system has not been cultured axenically successfully yet, hampering the functional characterization of both partners in the interaction. For EM mycoheterotrophs, culture in-vitro is also not possible, but transplantation experiments are feasible (Bidartondo & Bruns 2005). On the other hand, species delimitation in AM fungi is still a major challenge, impeding an objective way to evaluate the extent of diversity of their mycorrhizal interactions. The mycoheterotrophic interactions with AM fungi have been described to vary from very specialized to relatively general targeting a wide variety of taxa within the Glomeromycotina (Merckx *et al.* 2012). Moreover, AM fungi from different families within Glomeromycotina have been occasionally sequenced in the roots of some mycoheterotrophs, suggesting an ample diversity in their associations. Yet, due to the non-consistent recovery of more diverse communities in the majority of individuals, the hypothesis is that they may represent facultative associations (Merckx *et al.* 2012; Renny *et al.* 2017; Merckx *et al.* 2017). Specificity in mycorrhizal associations within AM mycoheterotrophs have been mostly investigated from a phylogenetic perspective. *Petrosavia* species show higher mycorrhizal specificity comparing to their green plant relatives suggesting a selection and specialization towards their AM partners (Yamato *et al.* 2014). Similarly, certain *Burmannia* species have been found to associate with narrow and unique phylogenetic ranges of AM fungi (Yamato *et al.* 2011; Ogura-Tsujita *et al.* 2013), as well as in *Voyria* (Bidartondo *et al.* 2002) and the temperate *Thismia* clade, where species were found to target very narrow fungal lineages (Merckx *et al.* 2017).

Additionally, the African genera such as *Kupea* and *Afrothismia* were also found to be quite specific in their fungal interactions (Franke *et al.* 2006; Merckx & Bidartondo 2008).

Distribution of mycoheterotrophic plants

Fully mycoheterotrophic plant species are almost always found in forests, usually with a closed canopy that produces deep shade. As a consequence, their non-photosynthetic mode of life is often regarded as an adaptation to the low-light conditions of forest understories (Bidartondo *et al.* 2004; Bidartondo 2005b). Nevertheless, mycoheterotrophy is not exclusive to forest habitats. In the neotropics, several arbuscular mycorrhizal mycoheterotrophic species are reported to grow in wet grasslands (Maas *et al.* 1986). Specimens of *Arachnitis* have been found on the treeless East Falkland Island, 'growing in sand amongst rocks on an eroded sandstone ridge' (Cribb *et al.* 1995). In Africa, mycoheterotrophic orchids occur in woodland and wooded grassland (Cheek & Williams 1999). And, in the northern hemisphere, mycoheterotrophic Ericaceae species are often found in open vegetation, such as dune slacks (Wallace 1985; Leake 1994). Besides their general preference for forest habitats, mycoheterotrophic plants seem to prefer microhabitats with high soil moisture and humidity, acidic soils, and a thick layer of decaying leaf litter (Wallace 1985; Maas *et al.* 1986; Merckx 2013). Due to the latter, they have been traditionally described as 'saprophytes' (Leake 2005).

The majority of fully mycoheterotrophic species are found in tropical rainforests, but they have a global distribution, occurring on all continents except Antarctica. The species in the families Burmanniaceae, Thismiaceae, Corsiaceae, Triuridaceae, Petrosaviaceae, Iridaceae, Polygalaceae, and Gentianaceae, are – a few exceptions notwithstanding – all restricted to tropical and subtropical forests, where they grow on arbuscular mycorrhizal fungi (Leake 1994; Merckx 2013). Mycoheterotrophic Orchidaceae species also occur in tropical and subtropical forests where they are associated either with ectomycorrhizal (Roy *et al.* 2009) or saprotrophic fungi (Martos *et al.* 2009). In terms of species diversity, tropical Asia harbors most fully mycoheterotrophic species, closely followed by tropical South America. In comparison, tropical Africa is relatively poor in fully mycoheterotrophic species. While some tropical mycoheterotrophic species have very restricted occurrences, many species reach relatively widespread distributions, spanning many countries and in some cases almost an entire continent (Maas *et al.* 1986; Cheek & Williams 1999). Each species, however, is confined to a single continent. On the other hand, many genera and families (e.g. Burmanniaceae, Triuridaceae, Thismiaceae)

occupy multiple continents. Outside the tropics, fully mycoheterotrophic plants species of Orchidaceae and Ericaceae occur throughout the temperate and boreal forests of the northern hemisphere, where they associate mainly with ectomycorrhizal fungi. In contrast, the temperate forests of the southern hemisphere lack mycoheterotrophic Ericaceae species, and within these forests mycoheterotrophic species of Orchidaceae grow on saprotrophic fungi. The liverwort *Aneura mirabilis*, the only non-angiosperm full mycoheterotroph, is restricted to temperate forests in Europe, where it obtains carbon from surrounding trees through shared ectomycorrhizal fungi (Bidartondo *et al.* 2003).

Despite the relatively widespread distributions of some species, most mycoheterotrophic plants have highly patchy distributions and are locally rare (Wallace 1985; Leake 1994; Merckx 2013). The fact that multiple mycoheterotrophic species are often found growing at the same site (see, for example, Maas *et al.* 1986; Cheek & Williams 1999) but in association with different fungi, possibly indicates that distribution of mycoheterotrophic species is restricted by adaptations to similar microhabitats (Merckx 2013). These microhabitats can be characterized by certain abiotic factors such as soil type, humidity, and water availability, or biotic factors, such as the presence of certain mycorrhizal fungi, pollinators, or seed dispersers (Swarts & Dixon 2009).

Challenges in mycoheterotrophy research

As expected for cheaters in general, mycoheterotrophic plants are commonly considered to have specialized fungal interactions. In fact, evidence indeed piles towards a general increase of specificity in fungal interactions by mycoheterotrophs, but also with clear exceptions (Hynson & Bruns 2009; Roy *et al.* 2009). At the same time, the fungal diversity harbored by these plants is known for a few species only, and for the large majority of species, information on the identity of the fungal partners and range of associations is still lacking (see, for example, Bidartondo *et al.* 2002; Yamato *et al.* 2011; Renny *et al.* 2017). The high observed specificity can be potentially biased by the sampling of only few specimens, or populations, due to the rarity of these plants, and thus limited to the fungal pool available locally. Broader inventories of fungal diversity in areas where mycoheterotrophic plants occur are needed to address questions regarding for example partner choice.

Furthermore, the placement of these cheater plants in the mycorrhizal network that links autotrophic plants is still obscure. Evidence for the presence of this mycorrhizal

network supporting mycoheterotrophic plants is now being unraveled (Bidartondo *et al.* 2002; Yamato *et al.* 2011). Due to the reliance of mycoheterotrophic plants on their fungal partners, this type of information is of importance to understand how biotic interactions shape the distribution of mycoheterotrophic plants, including their typical co-occurrence patterns where distantly related species are often seen in the vicinity of each other (Merckx 2013). This may be due to sharing similar fungi, or by integrating the mycorrhizal networks in different modules. Thus, having a broader overview on the fungal interactions of these plants, including the available pool of fungal species found locally, can give us valuable insights about the influence of the biotic interactions for the mechanisms promoting the occurrence and coexistence of these plants.

Compared to their biotic interactions, our knowledge on the abiotic preferences of mycoheterotrophic plants is even more fragmented. Their rare and patchy distribution, together with their ephemerons flowering periods hinder the effective design of studies to target this subject. There is a general idea that mycoheterotrophic plants are adapted to low light conditions inside deep dark, humid forests, but no empirical data on this topic has been presented yet (Leake 1994; Bidartondo *et al.* 2004; Bidartondo 2005b; Merckx 2013). Moreover, little is known about the environmental conditions that influence the outcome of symbiotic mycorrhizal interactions. Even less is known about the conditions where plants are able to subvert the mutualistic interaction with their associated fungi.

Moreover, knowing the range of ecological settings under which cheating is prone to occur is essential to predict whether certain adaptations promoting mutualistic or antagonistic relationships are likely to evolve in a given interaction. Deepening our knowledge on the biotic and abiotic factors that drive the occurrence of mycoheterotrophic plants will allow us to obtain novel insights on the ecological preferences of these plants, and also allow us to predict the environmental conditions where the mutualistic mycorrhizal interaction has more chances to be cheated.

THESIS OUTLINE

The aim of my thesis is to shed light on the diversity, ecology and distribution of mycoheterotrophic interactions. For that, I combine perspectives from different levels of ecological complexity in mycoheterotrophic plants' systems which can give us valuable insights into the occurrence of cheating in mycorrhizal interactions (Figure

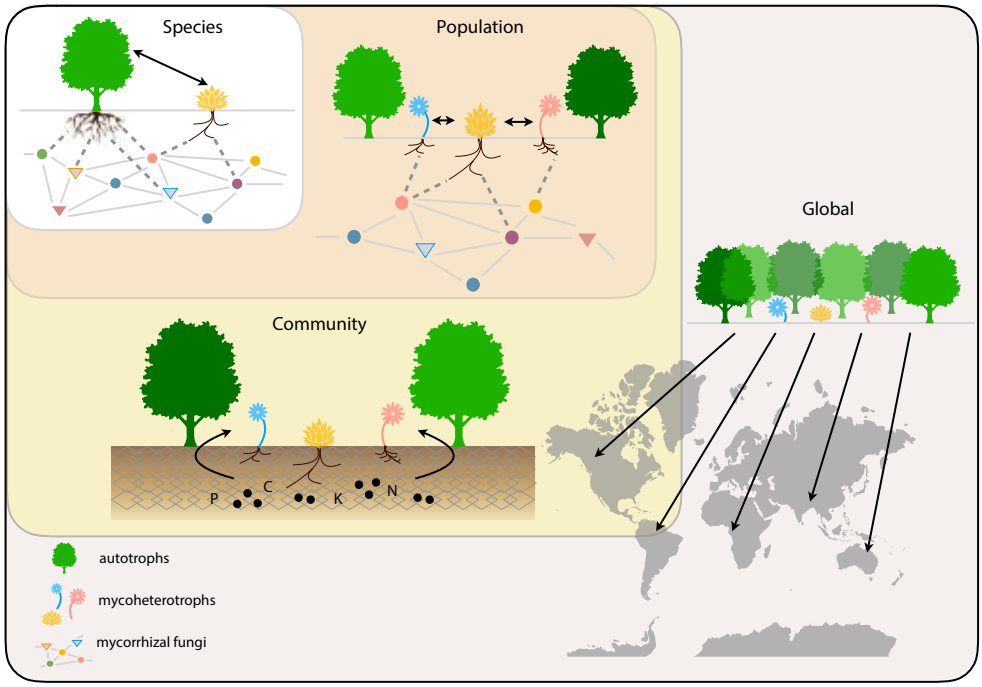


Figure 4 | Illustration of the different levels of ecological organization based on the chapters of this thesis to study the diversity, ecology and distribution of mycoheterotrophy. From single organism and its biotic interactions (chapter 2), to populations where species of mycoheterotrophic plants coexist with each other (chapter 3), to communities of mycoheterotrophic plants integrated in the environment (chapter 4), to finally niches at a global scale preferred by these plants (chapter 5).

4). Plant species together with their obligatory interactions with mycorrhizal fungi comprise the smallest organisational level (chapter 2). Followed by the interactions among distantly-related mycoheterotrophic plants searching for general mechanisms explaining fungal interaction patterns at the population level (chapter 3). Next, I investigate the edaphic properties where communities of mycoheterotrophic plants occur (chapter 4). Finally, I am interested in the general environmental drivers for the distribution of mycoheterotrophy at global scale (chapter 5).

Chapter 2

Mycoheterotrophic plants have an obligatory dependence on their mycorrhizal fungal partners. Previous studies have suggested a general predisposition for increased specialization in the associated AM fungi in the course of evolution from autotrophic

to parasitic interactions (Yamato *et al.* 2014). These studies have compared the fungal range of interactions of mycoheterotrophic taxa with their closely-related autotrophic relatives. However, most of these studies disregard the local ecological conditions where the plants occur, which can directly influence the range of available mycorrhizal partners, since the habitats of closely-related autotrophs are often divergent. In the chapter: 'Arbuscular mycorrhizal interactions of mycoheterotrophic *Thismia* are more specialized than in autotrophic plants' (Gomes *et al.* 2017a), I target a highly specialized clade of mycoheterotrophic plants, confined to Australia and New Zealand, and compare the arbuscular mycorrhizal interactions of these plants with the surrounding autotrophic plants, testing the hypothesis of phylogenetic niche conservatism in their fungal interactions.

Chapter 3

Curious enough is the patchy distribution displayed by mycoheterotrophs, but the co-occurrence of distantly-related taxa within these patches is even more remarkable (Leake 1994; Merckx 2013). Possible explanations for this phenomenon range from the existence of suitable environmental conditions compatible with the heterogeneity of soil resources availability (which will be dealt with in chapter 4) to a constrained distribution due to the local availability of selected fungi that can be cheated. Yet, if the presence of the AM partners is the main driving force for the co-occurrence of mycoheterotrophic plants, then we would expect that some cheater plants would outcompete others. Alternatively, these plants may rely on mechanisms to avoid competition and allow for a stable coexistence. This chapter entitled 'Fungal-host diversity among mycoheterotrophic plants increases proportionally to their fungal-host overlap' (Gomes *et al.* 2017b) explores the mycorrhizal host range of 20 mycoheterotrophs collected in French Guiana, and proposes a comprehensive framework of their co-occurrence.

Chapter 4

Abiotic factors, specifically edaphic characteristics, can be very heterogeneous even at a small scale. Due to the patchy distribution that mycoheterotrophic plants often exhibit, it is likely that particular soil parameters play a role in their occurrence. With this chapter: 'Environmental drivers of cheating arbuscular mycorrhizas in tropical rainforests' (submitted), I intend to unveil the soil preferences that allow the occurrence of mycoheterotrophic plants at local scale. For that, I focus on tropical mycoheterotrophic

plants occurring in two bioregions in Colombia, particularly including the Amazon and the Chocó rainforests, in the northwest part of South America separated by the Andes mountains. These two regions have peculiar biogeographical histories and harbour quite unique flora and fauna. In this chapter, I compare soil nutrients and chemistry of plots where mycoheterotrophs are found with plots where they are absent. By integrating both sites, I expect to find a more general overview on the local drivers of mycoheterotrophic plants' occurrence.

Chapter 5

Going from local to global scale, the environmental drivers that drive the distribution of mycoheterotrophic plants are expected to vary. Thus, in the chapter entitled 'Global distribution of mycoheterotrophic plants' (submitted), I examine large-scale patterns of distribution of these plants considering the environmental preferences of the 15 independent lineages where mycoheterotrophy evolved within the flowering plants (Figure 3).

Chapter 6

In this final chapter, I integrate the knowledge about mycoheterotrophy obtained within my PhD study. Final considerations are made and future steps that in my view would greatly contribute to increase the understanding of this fascinating system are proposed.

CHAPTER 2

Arbuscular mycorrhizal interactions of mycoheterotrophic *Thismia* are more specialized than autotrophic plants

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Abstract

The belowground interaction between plants and arbuscular mycorrhizal (AM) fungi is one of the most widespread mutualisms on Earth. In general, plants and AM fungi exchange photosynthetically fixed carbon for soil nutrients, but occasionally non-photosynthetic plants obtain carbon from AM fungi. These mycoheterotrophic plants are suggested to have more specialized interactions than green plants, although comprehensive comparisons between their AM communities are lacking.

We used next-generation sequencing to compare the AM communities from five closely related mycoheterotrophic species of *Thismia* (Thismiaceae), surrounding green plants, and soil, sampled over the entire temperate distribution of *Thismia* in Australia and New Zealand. We observed that fungal communities are phylogenetically more similar within each functional group, suggesting a specific association pattern according to the plant trophic mode. Similarly, both types of plants presented more clustered fungal communities when compared to the fungal pool in the soil. Moreover, the fungal communities of mycoheterotrophic plants are phylogenetically more restricted than in green plants, independent of geographic origin.

Our findings demonstrate that these mycoheterotrophic plants target more narrow lineages of fungi, despite the larger fungal pool available in the soil, and thus they are more specialized towards mycorrhizal fungi than autotrophic plants.

Introduction

The interaction between arbuscular mycorrhizal (AM) fungi and over 80% of the land plants is one of the most widespread mutualism on Earth (Smith & Read, 2008). The AM fungi, abundant in most terrestrial ecosystems, are obligatorily associated with the roots of plants and act like extensions of plant root systems for increasing nutrient uptake, especially phosphorus. However, despite the ubiquity of the interaction, the mechanisms that control its above- and belowground diversity are not well understood (van der Heijden *et al.*, 1998).

Plant diversity and productivity are significantly influenced by the AM fungal diversity in the soil (van der Heijden *et al.*, 1998; Vogelsang *et al.*, 2006). A key component in plant productivity is photosynthetic fixation of inorganic carbon. It is this carbon that plants transfer to their mycorrhizal partners in exchange for soil nutrients (Smith & Read, 2008). Occasionally, plants lineages lose the ability to perform photosynthesis but maintain belowground links with mycorrhizal fungi. This phenomenon has long fascinated researchers, because in such systems, the expected outcome is that the fungi would also withdraw their participation in the interaction (Sachs & Simms, 2006). Instead, these non-photosynthetic plants, known as mycoheterotrophs, still harbour AM fungi growing in their roots (e.g. Leake 1994; Bidartondo *et al.* 2002; Merckx *et al.* 2012).

Mycoheterotrophy is an evolutionarily stable mode of life present in more than 20,000 plant species (Merckx, 2013). It is characterized by the absence of photosynthesis, in which plants obtain carbon via the mycorrhizal fungi associated with their roots. The only known way AM fungi obtain their carbon is through symbiosis with a photosynthetic plant. Thus, mycoheterotrophic plants must rely on established mutualisms between photosynthetic plants and AM fungi, becoming cheaters within three-partite interactions (Bidartondo, 2005a; Sachs & Simms, 2006). Mycoheterotrophy can occur (i) during a short period of the life cycle of a plant, subsequently replaced by an autotrophic mode of life such as in most orchids, many ferns and lycopods, (ii) during the entire life cycle of a plant such as in some orchids and monotropes, or (iii) simultaneously with autotrophy – partial mycoheterotrophy as in some orchids (Merckx, 2013). Thus, mycoheterotrophy can be seen as a dynamic interaction along a continuum of possible outcomes. Because mycorrhizal associations are generally mutualistic (Smith & Read, 2008), it is intriguing why, and which, fungi are part of a mycoheterotrophic interaction. In particular, the differences between mycorrhizal

associations of mycoheterotrophic and green plants, and potential preference towards particular fungal lineages, remains poorly known. Many mycoheterotrophic plants are known to have more specialized interactions towards basidiomycete fungi (i.e. they interact with fewer fungal lineages) than ectomycorrhizal green plants, presumably to increase their fitness by optimizing host adaptation (Cullings *et al.*, 1996; Bidartondo, 2005a). However, this pattern of increased specificity remains speculative for arbuscular mycoheterotrophic interactions, since comprehensive direct comparisons between AM interactions of mycoheterotrophic and green plants have not been reported. To investigate this, data about the mycorrhizal partners of mycoheterotrophic plants needs to be generated and compared to the fungal communities associated with green plants.

In the past years, the study of fungal diversity patterns has become more important in understanding the mechanisms driving plant biodiversity (Öpik *et al.*, 2009; Davison *et al.*, 2011; Peay *et al.*, 2013). Next-generation sequencing techniques to identify AM fungi allow assessments of the complex fungal communities in soil and plant roots (Toju *et al.*, 2014). However, species delimitation of the ancient and apparently strictly assexual AM fungi has long been debated and no consensus has been achieved for suitable molecular markers with sufficient resolution for species-level identification, nor for the cut-off values to be used in clustering operational taxonomic units for species prediction (Bruns & Taylor, 2016). Thus, measuring species richness with standard methods may introduce a bias in the assessment of fungal communities' composition. To better understand how communities are structured, an integration of phylogenetic structure, trait information and community composition can offer relevant insights on the evolutionary and ecological processes shaping communities (Webb *et al.*, 2002). At the community scale, species should be segregated based on relative strengths of habitat filtering and competition among similar species. Community structure can be phylogenetically clustered, random, or over-dispersed on the phylogeny of the entire available pool of species. For example, Kembel & Hubbell (2006) have found that phylogenetic structure of rainforest tree communities varied among habitats in Panama. They found communities with more closely related taxa than expected by chance (phylogenetically clustered), suggesting a stronger habitat filtering as the driving force of community assemblages, while other communities were composed by more distant taxa (overdispersion), suggesting current or past competitive exclusion between closely related taxa, or convergent evolution of important traits for persistence in such habitats.

In this study, we consider a community to be composed by fungal OTUs belonging to the same trophic level and the same guild (AM fungi: mycorrhizal fungi from the Glomeromycota phylum) co-occurring spatially in the roots of a plant. We compare the phylogenetic structure of the fungal communities associated with *Thismia* plants and co-occurring green plants (comparing plant nutrition types: mycoheterotrophic and autotrophic) confined to the distribution area of the selected mycoheterotrophic lineage, by studying the fungal community composition in their roots using high-throughput DNA sequencing methods. We focus on the temperate mycoheterotrophic *Thismia* species to evaluate the mycorrhizal associations patterns within an entire mycoheterotrophic plant clade. Because specificity in biotic interactions may differ considerably over a species' distribution range (Thompson, 2005), we study the interactions over the geographic range of this *Thismia* clade. Soil samples were included to estimate the fungal pool available for these species. To evaluate general differences in fungal community structure between mycoheterotrophic and autotrophic plants, we use phylogenetic measures to infer community structure.

Materials and Methods

Sampling

We sampled temperate forest sites in Australia and New Zealand over the known distribution of the *Thismia* clade in the region. At each site, one to five *Thismia* specimens where sampled, at least 1m from each other. This resulted in sampling 18 sites within three broad areas: 4 in New South Wales (NSW), 10 in Tasmania (TAS) and 4 in New Zealand (NZ).

At each site, the entire root system of *Thismia* and root tips (c. 1 cm) of surrounding plants where taken and preserved on CTAB buffer. The sampling of the surrounding green plants was done by selecting up to eight root tips of green plants found in the same soil clump as *Thismia*. To estimate the fungal pool available for all plant species, soil was sampled from the soil clump as well. Soil was dried on silica gel before DNA extraction. The sampling effort resulted in 99 samples, including MH, green plants and soil (Supporting Information Table S1). All plant roots were identified using molecular methods (Supporting Information Methods S1).

Assessment of fungal communities using ION TORRENT

Fungal DNA was extracted from the CTAB preserved roots with the KingFisher Flex Magnetic Particle Processor (Thermo Scientific, USA) using the NucleoMag 96 Plant Kit (Machery-Nagel, Germany). Subsequently, amplicon libraries were created to amplify the internal transcribed spacer (ITS2), using the fungal specific primer fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) with a unique MID (Multiplex Identifier) label per sample, following the protocol described in Ihrmark *et al.* (2012). Sequencing was performed with a Personal Genome Machine (ION TORRENT; Life Technologies, Guilford, CT, USA) with 850 flows. Sequences obtained were processed using the UPARSE algorithm (Edgar 2013) incorporated in USEARCH v.7 (http://www.drive5.com/usearch/). Fastq files were screened for quality control and trimmed at the first base with Phred score of $Q < 20$. Followed by derreplication, singletons and sequences with less than 100 bp filtered out, the resulting sequences were clustered into OTUs at 97% similarity (Blaalid *et al.*, 2013). The taxonomy was assigned to the OTUs with UPPARSE, based on the UNITE + INSD database (10.09.2014) implemented with the current Index Fungorum identification. Only OTUs belonging to the Glomeromycota were kept for further analysis. The rawdata were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession SRP083901. Because of the imbalanced number of specimensobtained for mycoheterotrophic and green plants, we calculated the species richness estimate CHAO2 (Chiu *et al.*, 2014) for each plant group, using the function SPECPOOL in the VEGAN R package (Oksanen *et al.*, 2015).

Fungal community dissimilarities among samples

We calculated the phylogenetic relatedness between the OTUs to measure community differences between samples. An alignment of the OTU sequences and several reference Glomeromycota taxa from (Krüger *et al.*, 2012) was constructed with MAFFT (Katoh & Standley, 2013). Phylogenetic inference on the OTU sequences was performed with RAXMLHPC-SSE3 (Stamatakis, 2014) using the GTR+G+I model of substitution as determined by JMODELTEST v2.1.5 using the Akaike information criterion (AIC; Darriba *et al.*, 2012). The phylogenetic distances among fungal OTUs given the highest-likelihood tree were used to obtain a fungal community dissimilarity matrix between all the pairs of samples, using the function COMDIST in the PICANTE R package (R Development Core Team, 2008; Kembel *et al.*, 2010). This algorithm finds for each fungal OTU in

one sample the average distance to all the OTUs in the other sample, and calculates the mean of these phylogenetic distances. The fungal community dissimilarities were visualized by performing a metaMDS in the vegan R package (Oksanen *et al*., 2015). We investigated whether these fungal community dissimilarities differed between the 'type' of material (MH: mycoheterotrophic plants; green: green plants; and soil) and 'region' (New South Wales, Tasmania and New Zealand) with a permutational MANOVA using the function adonis in the vegan R-package (Oksanen *et al*., 2015).

In addition, we explored whether the community dissimilarity patterns observed in the *Thismia* species were correlated with the plant evolutionary relationships. For that, we computed the Mantel test correlation between the fungal community dissimilarity matrix and the phylogenetic distance matrix among the *Thismia* species (see Supporting Information Methods S2 for detailed methods).

Fungal phylogenetic community structure

To investigate the fungal community structure, we calculated the phylogenetic community structure indices developed by Webb (2000) for community assessment of rainforest trees, which have also been successfully applied for fungal community studies (e.g. Peay *et al*. 2010; Maherali & Klironomos 2012). The net relatedness index (NRI) and the nearest taxa index (NTI) measure the degree of phylogenetic clustering of a group of taxa over the whole pool of taxa in a phylogenetic tree or within particular terminal clades, respectively. Positive values indicate that the fungal OTUs are more closely related to one another than expected by chance (phylogenetic clustering), and negative values indicate that the fungal OTUs are more distantly related (phylogenetic evenness). The NRI measures the overall clustering across the phylogeny using the average pairwise distance of all taxa from a community. NRI is then equal to $1 - (MPD_{observed} - MPD_{random})/SD(MPD_{random})$, where MPD stands for mean phylogenetic distance, which measures phylogenetic distance among taxa using the pairwise branch lengths distances. The pairwise phylogenetic distances among the fungal taxa were obtained from the fungal OTUs phylogeny. Numerically, NRI is the inverse of the standardized effect size of the MPD, which compares the average phylogenetic relatedness in the observed and null communities, under a null model of randomizations, standardized by the standard deviation (SD) of phylogenetic distances in the null community (Webb *et al*., 2002). We obtained 999 randomizations shuffling the tips of the phylogeny from the total pool of fungal taxa. The NTI measures the terminal clustering among the taxa from the

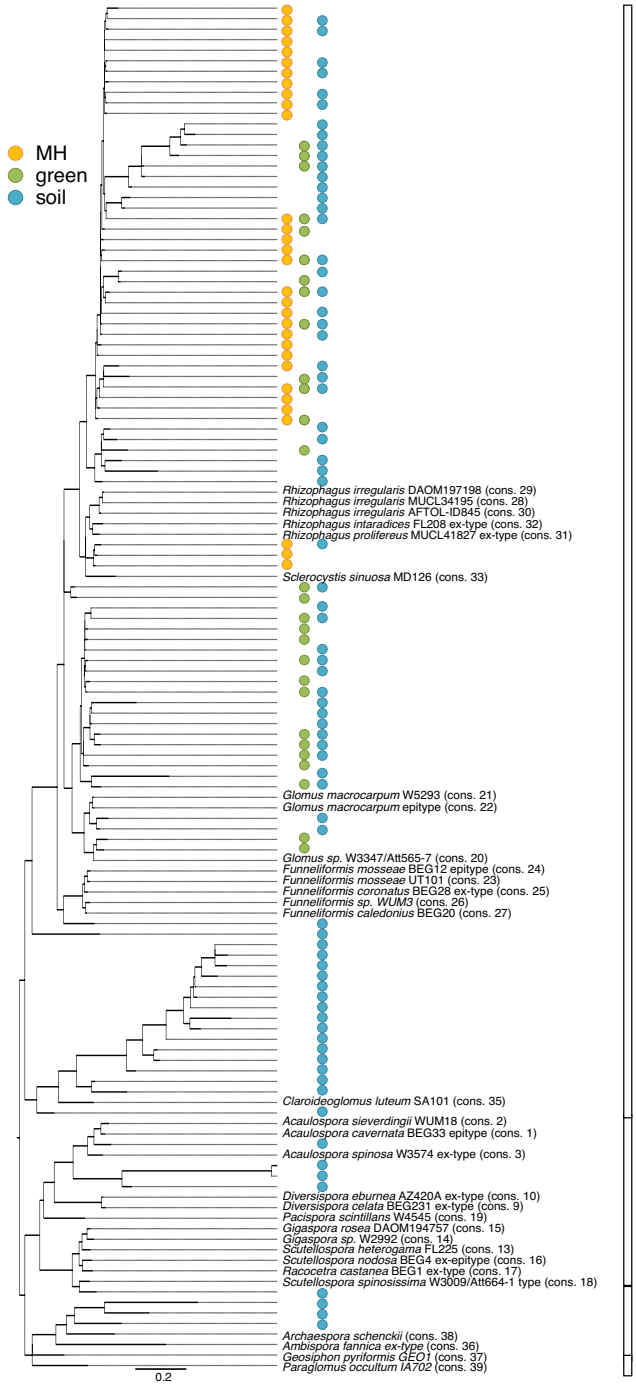
community. NTI is then equal to 1 – ($MNTD_{observed}$ – $MNTD_{random}$)/SD($MNTD_{random}$), where MNTD stands for mean nearest phylogenetic taxon distance, which measures the minimal distance separating each species in the community. Numerically, NTI is the inverse of the standardized effect size of the MNTD, calculated similarly as MPD (Webb *et al*., 2002). The standardized effects of the MPD and MNTD measures were calculated using PICANTE R-package (Kembel *et al*., 2010).

In addition, for *Thismia* we reconstructed the NRI value of themost recent common ancestor of the clade based on the plant phylogenetic tree (pruned to contain only one taxon per species) and NRI values per species. Reconstruction was performed using phylogenetic independent constrasts (Felsenstein, 1985) as implemented in APE (Paradis *et al*., 2004).

General patterns of fungal community structure

Because we were interested in general patterns of community structure, such as the specificity of interactions per trophic strategy, we focused on the NRI for an overall view of community clustering along the phylogeny. The observation of an overall phylogenetic clustered pattern indicates more specialized interactions, where the targeted fungal OTU taxa are more closely related than expected by chance. An overall phylogenetic overdispersion pattern suggests that the interactions are more generalist,where the targeted taxa are more spread out over the phylogenetic tree than expected by chance. In order to test the effects of the 'type' of material (mycoheterotrophic, green plants, and soil) on the NRI, we constructed a linear mixed-effects model with NRI as the response variable and 'type' of material as the predictor variable. We considered 'region' as a random factor to account for the nonindependence of the collections within and across regions. We then used a post hoc pairwise comparison test (Tukey's honest significant difference (HSD)) to assess whether the three types of material differed significantly from each other in their NRI.

Figure 1 (next page) | Highest likelihood tree (*LnL* = 10519.28) showing the phylogenetic relationships among the Glomeromycota operational taxonomic units (OTUs) found in all the samples, including several reference sequences. The colored circles indicate the presence of the fungal OTUs according to plant group (mycoheterotrophic, yellow; autotrophic, green) and the pool of fungal OTUs present in the soil (blue). Mycoheterotrophic plants of the genus *Thismia* are associated with fungi in the Glomerales family (one subclade: *Rhizophagus*/*Sclerocystis* sp.); and green plants are also associated with fungi in the Glomerales family (two subclades: *Rhizophagus* / *Sclerocystis* sp. and *Glomus* sp.). The soil also harbors fungi from the Glomerales family, and also from the Diversisporales and Archaeosporales families within the Glomeromycota phylum.


MH
green
soil
Rhizophagus irregularis DAOM197198 (cons. 29)
Rhizophagus irregularis MUCL34195 (cons. 28)
Rhizophagus irregularis AFTOL-ID845 (cons. 30)
Rhizophagus intaradices FL208 ex-type (cons. 32)
Rhizophagus prolifereus MUCL41827 ex-type (cons. 31)
Sclerocystis sinuosa MD126 (cons. 33)
Glomus macrocarpum W5293 (cons. 21)
Glomus macrocarpum epitype (cons. 22)
Glomus sp. W3347/Att565-7 (cons. 20)
Funneliformis mosseae BEG12 epitype (cons. 24)
Funneliformis mosseae UT101 (cons. 23)
Funneliformis coronatus BEG28 ex-type (cons. 25)
Funneliformis sp. WUM3 (cons. 26)
Funneliformis caledonius BEG20 (cons. 27)
Claroideoglomus luteum SA101 (cons. 35)
Acaulospora sieverdingii WUM18 (cons. 2)
Acaulospora cavernata BEG33 epitype (cons. 1)
Acaulospora spinosa W3574 ex-type (cons. 3)
Diversispora eburnea AZ420A ex-type (cons. 10)
Diversispora celata BEG231 ex-type (cons. 9)
Pacispora scintillans W4545 (cons. 19)
Gigaspora rosea DAOM194757 (cons. 15)
Gigaspora sp. W2992 (cons. 14)
Scutellospora heterogama FL225 (cons. 13)
Scutellospora nodosa BEG4 ex-epitype (cons. 16)
Racocetra castanea BEG1 ex-type (cons. 17)
Scutellospora spinosissima W3009/Att664-1 type (cons. 18)
Archaespora schenckii (cons. 38)
Ambispora fennica ex-type (cons. 36)
Geosiphon pyriformis GEO1 (cons. 37)
Paraglomus occultum IA702 (cons. 39)
0.2
Glomerales
Diversisporales
Archaeosporales
Paraglomales

Results

Plant identification

We successfully obtained sequences from the roots of 60 specimens of five *Thismia* species, 24 specimens of 11 green plant species and 25 soil samples (see Table S1 for details). The *Thismia* species were identified as *Thismia clavarioides* K. R.Thiele, *Thismia hillii* (Cheeseman) N. Pfeiff., *Thismia megalongensis* C. Hunt, G. Steenbeeke & V. Merckx, *Thismia rodwayi* F. Muell., and a fifth species that remains to be described, here termed *Thismia* sp. For the green plants, we identified the following species (Methods S1): Apocynaceae sp.; *Laurelia novae-zelandiae* A. Cunn., and *Doryphora sassafras* Endl. (Atherosper-mataceae); Bignoniaceae sp.; *Ceratopetalum apetalum* D. Don (Cunoniaceae); *Beyeria viscosa* Labill. (Euphorbiaceae); *Acacia* sp.(Fabaceae); *Beilschmiedia tawa* (A. Cunn.) Kirk (Lauraceae); *Pomaderris apetala* Labill. (Rhamnaceae); *Nematolepis* sp. Turcz. (Rutaceae); and Vitaceae sp. (Table S1). The success rate of sequencing Glomeromycota fungi from the autotrophic plants was considerably lower than for *Thismia*, and for several surrounding root samples we failed to obtain Glomeromycota OTUs. Some of the autotrophic plants are putatively ectomycorrhizal, which may explain the absence of Glomeromycota OTUs in surrounding roots. *Pomaderris apetala* and *Acacia* sp. can be both ectomycorrhizal and AM, and all other species are described as AM (Brundrett, 2008), except for *Beyeria viscosa* and *Nematolepis* sp. for which the mycorrhizal status is unknown, making them suitable for the comparisons in the downstream analysis.

Fungal sequences

Ion Torrent sequencing produced 4 038 169 raw sequences, of which 3 836 916 passed the quality filtering. After the quality control steps, the resulting sequences were clustered at 97% similarity, generating 466 OTUs, of which 99 OTUs were assigned to Glomeromycota and kept for subsequent analysis. Of these, 31 OTUs were found in the mycoheterotrophic plants, 28 OTUs were found in the green plants and 69 OTUs were found in the soil. The number of OTUs was not linearly correlated to the variable number of reads per sample, and thus neither is it linearly correlated to the number of OTUs per type of material (see Fig. S2). Using the Chao2 estimator, we obtained richness estimates of 32.26 ± SD 1.77 for mycoheterotrophic plants, 36.61 ± SD 6.30 for green plants, and 101.67 ± SD 15.12 for soil. Fig. 1 shows the highest likelihood phylogeny among the fungal OTUs and respective presence in mycoheterotrophic

plants, green plants andsoil. The fungal communities of the five *Thismia* species included Glomeromycota in the *Rhizophagus/Sclerocystis* sp. subclade; for the green plants, the same clades of fungi were present with the addition of the *Glomus* sp. subclade; in the soil, Glomerales, Diversisporales and Archaeosporales fungi were present.

Fungal community dissimilarities

Fungal community dissimilarities were calculated among all the samples, including mycoheterotrophic plants, green plants and soil. In Fig. 2, a nonmetric multidimensional scaling plot shows an ordination of the fungal community dissimilarities. Furthermore, we found no phylogenetic signal on the fungal community dissimilarities among the different *Thismia* species (Mantel test: $r = 0.092$; $P = 0.196$). Thus, we proceeded with the fungal community dissimilarity analysis including the green plants and looked for patterns within the 'type' of material (mycoheterotrophic plants, green plants and soil), and we also looked for geographic patterns ('region': Tasmania, New South Wales and New Zealand). Permutational MANOVA (Adonis) showed significant fungal

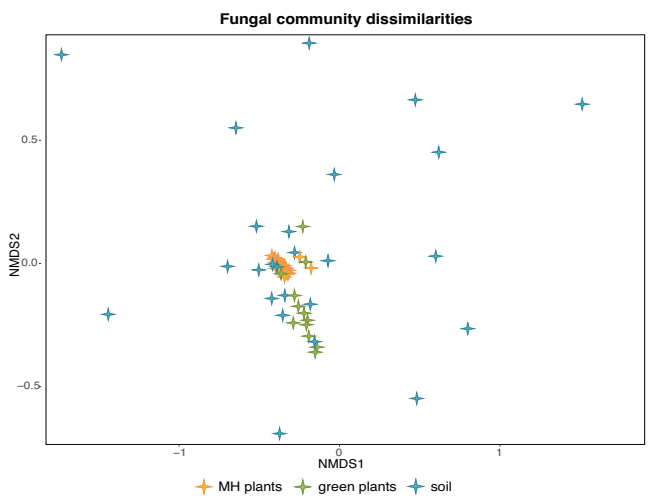


Figure 2 | Nonmetric multidimensional scaling plot (metaMDS) showing an ordination of the fungal community dissimilarities (Comdist) among all the samples. The fungal community dissimilarities are calculated based on the average phylogenetic distance between each fungal operational taxonomic unit (OTU) in one sample and the total OTUs in the other sample. Each symbol represents the Comdist of the fungal communities including all the OTUs found in each species per site. Permutational MANOVA (Adonis) showed significant fungal community dissimilarity between mycoheterotrophic (MH) *Thismia* plants, green plants, and soil ($F = 25.4$; $R^2 = 0.486$; $P = 0.001$).

Table 1 | Net relatedness index (NRI) and nearest taxa index (NTI) results for the fungal communities of mycoheterotrophic (MH) plants (*Thismia*), green plants and soil.

Type	Samples	N	NRI	RGR	NTI	RGR
MH plants	*T. rodwayi* 1 TAS	12	4.14**	999	2.38**	999
	T. rodwayi 2 TAS	4	2.16**	996	1.77**	996
	T. rodwayi 3 TAS	9	3.42**	999	2.13**	999
	T. rodwayi 4 TAS	14	4.49**	999	2.44**	999
	T. rodwayi 5 TAS	12	4.13**	999	2.34**	999
	T. rodwayi 6 TAS	3	1.79**	999	1.65**	998
	T. rodwayi 7 TAS	8	3.23**	999	2.12**	999
	T. rodwayi 8 TAS	9	3.41**	999	2.14**	999
	T. rodwayi 9 TAS	12	4.20**	999	2.41**	999
	T. rodwayi 10 TAS	8	3.24**	999	2.09**	999
	T. clavarioides NSW	5	2.53**	999	1.72**	995
	Thismia sp. NSW	3	1.80**	997	1.55**	998
	Thismia sp. NSW	4	1.74**	979	1.44**	957
	T. billii NSW	7	3.00**	999	2.08**	999
	T. megalongensis NSW	6	2.67**	999	2.08**	999
	T. billii 1 NZ	9	3.29**	999	2.17**	999
	T. billii 2 NZ	4	1.95**	991	1.65**	994
	T. billii 3 NZ	6	2.68**	998	2.04**	999
	T. billii 4 NZ	6	2.61**	999	1.96**	999
green plants	*Acacia* sp. TAS	2	0.92	818	0.92	808
	Beyeria viscosa TAS	2	0.53	567	0.54	545
	Pomaderris apetala TAS	4	1.13	788	1.11	840
	Nematolepis sp. TAS	2	1.24*	919	1.21*	935
	Acacia sp. NSW	2	1.20	892	1.19	868
	Bignoniaceae sp. NSW	3	1.66**	986	1.52**	975
	Ceratopetalum apetallum NSW	3	1.02	774	0.97	788
	Doryphora sassafras NSW	10	2.97**	999	1.70**	975
	Vitaceae sp. NSW	7	2.15**	988	1.44*	921
	Apocynaceae sp. NSW	6	1.30	887	0.84	739
	Beilschmiedia tawa NZ	3	0.75	656	0.55	624
	Laurelia novae-zelandiae NZ	13	2.28**	983	1.93**	993
Soil	Soil 1 TAS	9	0.06	523	-0.91	181
	Soil 2 TAS	2	1.19	890	1.19	892
	Soil 3 TAS	2	-1.13	153	-1.14	157
	Soil 4 TAS	6	2.65**	999	2.07**	999
	Soil 5 TAS	11	-1.26	105	-1.85	38
	Soil 6 TAS	5	0.59	735	0.46	669
	Soil 7 TAS	2	-1.43	69	-1.46	67
	Soil 8 TAS	3	0.74	679	0.84	777
	Soil 9 TAS	6	1.16	829	0.67	722
	Soil 10 TAS	9	-1.86	30	2.02**	994
	Soil 11 TAS	2	1.32**	993	1.39**	995
	Soil 12 TAS	13	-0.26	385	1.86**	982
	Soil 13 TAS	14	-0.92	184	1.04	855
	Soil 14 TAS	2	-1.49	70	-1.52	71
	Soil 15 TAS	7	0.65	717	0.61	709
	Soil 16 TAS	4	2.20**	996	1.77**	997
	Soil 17 TAS	5	0.79	757	0.57	717
	Soil 18 TAS	3	0.04	561	0.46	648
	Soil 1 NSW	4	-0.67	273	-0.44	312
	Soil 2 NSW	16	-0.38	376	2.36**	998
	Soil 3 NSW	3	1.34*	933	1.36*	945
	Soil 4 NSW	3	1.65**	971	1.50**	993
	Soil 5 NSW	2	0.33	496	0.30	474
	Soil 6 NSW	3	0.80	667	0.95	812
	Soil NZ	6	1.30	864	1.29	893

Samples, species per site; *n*, number of OTUs in a community; *RGR*, number of times the observed NRI or NTI was greater than the value obtained for the random permuted communities.

*Communities significantly structured at the $P = 0.10$ level.
*Communities significantly structured at the $P = 0.10$ level.

community dissimilarity for 'type' of material (F = 25.4; R^2 = 0.486; P = 0.001), but not for 'region' (F = 0.925; R^2 = 0.018; P = 0.427). These results suggest a distinctive andspecific association pattern of the fungal communities for mycoheterotrophic plants, green plants and soil, regardless of the region in which they occur.

Fungal phylogenetic community structure

We observed that all the mycoheterotrophic plants exhibited positive and significant NRI and NTI values (Table 1), which indicates a significant phylogenetic structure of the fungal communities. The two indices were correlated (Fig. S3). By contrast, most of the green plants and soil communities were phylogenetically randomly structured for both indices (Table 1). The roots of mycoheterotrophic plants tended to be colonized by AM fungi that were more closely related than expected by chance. The green plants tended to show no clear pattern in general, except for five species that presented phylogenetic structure. The soil also seemed to be mostly randomly phylogenetically structured. Overall, the two indices were concordant. The NRI of the most recent common ancestor of the *Thismia* clade was reconstructed to be 4.00 (95% confidence interval (CI) 3.26–4.74; see Fig. S4).

General patterns of fungal community structure

The mixed-effects model results showed that the fungal community structure was significantly explained by the 'type' of material. The fungal communities associated with the mycoheterotrophicplants were significantly more closely related to each other than in the case of the green plants and the soil. Likewise, for the green plants, the fungal communities were also significantly more closely related to each other than in the soil (see Fig. 3; Table S2).

Discussion

The plant sampling was designed to investigate the fungal community structure of closely related mycoheterotrophic plant species over their entire geographic range and, at the same time, compare their fungal community structure with that of the surrounding autotrophic plants, as a proxy for mycoheterotrophic and autotrophic types of nutrition, respectively. The soil data were used as a proxy for the diversity of local AM

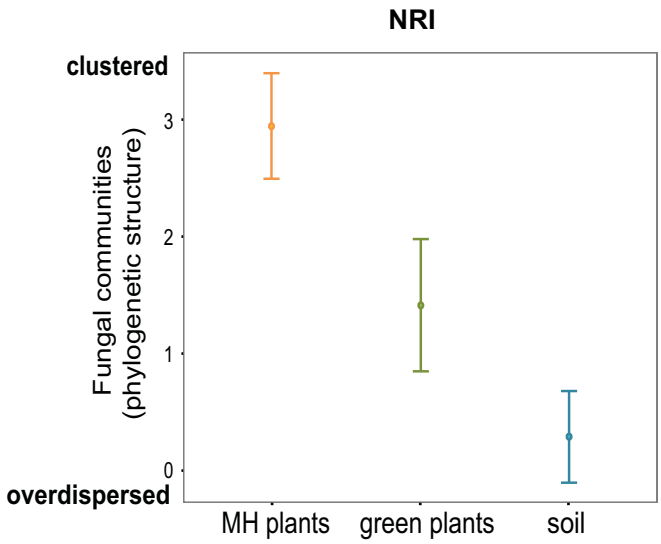


Figure 2 | Fungal community structure based on the net relatedness index (NRI) for each species per site. The graph represents the fungal communities' phylogenetic dispersion patterns as explained by the 'type'of material (mycoheterotrophic (MH) plants, green plants and soil). Negative NRI values indicate that the fungal communities are overdispersed in the phylogenetic tree, while positive NRI values indicate phylogenetic clustering. The NRI was significantly different in MH plants compared with green plants and soil. MH plants harbor more phylogenetically clustered AM fungal communities in their roots than green plants and the soil. Green plants also have significantly more clustered fungal communities than the soil. The mixed-effects model estimates with 95% confidence intervals are shown. See Supporting Information Table S2 for statistical details.

fungi. As expected, the soil presented a higher fungal diversity compared with individual plants, as it harbors the fungal reservoir from which the plant species obtain their fungal partners (Table S1). Our results indicate that, in general, mycoheterotrophic and green plants have distinct fungal community compositions with no geographic pattern (Fig. 2; Adonis test). In addition, the five closely related *Thismia* species tended to associate with more closely related AM fungi more often than expected by chance. Observations of other cases of mycoheterotrophic species growing on narrow phylogenetic lineages of AM fungi have been reported previously, for example *Arachnitis* (Bidartondo *et al.*, 2002), *Afrothismia* (Merckx & Bidartondo, 2008), *Burmannia* (Ogura-Tsujita*et al.*, 2013) and *Petrosavia* (Yamato *et al.*, 2011). Moreover, we observed that the phylogenetic structure of the fungal communities can vary according to the type of nutrition of aplant (i.e. mycoheterotrophic vs autotrophic; see Fig. 3).

For the mycoheterotrophic plants, we detected significant NRI and NTI values (Table 1). These two indices provide information about community structure that is

different from that provided by richness or taxonomic composition. In view of the unequal number of specimens of mycoheterotrophic and green plants and differences in sequencing success, we calculated the improved richness estimator Chao2 of Chiu *et al.* (2014), incorporating a small sample correction. This estimator reduces the bias when the heterogeneity of species detection probabilities is relatively high (Chiu *et al.*, 2014). While the estimated richness was higher for the green plants than for the mycoheterotrophic plants, the observed richness was higher for the mycoheterotrophic plants. Considering phylogenetic relatedness among the taxa, we found that, within the Glomeraceae family, the fungi associated with mycoheterotrophic plants belonged to one subclade, while green plants had fungal partners in two subclades (Fig. 1). Thus, the higher estimated richness for the green plants corresponded to a higher phylogenetic diversity compared with the mycoheterotrophic plants.

The phylogenetic clustering pattern observed in the mycoheterotrophic plants' fungal communities reflected ecological rather than biogeographic patterns, as there was no geographical structure of the fungal communities. Moreover, the tendency of *Thismia* species to target the same narrow clades of AM fungi (Fig. S5), and their similar levels of mycorrhizal specificity (Table 1), also reconstructed to have been present in the most recent common ancestor of the clade (Fig. S4), strongly suggest that the high level of mycorrhizal specificity is prone to phylogenetic niche conservatism (Harvey & Pagel, 1991; Lord *et al.*,1995), that is, the tendency of these *Thismia* species to retain similar ecological traits (i.e. similar fungal communities) overtime (Wiens & Graham, 2005; Wiens *et al.*, 2010). The phylogenetic niche conservatism observed in *Thismia* may be attributable to a reduction in the potential range of ecological character evolution caused by fixation of ancestral traits, enabling the descendants within this plant lineage to be more successfully adapted inparticular and similar habitat types (Lord *et al.*, 1995). The reason for the preference for targeting certain lineages of AM fungi in this mycoheterotrophic interaction is still not well understood. It is certainly not caused by a limited local availability of AM fungi, because we detected a much larger and phylogenetically broader pool of available fungi in the soil. Similar to the explanation for the high host specificity of many parasites, the mycoheterotrophs may fine-tune their physiology on particular lineages of fungi to maximize their carbon uptake (Leake & Cameron, 2010). Alternatively, the mycoheterotrophic plants may be rejected by most fungal lineages in the pool of available fungi, and therefore the pattern would result from an evolutionary arms race (Bidartondo, 2005). Therefore, it is our interpretation that the fungal communities associated with these mycoheterotrophic plants might

have been shaped not only by habitat filtering (occurrence of the fungal partners in space), but also by an effect of the ancestry of the plant species, which allow this local third-party cheater (*Thismia*) to participate in the globally mutualistic AM interaction with autotrophic plants.

For the green plants, some species showed significantly phylogenetically clustered AM fungal communities (Table 1). Specific patterns in the fungal associations of green plants have been previously reported in other studies (e.g. Öpik *et al.*, 2009; Davison *et al.*, 2011; Peay *et al.*, 2013). Nonetheless, other green plants in our study presented a randomly assembled fungal community. This may reflect a different community structure according to plant species, but it may also be an effect caused by an underrepresentation of the fungal communities, which was more likely to occur in the green plants than in the mycoheterotrophic plants because of sampling method limitations. For the green plants we could only collect a few centimeters of the extensive root system, so, because of the scattered pattern of AM fungal colonization along the roots, we may have assessed a limited fraction of the whole diversity, while for the mycoheterotrophic plants, we collected the entire small root system. Nevertheless, we do not think that this underrepresentation of green plants' fungal communities introduced bias to our results, because although it could be assumed that we were observing partial diversity, we obtained less phylogenetic clustering in green plants than in mycoheterotrophic plants. The phylogenetic clustering of these communities would become even more diluted with the introduction of more phylogenetically different taxa in the analysis, and therefore the specificity would decrease (Webb, 2000). Generally, the comparison of fungal communities associated with mycoheterotrophic and autotrophic plants showed that this particular lineage of mycoheterotrophic *Thismia* species have significantly more specialized interactions than the green plants living in the same regions (Fig. 3). Mycoheterotrophic plants had significantly more specialized fungal interactions than green plants, because the mycoheterotrophs showed higher NRI values almost exclusively. Similarly, mycoheterotrophic plants also had generally higher ranks of NTI values (Table 1). This suggests that, within the Glomerales subclade targeted by mycoheterotrophic plants, these plants also tend to target specific lineages at a lower taxonomic level. These results support the view that mycoheterotrophic mycorrhizal interactions are highly specialized. By contrast, green plants did not always show significantly clustered patterns. If we excluded the green plants for which we detected fewer than three OTUs (minimum number of OTUs found in the *Thismia* species), we found that half of the autotrophic plants (*Doryphora sassafras*, Bignoniaceae sp., *Laurelia novae-*

zelandiae and Vitaceae sp.) tended to associate with more closely related main lineages of AM fungi than expected by chance, but generally with lower ranks of positive NRI and NTI values compared with *Thismia*. We also found that the other half (Apocynaceae sp., *Ceratopetalum apetalum*, *Beilschmiedia tawa* and *Pomaderris apetala*) did not present a significantly clustered pattern. In conclusion, even though some green plants may also tend to target more closely related AM fungal taxa than expected by chance, in general these green plants have less specialized interactions compared with *Thismia*. In this study, we tested the association between these two ecological traits (type of plant nutrition (mycoheterotrophic vs autotrophic) and phylogenetic fungal community structure) for these *Thismia* species and surrounding green plants. The study of fungal community structure needs to be extended to other distantly related lineages of mycoheterotrophic plants before we make generalizations about the processes shaping the fungal interactions involved in mycoheterotrophy. Moreover, understanding how the fungal communities associated with plants in general are assembled can provide us with knowledge of how belowground ecological processes influence the global distribution of plants in ecosystems.

Acknowledgements

The authors thank Wendy Potts and Micah Visoiu (Tasmanian Department of Primary Industries, Parks, Water & Environment), Tim Wardlaw and Daniel Hodge (Forestry Tasmania), Neill Klaer, the National Parks and Wildlife Service NSW, the Blue Mountains City Council for permissions to sample *Thismia* in Australia. We thank Cheri Van Schravendijk and Terina Rakena (Waikato Tainui), Hannah Rainforth (Ngati Rangi) and Don Robinson (Uenuku Tribal Authority) for their time and consideration in consulting with us over *Thismia* collection on their respective lands, and the New Zealand Department of Conservation for authorisaton to collect under concession number CA-31615-OTH. Brian French, David Mabberley, James Wood, Peter de Lange, Mark Wapstra, Colin Hunt, Greg Steenbeeke, Rob Smissen, the staff at the National Herbarium of New South Wales, and the staff of the Royal Tasmanian Botanical Gardens are thanked for their assistance. This research is supported by a Veni grant of NWO to VSFTM (863.11.018) and by travel grants of the Alberta Mennega Foundation.

Author Contribution

S.I.F.G. and V.S.F.T.M planned and designed the research, V.S.F.T.M. collected the samples, S.I.F.G. generated the data and performed the analysis, J.A.-.G. participated in the data analysis, M.I.B. contributed to the interpretation of the data, S.I.F.G. wrote the manuscript. All the authors commented on the final version of the paper.

SUPPORTING INFORMATION

Methods S1

The plants collected in this study consisted of mycoheterotrophic and green species. The mycoheterotrophic species were identified by the genetic markers *ITS*, using the primers ITS1 and ITS4 (White *et al.*, 1990) and *cob*, using the primers COB1F and COB1R (Petersen *et al.*, 2006; GenBank accessions KX790794–KX790923). Partial *matK* sequences were obtained from the root tips DNA extractions of the surrounding plants and leaf samples of identified species collected at the sites using primers trnK685F and matK1777R (Hu *et al.*, 2000). For several plant samples from sites in New South Wales this did not result in amplification products. For these plants partial *trnL* sequences were obtained with primers trnL-f and trnL-c (Taberlet *et al.*, 1991). Root tips were identified based on their sequence similarity with the leaf samples and / or BLAST searches on GenBank.

Figures

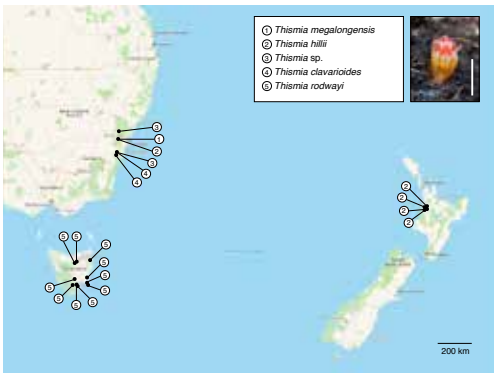


Figure S1 | Map of sampling localities. A total of 18 sites were sampled within three broad areas: 4 in New South Wales, 10 in Tasmania and 4 in New Zealand. Inset shows a flower of *Thismia rodwayi* as illustration of one of the species (bar, 1 cm).

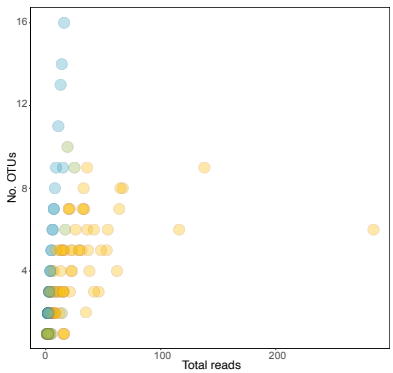


Figure S2 | Plot of the total number of OTUs against the total number of reads. The total number of reads originated by the Ion Torrent run after the quality control steps (excluding sequences with $Q < 20$) was plotted against the number of OTUs after clustering at 97% similarity, across all samples. Pearson correlation test ($r = 0.31$, $P < 0.05$) shows a weak correlation between the number of reads and the number of OTUs generated, but there is not a linear relationship ($r^2 = 0.096$, $P < 0.05$).

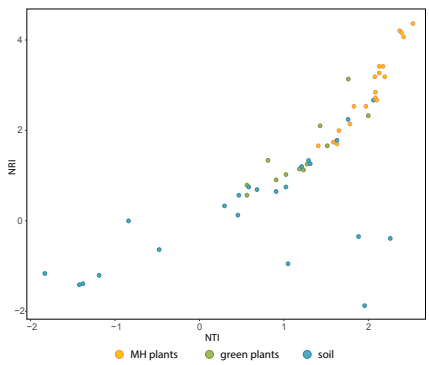


Figure S3 | Relationship between the net relatedness index and nearest taxa index. Pearson correlation test ($r = 0.77$, $P < 0.001$) shows that both indices (see Table 1) are correlated, indicating that an overall fungal communities' clustering or dispersal on the deeper nodes of the tree (NRI) corresponds to a similar extent of terminal clustering or dispersal, i.e., near the tips of the tree (NTI).

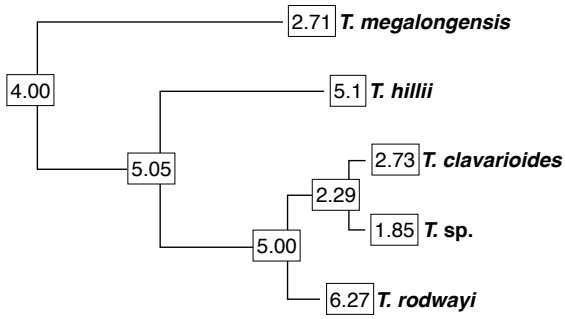


Figure S4 | Ancestral state reconstruction of the NRI on the species level *Thismia* phylogeny. For each species, the observed NRI is shown at the tips and the reconstructed values are shown on the nodes. The reconstructed NRI of the most common recent ancestor of this lineage (4.00; 95% CI: 3.26–4.74) is within the range of the extant species, which means that the ancestor had similar mycorrhizal specificity, and thus already showed specialized interactions.

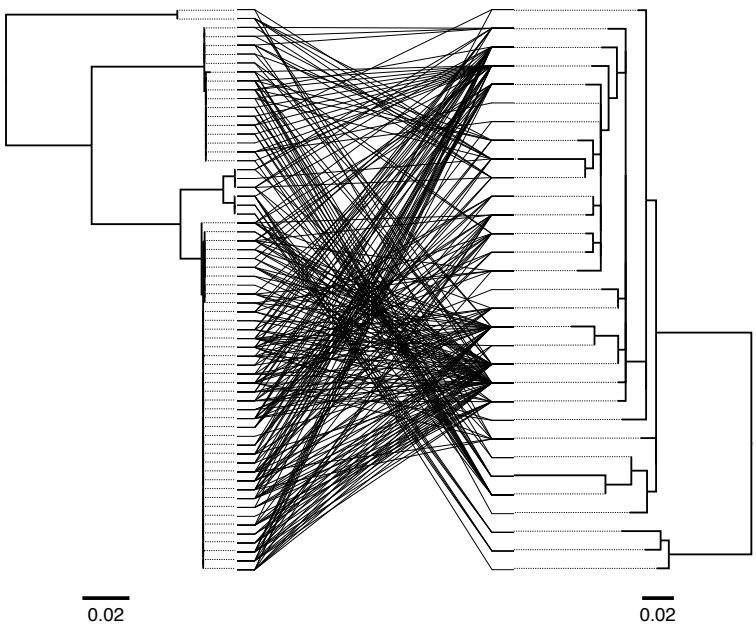


Figure S5 | Tanglegram of the interactions between mycoheterotrophic species of *Thismia* and AM fungal OTUs. The phylogenetic tree of *Thismia* is represented on the left side (see Supporting Information, Methods S2 for details on the phylogenetic relationships of the five species of *Thismia*), and the phylogenetic tree of the AM fungal OTUs on the right side (same as Fig. 1). The tanglegram was built using the APE R package. The figure shows extensive overlap in the fungal interactions within the five *Thimia* species.

Tables

Table S1 | Summary of the samples used in the analysis. In total, we found 99 Glomeromycota OTUs in 5 MH *Thismia* species and 11 green plant species. The table shows the number of samples (total 109), which were pooled in 61 samples for Ion Torrent sequencing. 'No. OTUs' corresponds to the number of unique OTUs found per plant species per site, or per locality in the case of soil samples. Species identification of the green plants is showed to the lowest taxonomical level possible to identify based on *matK* or *trnL* genetic markers.

Species	No samples	Pooled samples	Type	Location	No. OTUs
Thismia rodwayi	37	10	MH	Tasmania	23
Thismia megalongensis	2	1	MH	NSW	6
Thismia hillii	2	1	MH	NSW	7
Thismia clavarioides	2	1	MH	NSW	5
Thismia sp	3	3	MH	NSW	5
Thismia hillii	14	5	MH	NZ	16
Beyeria viscosa	1	1	green	Tasmania	2
Pomaderris apetala	4	1	green	Tasmania	4
Nematolepsis	1	1	green	Tasmania	2
Acacia sp	2	1	green	Tasmania	2
Ceratopetalum apetalum	2	1	green	NSW	3
Acacia sp	1	1	green	NSW	2
Doryphora sassafras	2	2	green	NSW	10
Bigoniaceae	1	1	green	NSW	3
Vitaceae	3	2	green	NSW	7
Apocynaceae	3	1	green	NSW	6
Beilschmiedia tawa	2	1	green	NZ	4
Laurelia novae-zelandiae	2	2	green	NZ	14
Soil	18	18	soil	Tasmania	56
Soil	6	6	soil	NSW	29
Soil	1	1	soil	NZ	7

Table S2 | Statistical results of the mixed-effects model and multiple comparison analysis explaining the fungal communities' phylogenetic dispersion patterns by the 'type' of material (MH plants, green plants, soil), using 'region' as a random factor. The multiple linear comparisons test whether the degree of phylogenetic dispersion of the fungal communities is significantly different among mycoheterotrophic plants, green plants and soil.

Comparisons	Coefficient	SE	P-value
MH plants	2.97	0.23	< 0.001
green plants	1.43	0.29	< 0.001
soil	1.30	0.20	0.433
MH plants - green plants	1.54	0.37	<0.001
MH plants - soil	2.68	0.31	<0.001
green plants - soil	1.13	0.36	0.007

CHAPTER 3

Fungal-host diversity among mycoheterotrophic plants increases proportionally to their fungal-host overlap

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Abstract

The vast majority of plants obtain an important proportion of vital resources from soil through mycorrhizal fungi. Generally, this happens in exchange of photosynthetically fixed carbon, but occasionally the interaction is mycoheterotrophic, and plants obtain carbon from mycorrhizal fungi. This process results in an antagonistic interaction between mycoheterotrophic plants and their fungal hosts. Importantly, the fungal-host diversity available for plants is restricted as mycoheterotrophic interactions often involve narrow lineages of fungal hosts. Unfortunately, little is known whether fungal-host diversity may be additionally modulated by plant-plant interactions through shared hosts. Yet, this may have important implications for plant competition and coexistence.

Here we use DNA sequencing data to investigate the interaction patterns between mycoheterotrophic plants and arbuscular mycorrhizal fungi. We find no phylogenetic signal on the number of fungal hosts nor on the fungal hosts shared among mycoheterotrophic plants. However, we observe a potential trend towards increased phylogenetic diversity of fungal hosts among mycoheterotrophic plants with increasing overlap in their fungal hosts. While these patterns remain for groups of plants regardless of location, we do find higher levels of overlap and diversity among plants from the same location.

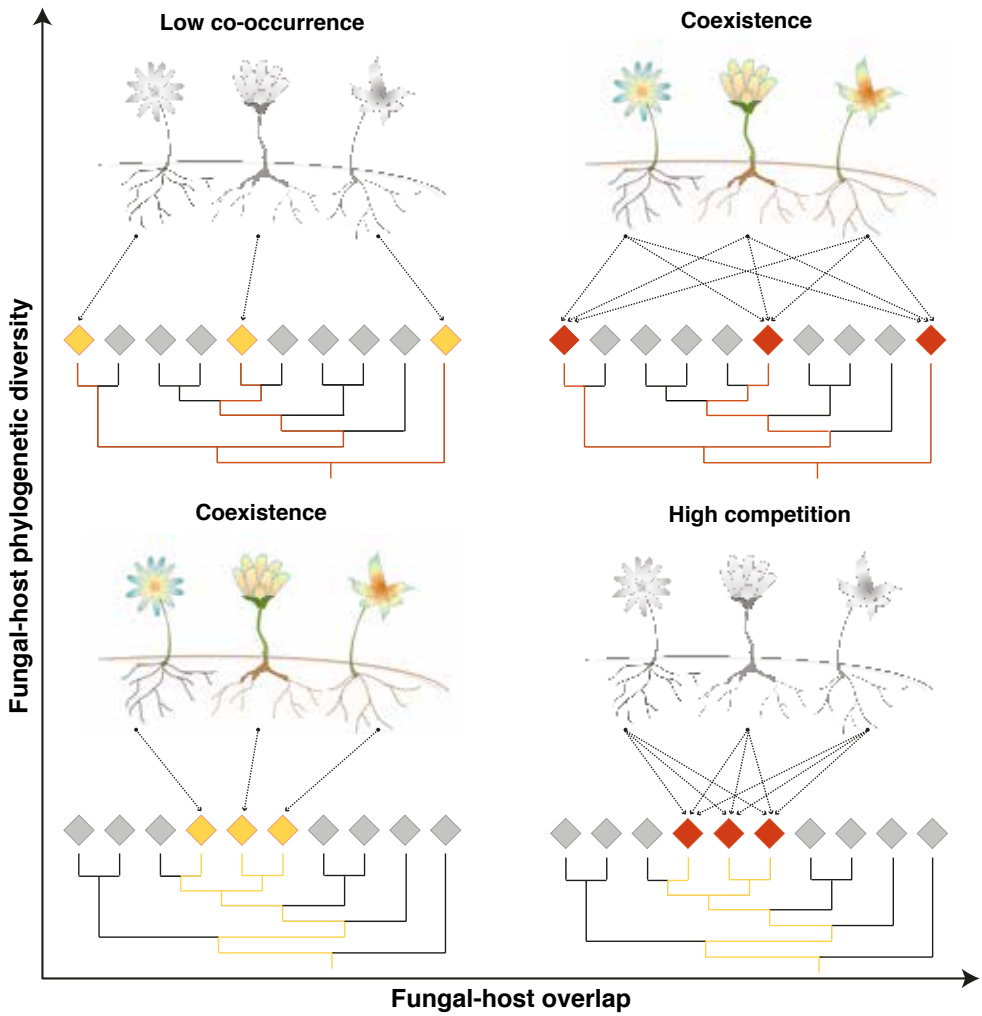
These findings suggest that species coexistence cannot be fully understood without attention to the two sides of ecological interactions.

Introduction

Mycorrhizal fungi play a crucial role for plant survival (Smith & Read, 2008). In mycorrhizal interactions, mycorrhizal fungi facilitate the uptake of essential resources for plant metabolism, such as water and soil minerals (Raven *et al.* 1999). Generally, in exchange, plants transfer photosynthethically fixed carbon to their mycorrhizal partners (Smith & Read, 2008). Occasionally, however, plants do not give back carbon, but instead obtain it from the mycorrhizal fungi as replacement for photosynthesis (Leake, 1994; Merckx *et al.* 2009). This results in an antagonistic interaction between plants and their fungal hosts. Specifically, these interactions are called mycoheterotrophic (MH) interactions and can occur in a single developmental stage (e.g. in orchids, and some ferns and lycopods) or during the entire life cycle of a plant (fully mycoheterotrophic plants) (Winther & Friedman, 2008; Merckx & Freudenstein, 2010). MH interactions represent a non-mutualistic mode of life that occurs in nearly all major lineages of land plants, involving more than 20,000 plant species (Merckx, 2013). In general, the fungal-host diversity available for these plants is restricted as MH interactions often involve more narrow lineages of mycorrhizal fungi than non-MH interactions (Bidartondo *et al.* 2002). Unfortunately, little is known whether fungal-host diversity may be additionally modulated by plant-plant interactions through shared hosts. Yet, this may have important implications for plant competition and coexistence (Bever *et al.* 2010).

Recent studies have shown that the diversity of mycorrhizal fungi is strongly associated with plant community composition (Davison *et al.* 2011; Peay *et al.* 2013; Martínez-García *et al.* 2015) and habitat conditions (Hazard *et al.* 2013). For instance, in the case of MH interactions, a given group of plant species can be exploiting either closely or distantly related fungal hosts (see Figure 1). Additionally, this same group of plants can have either a weak or a strong fungal-host overlap (see Figure 1). The combination of these two factors depends on plant niche, and have been shown to be determinant for plant coexistence (Levins, 1968; Levine & HilleRisLambers, 2009; Rohr *et al.* 2016). According to niche theory (MacArthur & Levins, 1967; Loreau, 2010), species coexistence is a function of their their niche width and niche overlap (Chesson, 2000). Competitive exclusion among species is high when their potential niche overlap is large and their combined niche width is small. Similarly, the chances of co-occurrence among species in the same niche space is low when their potential niche overlap is small and their combined niche width is large. Species coexistence (co-occurrence and no exclusion) then is expected to happen when niche overlap and

niche width are symmetric (Chesson, 2000; Tilman, 2011) (see Figure 1 - diagonal). Niche delimitation is never straightforward due to our often lack of a priori knowledge about the resources and functional traits defining the niche dimensions of a species (Kraft *et al.* 2015). Defining the niche of fungal hosts of mycoheterotrophic plants is as challenging as for other groups of organisms, but one potential hypothesis is that the higher the fungal-host diversity of mycoheterotrophic plants, the broader their niche. Thus, species coexistence may be favored under symmetric patterns of fungal-host overlap and diversity.



To work on the above hypothesis, we use a system where the mycorrhizal interaction involves mycoheterotrophic plants. In addition, these plants are associated with arbuscular mycorrhizal fungi (phylum Glomeromycota), which are associated with more than 80 % of land plants. Therefore, this association represents one of the most ancient and abundant mycorrhizal interaction among plants on a global scale (Smith & Read, 2008; Strullu-Derrien *et al.* 2014). Here, we investigate MH interactions by analyzing the observed patterns of associations between MH plants and their fungal hosts in a niche framework. In particular, we study how the phylogenetic diversity of arbuscular mycorrhizal hosts varies among individual MH plants, and how this diversity is modulated and shared among groups of MH plants.

Material and Methods

Sampling sites and mycoheterotrophic species

The geographic range of MH plants associated with arbuscular mycorrhizal fungi is mostly restricted to tropical rainforests worldwide (Leake, 1994). Neotropical forests harbor the largest species diversity compared to the paleotropical forests. In the neotropics, the two biomes with the highest diversity of MH species are the Amazon forest and the Atlantic forest (Merckx, 2013). We collected MH plants in these two biomes in French Guiana and Brazil, respectively (see Figure S3). The sampled sites in French Guiana were low land coastal plain forests (Guitet *et al.* 2015), and in Brazil were also low lands in ombrophilous dense coastal forests (Veloso *et al.* 1991). Due to the ephemeral nature of MH plants, it is only possible to collect them during their flowering period. Most MH species flower after the rainy season, from July until November. All collections were made during this period.

Figure 1 (previous page) | Illustration of possible fungal-host patterns among mycoheterotrophic plants. On the vertical and horizontal axes, the figure illustrates, respectively, an increase in fungal-host diversity and fungal-host overlap among MH plants. The bottom right panel represents a scenario for plants with high chances of competitive exclusion given by their large fungal-host overlap and their small fungal-host diversity (using similar functional traits). The top left panel represents a scenario for plants with low chances of co-occurring in the same space given by their small fungal-host overlap and their large fungal-host diversity (using different functional traits), which could be difficult to find in a common place. The diagonal panels then represent the scenarios for plants with a higher chance of coexistence given by their symmetry between fungal-host overlap and fungal-host diversity, which could lead to maximize co-occurrence (exploit available resources) and to minimize competitive exclusion

We visited 15 localities, 10 of which in the Amazon forests and 5 in the Atlantic forests. We considered all the individuals of the same species found within 4 x 4 m to be part of the same population. Populations of MH species were separated from each other with a minimum of 30 m. In each population, we collected at least one individual and a maximum of ten individuals per species. We focused on three of the four MH plant families distributed in the sampled area, namely Burmanniaceae, Gentianaceae and Triuridaceae. We did not target species of Thismiaceae, the fourth family of MH plants in the area, since all neotropical species are extremely rare. In the 15 localities, we identified 54 populations of MH species. In total, we collected root samples of 140 specimens of 20 MH plant species, covering more than a quarter of the described arbuscular mycorrhizal MH species for South America. See Supporting Information for further details about the sampling.

Fungal-host diversity in single mycoheterotrophic plants

To study fungal-host patterns, first we investigated the arbuscular mycorrhizal fungal-host diversity that can be potentially associated with single MH plants. This information was obtained through DNA sequencing of roots of arbuscular mycorrhizal MH plants. For each of the 140 specimens, immediately after collection, root samples were washed with distilled water and stored in 2% CTAB buffer at -20ºC until further processing. Subsequently, DNA was extracted using the NucleoSpin Soil kit (Macherey-Nagel Gmbh and Co., Düren, Germany). Next-generation DNA sequencing of each root sample was used to identify the arbuscular mycorrhizal hosts that can be potentially associated with each MH plant species. We sequenced the ITS2 region using the primers fITS7 (5'-GTGARTCATCGAATCTTTG-3') (Ihrmark *et al.* 2012) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990). In total, we found 138 operational taxonomic units (OTUs) identified as Glomeromycota by quering against UNITE database (version 6.0, 10.09.2014) using the BLAST algorithm. Hereafter, we refer to the fungal OTUs as fungal hosts. See Supporting Information for more details about the sequencing. Raw sequences are deposited in the NCBI Short Read Archive under the project number PRJNA339563.

To generate the phylogenetic tree for each family of MH plant species, we reconstructed the phylogenetic relationships between the species for each family by reanalyzing previously published datasets of Burmanniaceae (Merckx *et al.* 2010a), Triuridaceae (Mennes *et al.* 2013), and Gentianaceae (Merckx *et al.* 2013a). For

Triuridaceae we included newly sequenced data for *Soridium spruceanum* (GenBank accession number KX756649). We combined the resulting trees based on divergence ages taken from (Magallón *et al.* 2015). Only the 20 taxa from this study were kept in the phylogeny shown in Supplementary Figure S2.

To generate the host phylogenetic tree we used an alignment with the 138 Glomeromycota fungal OTUs with MAFFT 7.017 (Katoh & Standley, 2013) implemented in GENEIOUS PRO 6.1.4 (Biomatters, Auckland, New Zealand). Reference sequences of the accepted genera in the phylum were added as a backbone to the tree to support and better deduce the phylogenetic position of each OTU (Öpik *et al.* 2010; Krüger *et al.* 2012). We reconstructed a Maximum Likelihood tree using the GTR+I+G substitution model as selected with JMODELTEST 2.3.1 (Darriba *et al.* 2012) under the Akaike Information Criterion. The resulting highest-likelihood tree was transformed into an ultrametric tree using COMPUTE.BRLEN and VCV commands in the R-APE package. The phylogeny of the 138 Glomeromycota OTUs is shown in Supplementary Figure S3. The alignment and tree topology are archived in the database TREEBASE (http://www.treebase.org; submission ID 20259).

To calculate the effect of phylogenetic relatedness on the number of fungal hosts among MH plants (phylogenetic signal), we computed the Mantel test correlation between the phylogenetic distance matrix between plants and the dissimilarity matrix between the number of fungal hosts per plant. The phylogenetic distances were extracted from the plants phylogenetic tree, and the dissimilarity matrix was calculated by $|d_i - d_j|$, where d_i and d_j are the number of fungal hosts associated to plant *i* and *j*, respectively (Saavedra *et al.* 2014). Separately, phylogenetic relatedness on the number of fungal hosts was investigated among MH plants species that belong to the same location.

To calculate the phylogenetic signal on the shared fungal hosts among MH plants, we computed the Mantel test correlation between the phylogenetic distance matrix between plants and two dissimilarity matrices between the shared hosts. The phylogenetic distance matrix is the same as above, whereas the dissimilarity matrices here were calculated using two different measures. The Bray-Curtis measure $1 - (2Cij)/(di + dj)$, where C*ij* is the number of shared hosts between plant *i* and *j*, and d_i and d_j are the number of fungal hosts associated to MH plant *i* and *j*, respectively. Note that the Bray-Curtis measure corresponds to the number of shared fungal hosts relative to the total number of fungal hosts. The second measure we used is the overlap measure

$C_{ij}/\min(d_i,d_j)$, where the parameters are the same as above and $\min(d_i,d_j)$ refers to the smallest of the two values (Saavedra *et al.* 2013). The overlap measure corresponds to the number of shared fungal hosts relative to the maximum number of fungal hosts that can be shared. Correlations were computed using the function mantel in the R-VEGAN package. Mantel statistics were tested for significance by permutation 104 trials). Separately, phylogenetic signal on the shared fungal hosts was investigated among MH plants species that belong to the same location.

For each MH plant, the observed fungal-host diversity was calculated using the phylogenetic diversity (PD) of the observed hosts. Phylogenetic diversity was calculated by summing up the branch lengths in the fungal-host phylogenetic tree among all the fungal hosts associated to the MH plant or group. Because the number of fungal hosts determines the branch length, we normalized the PD by calculating the scaled PD as $PD' = (PD - PD_{MIN})/(PD_{MAX} - PD_{MIN})$, where PD_{MAX} and PD_{MIN} correspond, respectively, to the maximum and minimum PD values that can be generated from all the possible combinations of fungal hosts. These combinations are generated by creating groups of fungal hosts of the same number as in the observed case, but the identity of the hosts is changed using the pool of the 138 possible fungi. The MH plants from our study were only found to associate with these 138 fungi, which represent a subset of the total fungal diversity available in the soil. Note that this scaling does not assume a particular generative process, rather it compares the observed phylogenetic diversity to all the possible outcomes with the same number of fungal hosts.

Fungal-host diversity and overlap among mycoheterotrophic plants

We investigated the diversity and overlap patterns among observed co-occurring MH plants in the field, as well as among the artificially-generated groups. In particular, we observed six communities of MH plants that were found co-occurring in the field. To maximize the possibility of co-occurrence and to avoid small-scale niche segregation of mycorrhizal communities (Jacquemyn *et al.* 2014), plants were considered to co-occur when flowering specimens were found growing less than one meter from each other (see Supporting Table S6 for the composition of these communities). Two of the observed communities in the field had 2 plants, three communities had 3 plants, and one community had 5 plants. Additionally, to generate groups of potentially co-occurring plants, we formed all groups with n plant species using the 20 MH collected species. We generated artificial groups with 2, 3, 4 and 5 MH species (mimicking the size of the observed communities in the field).

In every single observed community and generated group, we calculated the combined phylogenetic diversity (PD) of the fungal hosts that can be associated with a given community/group of MH plants. Similarly, to investigate fungal-host overlap among MH plants, we calculated the overlap of fungal hosts among MH plants in a given community/group. This overlap is again calculated as $\sum_{i<j} C_{ij}/\min(d_i,d_j)$, where C_{ij} represents the number of fungal hosts shared between MH plant *i* and *j* that belong to a given community/group, min(di,dj) refers to the smallest of the two values, and the summation is done over all possible pairs of MH plants (Saavedra *et al.* 2013). Note that this overlap measure corresponds to the average number of shared fungal hosts among all pairs of MH plants in given community/group relative to the maximum number of fungal hosts that can be shared. To compare phylogenetic diversity and overlap across communities/groups, we used the scaled PD and scaled overlap, which are the values of the phylogenetic diversity and overlap measures within the range of possible phylogenetic diversity and overlap values generated by all the groups with the same number of plants.

Finally, to investigate the spatial influence of our sampling in the observed patterns of fungal hosts in MH plants, we compared the scaled PD and scaled overlap between MH plants belonging to the same location and MH plants belonging to different locations. Because in nine of the fifteen localities we visited, we found more than one MH plant species (see Figure S1), we generated two categories for each of the groups with 2, 3, 4 and 5 plant species generated above. Only if all plants in a given group were found in a common location, they were considered in category one. Otherwise, the group was considered in category two. For each group and category, we separately calculated the scaled PD and scaled overlap.

RESULTS

Fungal-host diversity in single mycoheterotrophic plants

We found that the number of fungal hosts in each of the 20 MH plant species varies from 2 to 42 (see Fig. 2A). Particularly, we found no phylogenetic signal on the number of fungal hosts among plants (Mantel test: r = -0.050, P = 0.766, df = 19) nor on the fungal hosts shared among plants (Mantel tests: Bray-Curtis r = -0.035, P = 0.682; overlap r = 0.047, P = 0.245; df = 19). Looking at the MH plants that belong to the

same location (Fig. S1), we found no phylogenetic signal on the number of fungal hosts among plants (Mantel test: $r = 0.17$, $P = 0.375$, $df = 3$ for Laussat; $r = -0.20$, $P = 0.650$, $df = 4$ for Elie; $r = -0.21$, $P = 0.717$, $df = 5$ for Singes; $r = 0.37$, $P = 0.089$, $df = 5$ for Virginie) nor on the fungal hosts shared among plants (Mantel test: Bray-Curtis $r = 0.03$, $P = 0.583$; overlap $r = 0.03$, $P = 0.512$; $df = 3$ for Laussat; Bray-Curtis $r = -0.54$, $P = 0.983$; overlap $r = 0.34$, $P = 0.150$; $df = 4$ for Elie; Bray-Curtis $r = -0.22$, $P = 0.794$; overlap $r = 0.08$, $P = 0.472$; $df = 5$ for Singes; Bray-Curtis $r = -0.09$, $P = 0.608$; overlap $r = 0.25$, $P = 0.161$; $df = 5$ for Virginie). Overall, these findings reveal an important variability in MH interactions that can be driven by mechanisms other than evolutionary relationships.

Additionally, we found that fungal-host diversity in each observed plant ranks among the highest when compared to the potential host diversity that can be expected by chance in a single MH plant with the same number of fungal hosts. The majority of plants (14 out of 20) lies in the upper half of the range of possible phylogenetic diversity values (scaled PD > 0.5; Figure 2). These findings imply that individual plants typically have a high fungal-host diversity by exploiting distantly related fungi, regardless of their number. This raises then the question of how plants are sharing their fungal hosts.

Fungal-host diversity and overlap among mycoheterotrophic plants

Mycorrhizal fungi create extensive underground networks that could make MH plants compete to obtain their belowground vital resources via their MH interactions. This makes necessary the study of how the diversity of MH interactions is modulated and shared within groups of plants.

We find that on average the fungal-host diversity (the combined phylogenetic diversity of the associated fungal hosts within the group) is proportional to fungal-host overlap (the average fraction of shared fungal hosts) in groups of MH plants. This pattern was present in both the observed communities in the field (Figure 3A) and in the generated group of plants (Figure 3B). In particular, there is a systematic positive association between scaled PD and scaled overlap in the observed communities (Pearson correlation: $r = 0.805$, $P = 0.053$, $df = 4$) and in the artificially-generated groups (Pearson correlation: $r = 0.497$, $P = 0.001$, $df = 21680$). This positive relationship does not depend on group size (Pearson correlation: $r = 0.377$, $df = 191$, $P = 0.001$ for 2 species, $r = 0.487$, $df = 1138$, $P = 0.001$ for 3 species, $r = 0.493$, $df = 4843$, $P = 0.001$ for

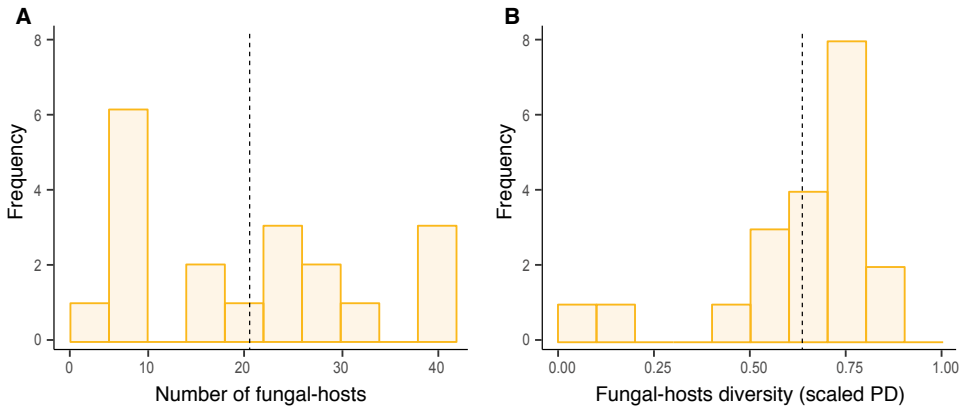


Figure 2 | Fungal- host patterns in single mycoheterotrophic plants. Panel (A) shows the distribution of the total number of fungal hosts associated with each of the 20 observed MH plants. Panel (B) shows the fungal-host diversity (scaled phylogenetic diversity) associated with each of the 20 observed plants. This shows that most of the observed MH plants have a fungal- host diversity that falls in the upper half of the potential range. The dashed lines correspond to the mean values in the distributions.

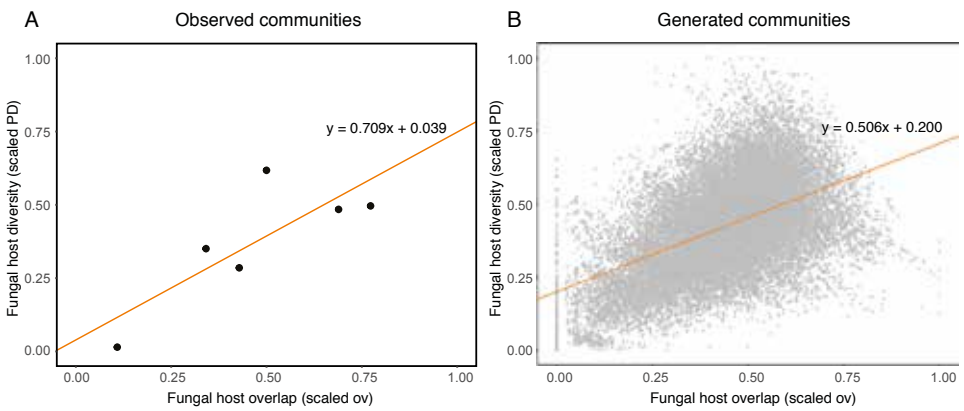


Figure 3 | Fungal-host diversity increases along with fungal-host overlap among mycoheterotrophic plants. The figures show the relationship between fungal-host diversity and fungal-host overlap for both the six observed communities in the field (panel A) and in the artificially generated groups of plants (of the 20 sampled MH species) (panel B). Both panels show the common positive relationship between fungal-host diversity (scaled phylogenetic diversity in y-axis) and fungal-host overlap (scaled overlap in x-axis). Fungal-host diversity and overlap correspond, respectively, to the combined phylogenetic diversity of the hosts associated with the plants in each group normalized by the number of fungal hosts, and the fraction of shared fungal hosts (see Section 2). The solid lines correspond to the linear regression between scaled PD and scaled overlap across all points.

4 species, $r = 0.478$, $df = 15502$, $P = 0.001$ for 5 species).

The results above are also qualitatively the same if scaled PD and scaled overlap values are replaced by their raw values while controlling for the total number of fungal hosts. Because the number of specimens and the OTU richness per MH species are variable among samples and may influence the results (see Supporting Table S1), we computed the partial Pearson correlations between scaled PD and scaled overlap controlling for the number of individuals sampled per species, number of OTUs, and variation in the number of individuals per species within a community (using the Herfindahl index). The obtained correlations remain positive and significant at the 95 % confidence, which confirm that fungal-host diversity within a group of plants increases together with their fungal-host overlap.

Finally, by dividing the categories of MH plants into one in which all plants belong to the same location and another one in which not all plants belong to the same location (see Methods), we found that typically the former group displays higher levels of both scaled PD and scaled overlap across the different group sizes (see Tables 1 and 2). These

Table 1 | Fungal-host diversity is higher in groups of plants that belong to the same location. The table shows the *t*-test results comparing the scaled PD in groups of MH plants (composed by two, three, four, or five species) that belong to the same location and in different locations.

Scaled PD	Mean in same location	Mean in different location	P-value	95 % CI
Two species	0.421	0.297	0.0012	0.05, 0.20
Three species	0.412	0.327	0.0002	0.04, 0.13
Four species	0.479	0.394	0.0009	0.04, 0.39
Five species	0.553	0.440	0.0023	0.05, 0.18

Table 2 | Fungal-host overlap is higher in groups of plants that belong to the same location. The table shows the *t*-test results comparing the scaled PD in groups of MH plants (composed by two, three, four, or five species) that belong to the same location and in different locations.

Scaled PD	Mean in same location	Mean in different location	P-value	95 % CI
Two species	0.358	0.220	$6.6\ e^{-6}$	0.07, 0.21
Three species	0.493	0.362	$3.2\ e^{-8}$	0.09, 0.17
Four species	0.512	0.404	$2.1\ e^{-8}$	0.08, 0.14
Five species	0.577	0.458	$1.3\ e^{-5}$	0.08, 0.15

results suggest that fungal-host diversity increases within a location as a response to a natural increase in fungal-host overlap, which can be expected from a niche framework perspective (MacArthur & Levins, 1967; Levine & HilleRisLambers, 2009; Rohr *et al.* 2016).

Discussion

Previous studies have investigated fungal-host diversity of MH plants in relation to the fungal diversity associated with the surrounding green plants (Cullings *et al.* 1996; Bidartondo *et al.* 2002, 2003; Bougoure *et al.* 2009; Roy *et al.* 2009b; Yamato *et al.* 2011). However, several MH species present vast geographic distributions despite being locally rare. Therefore, these surrounding plants may not be the exclusive factors determining fungal-host diversity in MH plants. Indeed, many studies have reported the occurrence of different species of arbuscular mycoheterotrophs in the field without a clear explanation for this phenomenon (e.g. van der Pijl, 1934; Jonker, 1938; van Royen, 1972; van de Meerendonk, 1984; Maas & Rübsamen, 1986; Cheek & Williams, 1999; Merckx, 2013).

In our study, we have considered potential neighboring effects of MH plants with each other as possible drivers of fungal-host diversity. Because many unmeasured factors can influence MH interactions, we opted to compared the observed patterns against all the possible fungal-host combinations (what we called artificially-generated groups of plants). We have found that individual MH plants have a tendency to exploit more distantly related fungi than expected by chance. This tendency of targeting distantly related fungi has been described in autotrophic plants (Giovannetti *et al.* 2004). Nevertheless, it has been suggested that MH plants have more restricted interactions, since they often show higher specificity towards their fungal-hosts (e.g. Bidartondo *et al.* 2003; Gomes *et al.* 2017a). For example, in *Afrothismia*, five closely related MH plants were found to specialize in five closely related lineages of Glomeromycota fungi (Merckx & Bidartondo, 2008). In contrast, in Monotropoideae, the five MH species in this clade associate with five different distantly related Basidiomycota fungi, but each within the same fungal lineage (Bidartondo & Bruns, 2005). Either way, and despite the processes leading to this extreme level of fungal specificity, it has been suggested that MH plants adapt to the suitable fungal partners that participate in this mycoheterotrophic interaction, and therefore host-jumps to distantly related fungal lineages are unexpected (Bidartondo & Bruns, 2002).

Building on niche theory, our results may reflect a MH plant strategy to increase its fungal-host diversity or niche width, as species with a wider niche may be more likely to obtain different resources and to establish successfully in new habitats (Levins, 1968; Tilman *et al.* 1996; Levine & HilleRisLambers, 2009). Mycoheterotrophic plants require established mycorrhizal networks to persist (Sachs & Simms, 2006; van der Heijden *et al.* 2015). Although each species tend to increase the phylogenetic diversity of their fungal hosts, it is still a limited fraction of the total diversity of arbuscular mycorrhizal fungi that can be part of this interaction (Douglas, 2008; Merckx *et al.* 2009; Gomes *et al.* 2017a), suggesting that these fungi appear to be under selection pressure to be resistant to these cheaters (Douglas, 2008). Therefore, the ability to increase its fungal-host diversity may confer an advantage to increase the opportunities to cheat mycorrhizal networks.

We have found that in communities of co-occurring MH plant species in the field the fungal-host diversity among MH plants appear to increase proportionally to their fungal-host overlap. This same tendency was confirmed among the artificially-generated groups of MH plants showing that the patterns observed are not an artifact of the reduced number of MH communities observed in the field. Moreover, we have found that both fungal-host diversity and overlap are significantly higher among plants that belong to the same geographical location, which could provide an explanation for the lack of phylogenetic signal on the fungal hosts among MH plants. These results indicate that fungus-plant interactions can be better explained by understanding plant-plant interactions generated by sharing resources or fungal hosts. Future studies could explain whether this symmetry between fungal-host diversity and overlap may respond to an ecological mechanism driven by maximizing co-occurrence and avoiding competitive exclusion among MH plants.

A potential bias in our study is the use of ITS2 sequences and future work should consider expanding these sequences (see Supporting Information for more details). Another aspect that deserves particular attention is the influence of abiotic factors that can affect the diversity of fungal hosts for the MH plants. In fact, many other factors can influence diversity, including the surrounding autotrophic plants. Taking everything into account is virtually impossible. However, our findings suggest that species coexistence cannot be fully understood without attention to the two sides of ecological interactions.

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Data accessibility

Data is publicly available as Supporting Information.

Author contribution

SS designed the overall study. VSFTM designed the sampling, DNA study, and provided data. SIFG performed the analysis. SIFG and SS wrote a first draft of the manuscript; all authors contributed to revisions.

Supporting Information

DNA sequencing

Fungal DNA was extracted from root material with KingFisher Flex Magnetic Particle Processors (Thermo Scientific, USA), using the NucleoMag 96 Plant Kit (Machery-Nagel Gmbh and Co., Düren, Germany). The internal transcribed spacer 2 (ITS2) was amplified using the fungal specific primer fITS7 (Ihrmark *et al.* 2012) and the universal primer ITS4 (White *et al.* 1990), which was labeled with 96 different Ion Torrent MID-labels to differentiate individual samples. ITS2 labelled amplicons were sequenced on a Personal Genome Machine with 850 flows (PGM; Life Technologies, Guilford, CT, USA). The next-generation sequencing of the 140 specimens were done in two runs, including other plant species not used for this work. The reads obtained then were processed using USearch v.7 using the UPARSE algorithm (Edgar, 2013). The Ion Torrent runs originated 9 547 370 raw sequences. From these, 156 517 passed our quality control steps (excluding sequences with $Q < 20$, length < 100 bp and global

singletons), originating 37 563 unique sequences. These sequences were clustered at 97% similarity. A chimera check was performed using Uchime Reference Database (3.07.2014 UNITE/INSD; (Edgar *et al.* 2011). Global OTUs singletons and doubletons were excluded, generating a total of 138 Glomeromycota OTUs (represented by 7,227 sequences). The 138 OTUs were identified by BLAST search using the UNITE+INSD database (version 6.0, 10.09.2014) in UPARSE implemented with the current Index Fungorum classification. See Table S2 in Supporting Information for information on the closest match for each OTU. We matched the 138 fungal hosts to the 20 MH plant species. All non-Glomeromycota OTUs were omitted, retaining 138 Glomeromycota OTUs for further analysis. Because the majority of the Glomeromycota hits (see Table S2) matched uncultured Glomeromycota species, we placed the obtained OTUs in a phylogenetic tree (see Figure S3) to better understand their phylogenetic relationships.

To avoid the conflicts that molecular assessments generate in the species delimitation of arbuscular mycorrhizal fungi, due to the current absence of species concept for the fungi in this phylum, we measured the diversity of MH interactions as the phylogenetic diversity among the fungi detected per plant species, instead of considering the number of OTUs.

A potential bias in our study is the use of ITS2 sequences. The marker regions often used for Glomeromycota phylogenetic studies are ribosomal DNA markers, including SSU, ITS and LSU genes, also because rDNA markers are the largest sampled within this group of fungi. Previous studies showed that SSU alone has a limited resolution power (Bruns et al. 1991; Hofstetter et al. 2007), which can introduce a bias towards an under-estimation of AM fungi (Krüger, 2011). The ITS region is known to be a highly variable region, which can also introduce a bias in the opposite direction of the SSU marker, towards an over-estimation of AM fungi. To overcome these problems, Krüger et al. (2009) suggested the amplification of a SSU-ITS-LSU fragment for a phylogenetic analysis with species-level resolution. However, the use of next-generation DNA sequencing techniques only allows amplification of short DNA fragments, which forces us to choose a fragment of one of the three markers. Due to the limited length of Ion Torrent sequencing, the better candidate region chosen was the ITS2. Preliminary data analysis (not shown) based on the SSU region has proven not to discriminate the different fungal lineages associated with these plants. Therefore, the use of ITS2, which is a more variable region, potentially delivers the most phylogenetic informative characters. Because ITS2 is a fast-evolving region we used a backbone alignment

including concatenated reference sequences (Krüger et al. 2012) of partial SSU, whole ITS and partial LSU representative of all the described AM fungi genera (Krüger et al. 2009), adding the two new genera described later on curated in the MaarjAM database (Öpik *et al.* 2010), for a more accurate phylogenetic placement of the generated fungal sequences in this study. To reduce the potential bias due to the under- or over-splitting of fungal taxa in OTUs, we used the phylogenetic distances between the fungal taxa instead of richness of the samples in the downstream analysis.

Figures

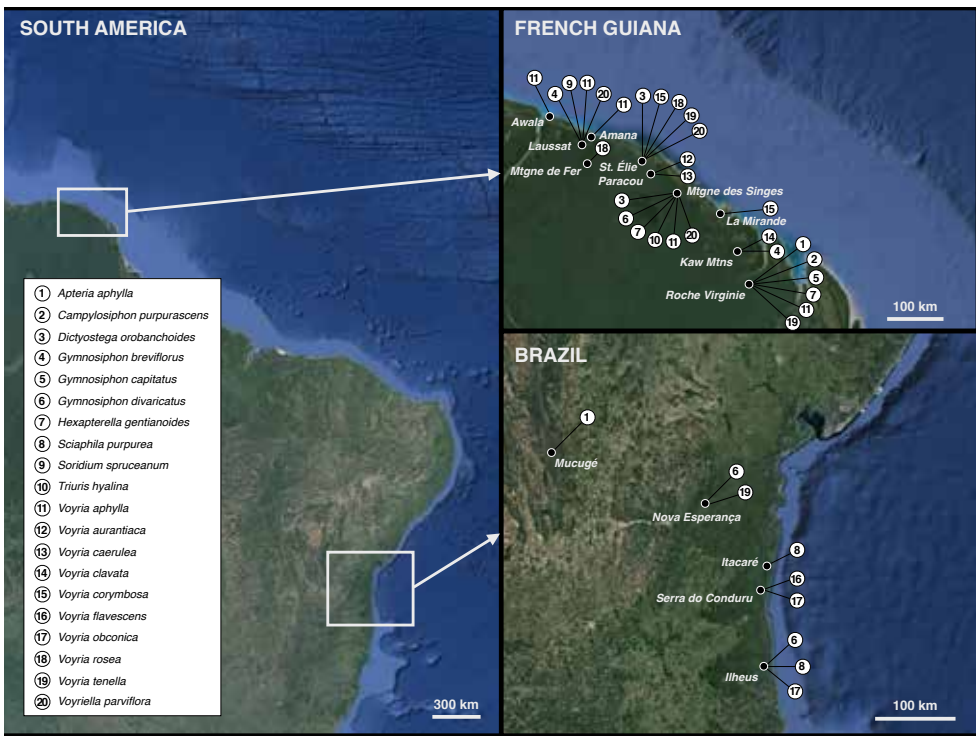

Figure S1 | Map of the 15 sampling locations of our study: 10 in French Guiana and 5 in Brazil. Mycoheterotrophic species (on the left) are represented by numbers in each location that were collected (on the right).

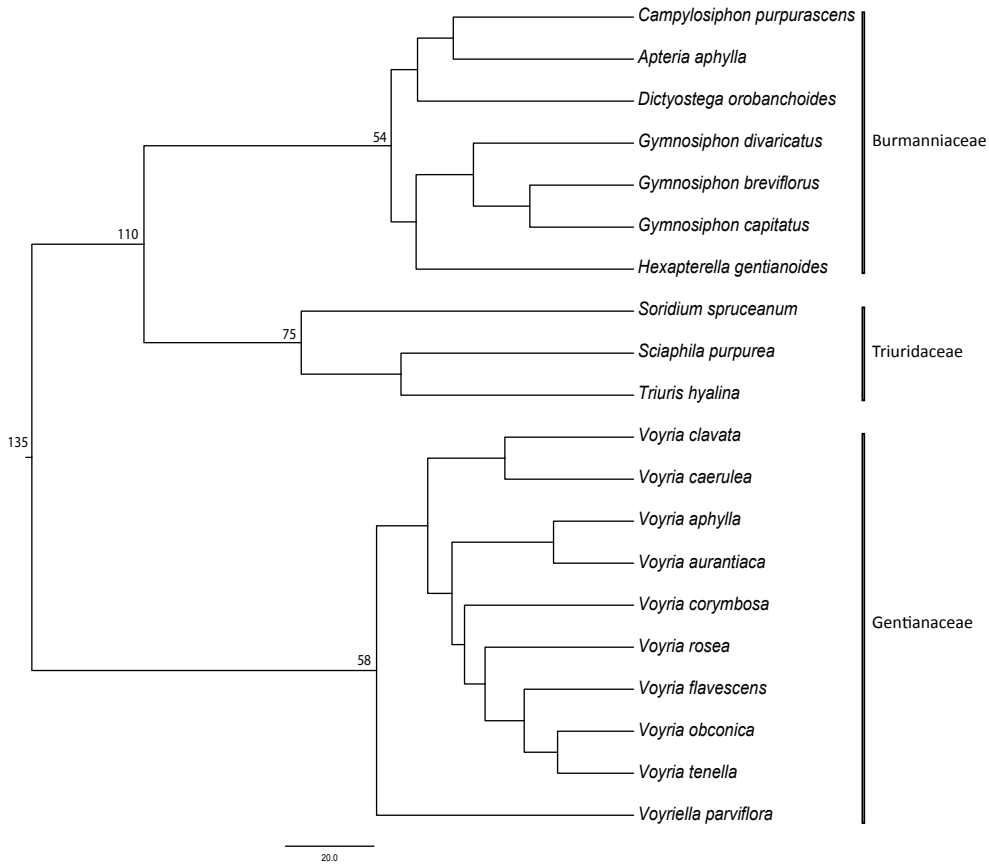

Figure S2 | Phylogeny of MH plants used to infer phylogenetic signal. Branch lengths represent divergence times. Root age and crown node ages of the sampled families are shown (in million years ago).

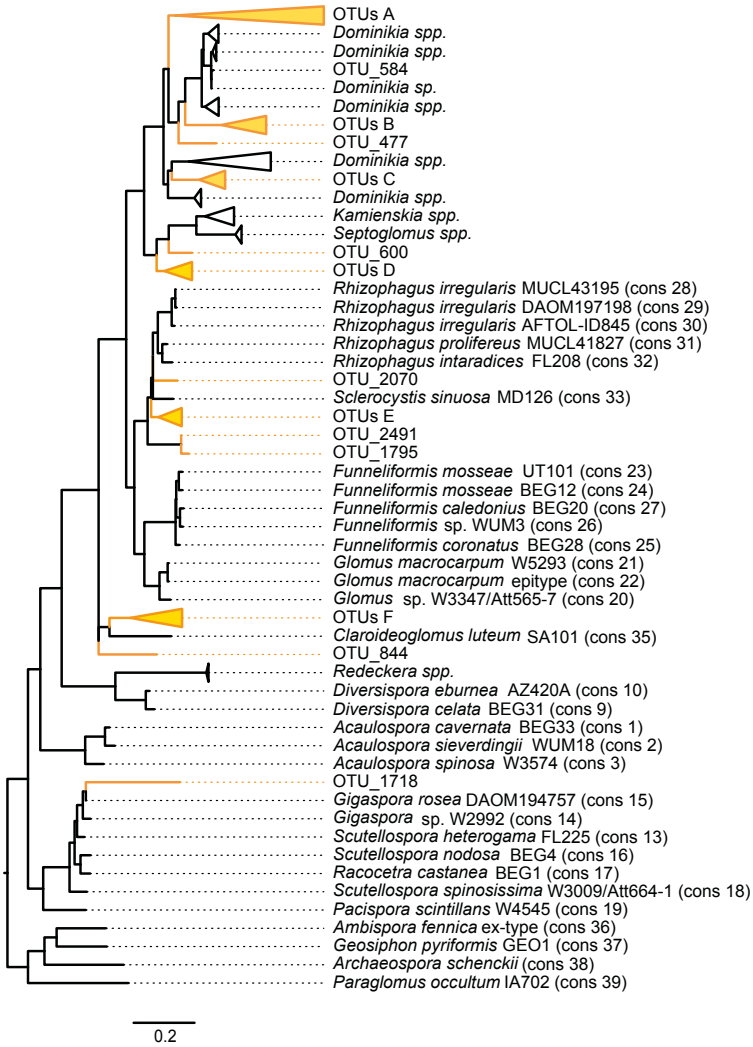

Figure S3 | Phylogeny of the Glomeromycota OTUs found in all the MH plants. Sequences with identification correspond to curated sequences of Glomeromycota (see DNA Sequencing in Supporting Information). Sequences indicated with cons were obtained from the reference dataset of AM fungi built by Krüger et al. (2011). We also included the following genera identified in the MaarjAM database (Öpik et al., 2010): *Dominikia* spp. (HG938301-HG938304, KJ564145-KJ564169, KM05657-KM05665, KR105638-KR105649), *Kamienskia* spp. (KJ564133-KJ564144), *Redeckera* spp. (HG518627-HG518629), *Septoglomus* spp. (HF548853-HF548862). The list of OTU numbers in the collapsed clades is the following OTUs A: 7, 36, 41, 42, 56, 65, 79, 82, 97, 100, 136, 170, 203, 210, 225, 293, 325, 438, 471, 497, 499, 508, 545, 641, 683, 765, 777, 798, 819, 956, 1048, 1109, 1255, 1257, 1353, 1355, 1362, 1594, 1939, 2129, 2182, 2186, 2191, 2239, 2266, 2443, 2509, 2518, 2586, 2660, 2847, 2898, 3037, 3062, 3094, 3239, 3386, 3515, 3581, 3700; OTUs B: 338, 873, 1377, 1625, 2613; OTUs C: 12, 45, 57, 80, 81, 159, 162, 211, 233, 253, 260, 320, 354, 364, 382, 506, 523, 553, 653, 686, 687, 769, 772, 997, 1002, 1034, 1052, 1064, 1079, 1086, 1135, 1357, 1381, 1492, 1512, 1633, 1664, 1788, 2072, 2198, 2304, 2312, 2319, 2402, 2424, 2772, 3171, 4185, 4203; OTUs D: 400, 590; OTUs E: 38, 299, 1349, 1656, 2112, 3260, 3355; OTUs F: 107, 112, 146, 383, 786, 1642, 1677.

Tables

Figure S1 | Identity of MH plant species.

Species	Family	Locality	Date
Apteria aphylla		French Guiana, Savanne Roche Virginie	02-08-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		Brazil, Mucugé	27-10-2014
Campylosiphon purpurascens		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
Dictyostega orobanchoides		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
Gymnosiphon breviflorus		French Guiana, Kaw, trail to caves	01-08-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
Gymnosiphon capitatus	Burmanniaceae	French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
		French Guiana, Savanne Roche Virginie	25-08-2014
Gymnosiphon divaricatus		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	02-11-2017
Hexapterella gentianoides		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		French Guiana, Montagne de Singes	22-07-2014
Sciaphila purpurea		Brazil, Bahia, Ilhaeus	04-11-2014
	Triuridaceae	Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014

Coordinates	Habitat	Code	No OTUs	unique OTUs
4°11'42.6"N, 52°08'58.5"W	Edge of woody vegeation on rock outcrop	GM072_1	4	
4°11'42.6"N, 52°08'58.5"W	Edge of woody vegeation on rock outcrop	GM072_2	2	7
12°59'56"N, 41°20'55"W	In *Sphagnum* on rocks near stream	PM3780_1	4	
4°11'42.6"N, 52°08'58.5"W	Primary forest on swampy soil	Bk1_52	1	
4°11'42.6"N, 52°08'58.5"W	Primary forest on swampy soil	Bk1_53	7	10
4°11'42.6"N, 52°08'58.5"W	Primary forest on swampy soil	Bk1_54	3	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	GM009_1	5	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	GM009_2	1	22
5° 8'7.87"N, 53°2'56.77"W	Primary lowland rainforest	GM012	5	
5° 8'7.87"N, 53°2'56.77"W	Primary lowland rainforest	GM018	12	
4°33'2.13"N, 52°10'28.71"W	Primary rainforest on lateritic rocks	GM057	6	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	GM048	7	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_GB1	13	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_GB4	4	42
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_GB1	5	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_GB2	6	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_GB3	31	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_GB4	12	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	GM004	10	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest on steep slope	Bk2_16	6	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest on steep slope	Bk2_31	2	29
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest on steep slope	Bk2_63	7	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest on steep slope	Bk2_70	17	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest on steep slope	Bk2_104	9	
15°14."53"S, 39°04'05"W	Primary Atlantic rain forest	PM3931B	4	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_1	7	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_2	10	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_3	1	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_4	15	42
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_5	13	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_6	12	
13°34'48"S, 39°41'44"W	Primary Atlantic rain forest	PM3854_7	5	
13°34'05"S, 39°42'25"W	Primary Atlantic rain forest	PM3890	7	
5°28'18"N, 53°34'38"W	Primary Atlantic rain forest	GM005_1	13	
5°28'18"N, 53°34'38"W	Primary Atlantic rain forest	GM005_2	1	23
4°11'42.6"N, 52°08'58.5"W	Primary Atlantic rain forest	GM065	3	
5°03'59"N, 52°41'50"W	Primary Atlantic rain forest	P3_HG1	15	
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_1	1	
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_2	7	26
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_3	15	

Figure S1 | Identity of MH plant species (continued).

Species	Family	Locality	Date
Sciaphila purpurea		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Itacaré	06-11-2014
		Brazil, Bahia, Itacaré	06-11-2014
		Brazil, Bahia, Itacaré	06-11-2014
Soridium spruceanum		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
	Triuridaceae	French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
Triuris hyalina		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
Voyria aphylla		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Reserve Amana, zone B	26-07-2014
		French Guiana, Reserve Amana, zone B	26-07-2014
		French Guiana, Reserve Amana, zone B	26-07-2014
		French Guiana, Reserve Amana, zone B	26-07-2014
		French Guiana, Reserve Amana, zone B	26-07-2014
		French Guiana, Kiwala, Awala Reserve	27-07-2014
	Gentianaceae	French Guiana, Kiwala, Awala Reserve	27-07-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		French Guiana, Laussat	28-07-2014
Voyria aurantiaca		French Guiana, Paracou	24-07-2014
		French Guiana, Paracou	24-07-2014
		French Guiana, Paracou	24-07-2014
		French Guiana, Paracou	24-07-2014
		French Guiana, Paracou	24-07-2014
		French Guiana, Paracou	24-07-2014
Voyria caerulea		French Guiana, Paracou	24-07-2014

Coordinates	Habitat	Code	No OTUs	unique OTUs
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_4	6	
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_5	1	
15°14'53"S, 39°04'05"W	Primary Atlantic rain forest	PM3928_6	1	
14°13'40"S, 39°00'58"W	Coastal Atlantic forest	PM3994_1	2	
14°13'40"S, 39°00'58"W	Coastal Atlantic forest	PM3994_2	2	
14°13'40"S, 39°00'58"W	Coastal Atlantic forest	PM3994_3	3	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	GM049_1	7	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	GM049_2	12	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_SS1	7	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_SS2	13	30
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_SS3	5	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_SS6	10	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_SS7	8	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM006_1	4	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM006_2	13	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH1	7	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH2	8	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH3	2	24
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH4	1	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH5	2	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH6	1	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	P3_TH7	1	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM008_1	6	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM008_2	2	
5°32'38"N, 53°29'49"W	Coastal forest on white sand	GM037_1	6	
5°32'38"N, 53°29'49"W	Coastal forest on white sand	GM037_2	2	
5°32'00"N, 53°33'57"W	Coastal forest on white sand	GM040_1	13	
5°32'00"N, 53°33'57"W	Coastal forest on white sand	GM040_2	5	31
5°32'00"N, 53°33'57"W	Coastal forest on white sand	GM040_3	2	
5°44'45.1"N 53°56'07.0"W	Coastal forest on white sand	GM041	7	
5°44'45.1"N 53°56'07.0"W	Coastal forest on white sand	GM042	5	
4°11'42.6"N, 52°08'58.5"W	In shrubby vegetation on rock outcrop	GM070	6	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_VA1	2	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM028_1	5	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM028_2	5	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM032_1	4	9
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM032_2	1	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM034_1	6	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM034_2	5	
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM033_1	3	5

Figure S1 | Identity of MH plant species (continued).

Species	Family	Locality	Date
Voyria caerulea	Gentianaceae	French Guiana, Paracou	24-07-2014
Voyria clavata		French Guiana, Montagne de Singes	22-07-2014
Voyria corymbosa		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Savanne Roche Virginie	25-07-2014
Voyria flavescens		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
Voyria obconica		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Serra do Conduru	05-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
		Brazil, Bahia, Ilhaeus	04-11-2014
Voyria rosea		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Montagne de Fer	27-07-2014
		French Guiana, Montagne de Fer	27-07-2014
Voyria tenella		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		French Guiana, Savanne Roche Virginie	02-08-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014

Coordinates	Habitat	Code	No OTUs	unique OTUs
5°16'48.06"N 52°55'4.56"W	Primary lowland rainforest	GM033_2	3	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM053	2	2
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM015	3	8
4°51'58.7"N 52°20'45.6"W	Primary lowland rainforest	GM036	5	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_1	6	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_2	4	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_3	3	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_4	5	17
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_5	10	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_6	5	
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3974_7	5	
15°14'53"S, 39°04'05"W	Primary lowland rainforest	PM3931_1	3	
15°14'53"S, 39°04'05"W	Primary lowland rainforest	PM3931_2	1	
15°14'53"S, 39°04'05"W	Primary lowland rainforest	PM3931_3	2	
15°14'53"S, 39°04'05"W	Primary lowland rainforest	PM3931_4	4	
15°14'53"S, 39°04'05"W	Primary lowland rainforest	PM3947b	2	10
14°28'50"S, 39°06'29"W	Primary lowland rainforest	PM3947c	1	
15°14'53"S, 39°04'05"W	Restinga forest on sandt soil	PM3950_1	3	
15°14'53"S, 39°04'05"W	Restinga forest on sandt soil	PM3950_2	1	
15°14'53"S, 39°04'05"W	Restinga forest on sandt soil	PM3950_3	3	
15°14'53"S, 39°04'05"W	Restinga forest on sandt soil	PM3950_4	6	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM011_1	3	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM011_2	1	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM014	1	8
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM016	1	
5°18'0" N, 53°36'0" W	Primary forest on swampy soil	GM043_1	2	
5°18'0" N, 53°36'0" W	Primary forest on swampy soil	GM043_2	6	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM019_1	9	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM019_2	2	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM019_3	5	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM019_4	1	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM019_5	2	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest	GM063_1	1	
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest	GM063_2	5	41
4°11'42.6"N, 52°08'58.5"W	Primary lowland rainforest	GM063_3	7	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_1	7	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_2	4	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_3	3	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_4	1	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_5	3	

Figure S1 | Identity of MH plant species (continued).

Species	Family	Locality	Date
Voyria tenella		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	01-11-2014
		Brazil, Bahia, Nova Esperança	02-11-2014
		Brazil, Bahia, Nova Esperança	02-11-2014
		Brazil, Bahia, Nova Esperança	02-11-2014
Voyriella parviflora		French Guiana, Montagne de Singes	22-07-2014
		French Guiana, Piste de St. Ellie, Sentier Botanique	23-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
	Gentianaceae	French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014
		French Guiana, Laussat	28-07-2014

Table S1 | Identity of MH plant species. Detailed sample localities with GPS coordinatesand collection dates are presented for each sampled specimen. Specimens coded with thesame collection number followed by underscore and specimen number were collected lessthan 1 m apart from each other; different collection numbers indicate that specimens werecollected isolated. It is represented the number of reads generated by next generationsequencing (after filtering steps), OTUs detected per plant specimen and total unique OTUs per plant species (table above).

Table S2 | BLAST hits for the Glomeromycota OTUs based on the UNITE database. Foreach OTU, the closest match is presented (available at https://tinyurl.com/yajewo7p).

Table S3 | Overview of the number of OTUs and number of sequences generated per sample.Presence of each OTU is shown per sample (available at https://tinyurl.com/y7o5hoz4).

Coordinates	Habitat	Code	No OTUs	unique OTUs
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_6	6	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3865_7	1	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3889_1	10	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3889_2	7	
13°34'05"S, 39°42'25"W	Primary lowland rainforest	PM3889_3	9	
5°03'59"N, 52°41'50"W	Primary lowland rainforest	GM007	4	
5°18'7.87"N 53°2'56.77"W	Primary lowland rainforest	GM023	1	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_VP1	4	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_VP2	1	
5°28'25"N, 53°34'51"W	Primary lowland rainforest	P1_VP4	3	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP1	4	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP2	5	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP3	2	18
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP5	4	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP7	2	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP8	1	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP9	2	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP10	1	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP14	1	
5°28'18"N, 53°34'38"W	Primary lowland rainforest	P2_VP15	1	

Table S4 | Species composition of the co-occurring MH plant communities. Plants were considered to co-occur when flowering specimens were found growing less than 1 meter apart from each other. We observed six communities of co-occurring species.

Communities	Plant species
A	*G. breviflorus, V. parviflora*
B	*A. aphylla, V. aphylla*
C	*C. purpurascens, D. orobanchoides, V. aphylla*
D	*G. breviflorus, H. gentianodes, T. hyalina*
E	*V. clavata, V. corymbosa, V. rosea*
F	*D. orobanchoides, G. breviflorus, S. spruceanum, V. aphylla, V. parviflora*

CHAPTER 4

Environmental drivers for cheaters of arbuscular mycorrhizal symbiosis in tropical rainforests

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ABSTRACT

Hundreds of non-photosynthetic mycoheterotrophic plant species cheat the arbuscular mycorrhizal symbiosis. Their patchy local occurrence suggests a constraint by abiotic factors. Yet, little is known about the ecological conditions under which mycoheterotrophy is able to occur.

Here, we examine the edaphic drivers predicting the local-scale distribution of mycoheterotrophic plants in two lowland rainforests in South America. We compared soil chemistry and nutrients in plots where mycoheterotrophic plants were present to those without these plants. Soil pH, soil nitrate, and the interaction between soil potassium and nitrate concentrations were the best predictors for the occurrence of mycoheterotrophic plants in these tropical rainforests. Mycoheterotrophic plant occurrences decreased with a rise in each of these predictors. This indicates that these plants avoid high fertility patches. Such low-fertility conditions coincide with conditions that potentially favor a weak mutualism between plants and arbuscular mycorrhizal fungi according to the trade balance model. Hence, when local-scale mutualism is weak, cheating is likely to occur.

Our study uncovers the mechanisms favoring the cheating of arbuscular mycorrhizal networks in tropical forests. The patchy occurrence of mycoheterotrophic plants suggests that local soil heterogeneity causes the stability of arbuscular mycorrhizal networks to vary at a very small scale.

INTRODUCTION

Mycorrhizal symbiosis is one of the most widespread mutualisms on Earth (van der Heijden *et al.* 2015). Typically, it is a mutually beneficial interaction where plants transfer photosynthesized carbon to their mycorrhizal fungal partners, which in turn facilitate the uptake of mineral nutrients from the soil, enhancing plant nutrition (Smith & Read, 2008). The symbiosis is therefore extremely important in soils of low nutrient availability or where the distribution of nutrients is heterogeneous (Cavagnaro *et al.* 2005). Yet, mycoheterotrophic plants evolved a strategy where the carbon flux is reversed from their fungal partners to themselves so that the plants depend exclusively on their mycorrhizal partners to obtain carbohydrates (Leake 1994). It has been argued that mycoheterotrophic plants may provide other benefits to the fungi in exchange for the carbon, such as vitamins or protection but such evidence has not been yet presented (Selosse & Rousset, 2011). Thus, these plants managed to subvert the "biological market" established between plants and mycorrhizal fungi (Kiers *et al.* 2011), avoiding the costs of obtaining resources from symbiotic partners. There are over 500 fully mycoheterotrophic plant species, of which about half is associated with arbuscular mycorrhizal (AM) fungi (Merckx, 2013). Since these plants require the presence of an established mycorrhizal network to support their carbon demands during the entire life cycle, ultimately relying on the surrounding photosynthetic plants, mycoheterotrophy can be regarded as a mechanism enabling cheating on the mycorrhizal symbiosis.

Many species of mycoheterotrophic plants have remarkably widespread distributions, yet at local-scale their distribution is often highly patterned (Cheek & Williams, 1999; Bergman *et al.* 2006; Yamato *et al.* 2016). As general characteristics of mycoheterotrophic plants' habitat at global scale, Leake (1994) described that these plants often occur in humid forests with dense overstory in deep shade, with a thick layer of leaf litter on the forest floor and restricted occurrence of herbaceous plants. This leads to the hypotheses that mycoheterotrophic plants are probably adapted to low-light conditions, occurring mostly inside forests with close canopies, where autotrophic plants fail to grow (Bidartondo *et al.* 2004). Alternatively, mycoheterotrophs may require a particular forest floor microclimate, with potentially high vapor pressure, low evapotranspiration and small diurnal temperature variation (Leake 1994; Cheek & Williams, 1999; Klooster & Culley 2009). Furthermore, the availability of water seems to be a consistent feature of habitats of mycoheterotrophs and potentially explains their

preference for the humid tropics and moist temperate regions, and swamps periodically inundated or moist humus rich soils (Merckx, 2013).

The patchy occurrence of these plants suggests that besides fulfilling general requirements such as humidity, light availability or temperature suitability, the presence of mycoheterotrophic plants in a community is constrained by particular local-scale factors. Due to the reliance of these plants on mycorrhizal networks, both biotic (interactions with their fungi) and abiotic (soil conditions) factors can potentially contribute to their occurrence at local-scale. Previous studies showed highly species-specific interactions between these plants and their fungal partners from local to global scale (Yamato *et al.* 2016; Renny *et al.* 2017). This could indicate that the occurrence of their fungal associates may determine the distribution of mycoheterotrophic plants (Bougoure *et al.* 2009; Yamato *et al.* 2016). However, Merckx et al. (2017) suggested that the distribution of AM fungi does not drive the distribution of highly specialized mycoheterotrophic plants in the genus *Thismia*, since their specific fungal associates were found to occur beyond the range of the plants' distribution. Also other studies indicated inconsistent trends between the local occurrence of mycoheterotrophic plants and the abundance of their associated fungal partners (Yamato *et al.* 2016; Sheldrake *et al.* 2017). Hence the presence of specific fungi is not enough for cheaters to establish. Sheldrake et al. (2017) tested the impact of nitrogen and phosphorus on the occurrence of mycoheterotrophic plants along a fertility gradient across a 65-Km forest in Panama. Their results suggested that the occurrence of these plants is limited by high phosphorus concentration in the soil, which simultaneously reduces the presence and abundance of the AM fungi associated with mycoheterotrophic species. Hence, soil nutrient availability may have an impact on the occurrence of mycoheterotrophic plants by affecting them directly, or indirectly via the AM networks that these plants rely upon. Yet, which soil characteristics influence the occurrence of mycoheterotrophic plants at local scale, either directly or indirectly, is not known.

Here, we examine which soil drivers lead to the patchy distribution of AM mycoheterotrophic plants in tropical rainforests. We explore how the edaphic preferences of mycoheterotrophs reflect the stability of the AM symbiosis upon conditions under which cheating arises.

MATERIALS AND METHODS

Study area

This study was conducted in two forest sites in Colombia in the beginning of the wet season, where mycoheterotrophic plant species are known to occur. We spent five days sampling in each of the sites. The first site consisted of wet tropical lowland forest on terra firme, part of the Amazon rainforest near Leticia ('Amazon'; 4º00'30"S 70º06'12"W). The second site consisted of wet tropical coastal forest on terra firme, part of the Chocó rainforest, near Buenaventura ('Coast'; 3º55'24"N 77º18'56"W). Both sites have no human influence.

Large scale patterns of soil properties do not necessarily reflect the high heterogeneous profiles of soil at local scale, thus we opted for a paired plot sampling strategy where a "positive" plot with mycoheterotrophic plants was simultaneously selected alongside with a nearby "negative" plot without mycoheterotrophic plants. Through this design we were able to identify the effects of specific local differences in soil properties on the patchy occurrences of mycoheterotrophy, within the presumable large-scale variation in soil parameters among sites. We established a total of 16 pairs of plots of 4 x 4 m in the two forests, with five pairs of plots in the Amazon and eleven in the Coast. Positive and negative plots were 5-10 m apart. Pairs of plots were separated by at least 30 m. The number of mycoheterotrophic plants in the positive plots varied between 1 and 22 individuals, and we found up to 6 species per plot (Supporting Information Table S1).

Within each plot, we randomly collected six soil cores, and combined them into a 250 g composite sample per plot. The soil in both sites had clay texture. Soil cores were taken in the shallow top layer of the soil (0-5 cm depth) because we were interested in the chemical properties and nutrient abundance in the soil layer where the roots of the mycoheterotrophic plants are found. Big stones and roots were removed from the samples. The soil was homogenized and preserved on ice immediately after collection until transportation to the laboratory for further processing.

Soil chemistry and nutrient analyses

Soil chemical and nutrient properties were assessed for all 32 plots. Each composite sample was analyzed for soil pH. Total amounts of nitrogen (N_{TOT}) and phosphorus

(P_{TOT}) were estimated by the Kjeldahl method (Bremner, 1960). The available nitrogen (NH_4^+ and NO_3^-) in the soil was determined by spectrophotometry using 1 N potassium chloride (Maynard & Kalra, 1993). The available phosphorus (P_{AV}) was extracted using Bray II solution (Murphy & Riley, 1962). Exchangeable bases (Na, K, Ca and Mg) were measured by the ammonium acetate method (Hanway & Heidel, 1952) and determined by atomic absorption spectometry. The available micronutrients (Cu, Zn, Mn and Fe), available bore (B), sulfur (S), aluminum (Al), cation exchange capacity (CEC), and soil moisture (Humidity) were determined according to Carter & Gregorich (2006). Organic matter (OM) content in the soil was determined according to Walkley & Black (1934). All analyses were performed by the Centro Internacional de Agricultura Tropical in Colombia. Total soil C and N (on air-dried soil), and abundance of $\delta^{13}C$ and $\delta^{15}N$ were analyzed at the UC-Davis (University of California). To evaluate the influence of nutrient stoichiometry on soil processes, we calculated the N:P, N:K, C:P and C:N ratios.

Data analysis

We described and compared the general soil characteristics from the Amazon and Coast using a principal component analysis (PCA). We tested for differences in overall soil composition among positive and negative plots across both sites using a one-way permutational multivariate analysis of variance (perMANOVA with 999 permutations). We tested for homogeneity of dispersion among groups before performing the perMANOVA, and confirmed the assumption of homogeneous dispersion among sites ($P = 0.753$), and between negative and positive plots within the Amazon ($P = 0.198$) and the Coast ($P = 0.873$).

Because we were interested in the effect of soil properties that drive the presence of mycoheterotrophic plants, we calculated the difference in the soil parameter values within each paired negative and positive plot, which hereafter we refer to as delta (Δ). A negative delta indicates that the parameter is lower in the plots where mycoheterotrophic plants were absent, and a positive delta indicates that a specific parameter is lower in the plot where these plants were present. We tested whether there were significant differences across all deltas of the soil properties among sites using perMANOVA (homogeneity of dispersion: $P = 0.713$). We examined whether the individual delta of soil properties varied across and within sites using ANOVAs with "Site" as fixed factor. We also tested for differences in the individual deltas of soil properties while

considering the density of mycoheterotrophic plants found in each plot as a weighting factor to evaluate the effects on both occurrence and abundance of mycoheterotrophic plants simultaneously.

To assess which combination of soil properties mycoheterotrophic plants were selecting for, we selected all soil properties with significantly different deltas. Each predictor was standardized to mean = 0 and SD = 1 to avoid scaling variance issues due to different measurement scales. With all predictors, we built generalized mixed-effects models (GLMMs) to understand the soil parameters that mycoheterotrophic plants have preference for, with 'Plot' as a random effect term to account for the heterogeneity of soil reflected in the spatial clustering of the paired plots. Model selection was performed by adding terms to the model, including interactions between variables, and selecting the terms that gave the greatest improvement to the model likelihood, as assessed by the lowest Bayesian Information Criteria (BIC; Aho et al. 2014). The variables included in the final model were retained if they were significant, and had a variance inflation factor (VIF) < 4 (Zuur *et al.* 2010) and showed a Pearson correlation with all other modelled predictors < |0.70| (Dormann *et al.* 2013). Furthermore, to quantitatively examine the nature of the observed relationships, we used general linear models (GLMs) to tests for relationships between the predictors retained in the best model and the density of mycoheterotrophic plants found in each plot using the deltas of the soil variables.

All analyses were performed in R 3.4.1 (R Core Team, 2016), using the packages NLME, MULTCOMP and VEGAN.

Results

Soil characteristics

We obtained 21 soil parameters from the soil analyses (Table 1). Overall, soil characteristics were significantly different between the two sites ($F = 28.338$, $R^2 = 0.49$, $P = 0.001$; Supporting Information Fig. S1). Soil characteristics were not significantly different between positive and negative plots in the Amazon ($F = 0.738$, $R^2 = 0.08$, $P = 0.627$; Fig. 1a), but they did differ in the Coast ($F = 3.079$, $R^2 = 0.13$, $P = 0.044$; Fig. 1b). Yet, when considering each soil property individually, in the Amazon site the availability of NO_3, P_{AV} and CEC was significantly lower (Supporting Information Table S2) in the positive plots compared to the respective negative plots, while the main soil

Table 1 | Variation of the soil parameters measured in the plots within the Amazon and Coast calculated by the difference between negative and positive plots.

Soil parameters	Amazon mean Δ	P	Coast mean Δ	P
pH	0.226	0.278	0.183	0.172
MO	-15.936	0.790	-39.811	0.074
P_{TOTAL}	-39.600	0.279	-12.727	0.727
P_{AV}	1.257	0.900	-3.834	0.161
Ca	0.707	0.105	-0.376	0.223
Mg	0.163	0.482	-0.222	0.081
Al	0.392	0.541	0.032	0.990
CICE	1.262	0.083	-0.798	0.106
S	2.378	0.977	-13.095	0.252
B	-0.356	0.743	-0.740	0.097
Zn	-2.721	0.351	-3.723	0.031
Mn	70.543	0.011	-12.612	0.635
Fe	-42.925	0.531	-6.253	0.969
N_{TOTAL}	-487.674	0.402	-310.000	0.442
NH_4	9.260	0.459	-9.354	0.199
NO_3	21.504	0.000	0.278	0.991
Humidity	-35.408	0.846	-132.661	0.024
K	0.027	0.966	-0.232	0.020
Cu	-0.073	0.895	-0.042	0.923
$\delta^{13}C$	2.328	0.116	-0.034	0.999
$\delta^{15}N$	0.026	0.997	0.610	0.053
N:P	2.058	0.316	1.598	0.228
N:K	53.925	0.023	17.158	0.344
K:P	-0.007	0.961	-0.003	0.982
K:C	0.000	0.928	-0.001	0.158
C:P	-6.560	0.879	13.978	0.311
C:N	-1.434	0.946	-8.116	0.050

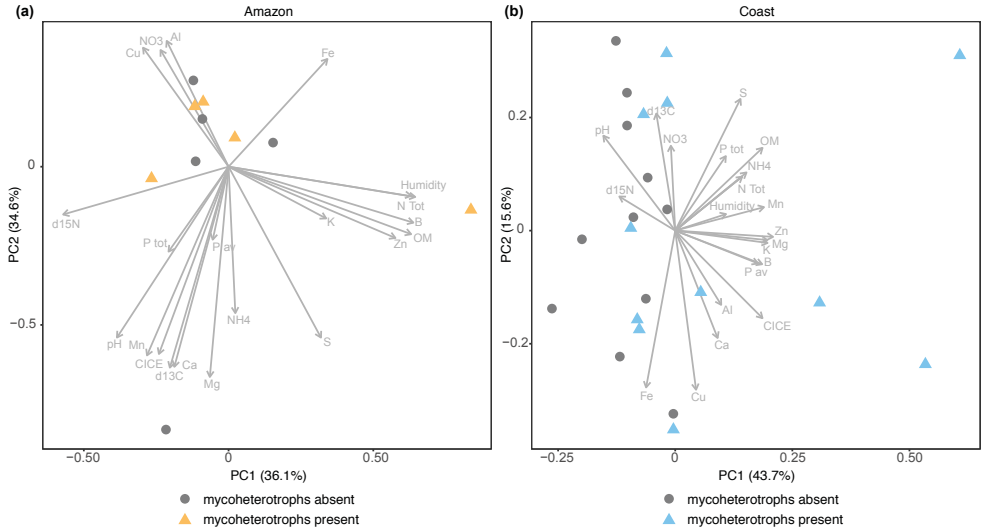

Figure 1 | Principal Components Analysis of the soil properties in the positive plots (triangles) and negative plots (circles) present in the Amazon (a) and the Coast (b). Length of the arrows represents the relative importance of predictors.

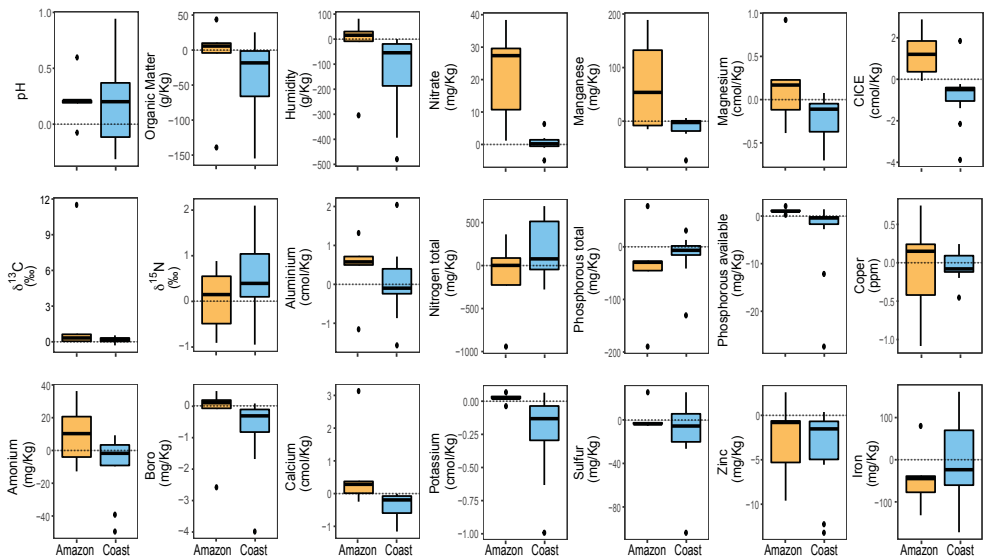

Figure 2 | Variation of soil properties between the negative and positive plots in the Amazon (yellow) and the Coast (blue). Positive values indicate higher availability of a soil property in the negative plots, while negative values indicate a higher availability in the positive plots.

properties that mostly vary within the whole site were OM, Humidity, N_{TOT}, $\delta^{15}N$, B and Zn (Fig. 1a). This indicates that the nutrients that are more variable within this site did not provide a clear separation between positive and negative plots. At the Coast site, positive plots had significantly higher availability of OM, Humidity, CEC, N_{TOT}, and positive ions such as K, Ca, Mg, B, Zn, Mn; and lower availability of $\delta^{15}N$ (Supporting Information Table S2), which correspond to the same soil properties that had more variation within this site (Fig. 1b).

The deltas of the soil characteristics observed between negative and positive plots showed a trend of difference among sites ($F = 2.266$, $R^2 = 0.14$, $P = 0.051$; Fig. 2). The deltas of OM, pH, NO_3, Humidity and Zn were significantly different for both sites combined. The deltas of NO_3 and Mn differed significantly within the Amazon plots, while the deltas of Humidity, Zn and K differed significantly within the Coast plots (Table 1). Thus, OM, pH, NO_3, Humidity, Zn, Mn and K were selected as predictors to build generalized mixed-effects models. 'Site' was also considered as predictor due to the interaction of some predictors with either the Amazon or the Coast plots.

Model selection

The best model showed a significant effect of NO_3, pH and the interaction between NO_3 and K (GLMM: $R^2 = 0.53$, BIC = 40.3; Table 2). We selected this model from a set of undistinguishable models which included the effect of the interaction of pH with Site, the interaction of pH with K, or the interaction of NO_3 with Site. The second best model showed a significant effect of Humidity and pH and the interaction between Humidity and OM (GLMM: $R^2 = 0.35$, BIC = 46.3; Table 2). OM was highly correlated with K (Pearson correlation: $R^2 = 0.67$) and Zn (Pearson correlation: $R^2 = 0.72$), and K correlated with Zn (Pearson correlation: $R^2 = 0.74$), not allowing to separate their impacts. Therefore we did not include these factors in the same model.

The ΔNO_3 was the best predictor for the density of mycoheterotrophic plants (GLM: $Z = -4.827$, $df = 15$, $R^2 = 0.48$, $P < 0.001$; Fig. 3). The number of mycoheterotrophic plants decreased with the increasing difference in concentration of NO_3, corresponding to an increase in total concentration of NO_3 (Pearson correlation between ΔNO_3 and NO_3: R2 = 0.77; Fig. 4).

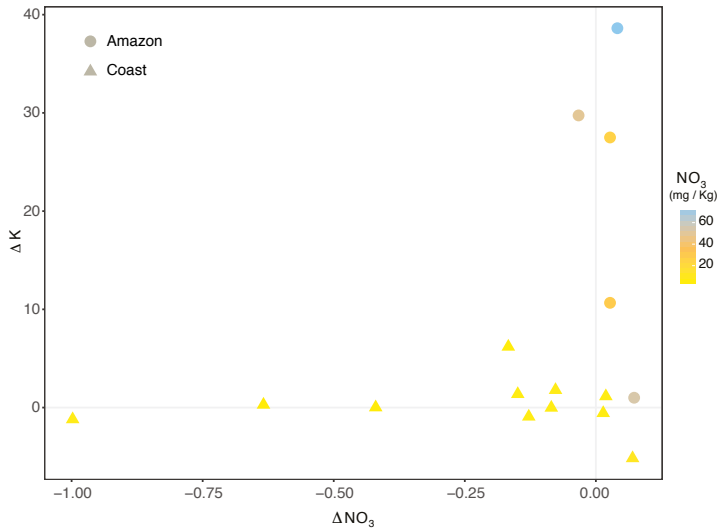

Figure 3 | Variation of K in relation to NO_3 per sample. Positive values indicate higher values of a soil property in the negative plots, while negative values indicate a higher values in the positive plots.

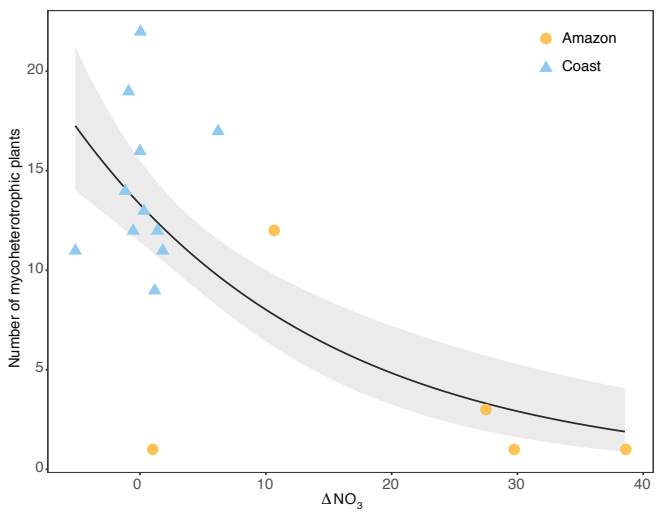

Figure 4 | Relationship between the number of mycoheterotrophic plants observed in the positive plots and the ΔNO_3 (negative plots minus positive plots).

Table 2| Outcomes of the Generalized Linear Mixed Effect modeling aimed to explain the occurrence of mycoheterotrophic plants. 1 is the best model. 2 is the best alternative model (ΔBIC = 6.0).

Model	Terms	Coefficient	SE	z value	P value
1	Intercept	1.038	0.710	1.463	0.144
	NO_3	-3.432	1.347	-2.548	0.011
	pH	-1.405	0.820	-1.713	0.087
	NO_3 : K	-11.165	4.264	-2.618	0.009
2	Intercept	-0.474	0.614	-0.772	0.440
	Humidity	5.052	2.377	2.126	0.034
	pH	-2.157	1.137	-1.897	0.058
	Humidity : OM	4.538	2.226	2.038	0.042

Discussion

In this study, we compared soil characteristics of paired plots with and without mycoheterotrophic plants to search for local-scale drivers that influence the occurrence of cheaters in the AM mycorrhizal symbiosis in tropical rainforests. We found that the strongest edaphic predictors of mycoheterotrophic species occurrences involved the interaction between NO_3 and K, and the individual effect of NO_3 and pH. The interaction between soil NO_3 and K is well known in crop responses. Crop response to added nitrogen fertilizers decreases when the exchangeable potassium content of a soil is below an optimal level, because plants deficient in potassium content are not able to produce proteins despite an abundance of available nitrogen (Ranade-Malvi, 2011). In addition, several studies have shown a negative impact of nitrogen addition in agriculture systems on the AM symbiosis performance (Kabir *et al.* 1998; Galvez *et al.* 2001; Oehl *et al.* 2004), because an increased availability of nitrogen to plant roots can lead to carbon limitation in the AM fungal network, which can in turn induce phosphorus deficiency due to carbon limitation to the fungi (Olsson *et al.* 2005). Moreover, excessive amounts of N reduce the plant uptake of P, K and other micronutrients (Ranade-Malvi, 2011). While the stoichiometry of nutrients in soils does not sufficiently reflect nutrient deficiency levels, nutrient stoichiometry in soils has been shown to be crucial in determining the relative availability of nutrients for plant uptake and the stability of the AM symbiosis (Johnson, 2010; Khan *et al.* 2015). Additionally, pH strongly influences the availability of nutrients in the soil, which in turn also impacts the efficiency of nutrient uptake by plants (Rippy *et al.* 2004).

Our study shows a distinct response of the occurrence of mycoheterotrophic plants to NO_3 and K according to the degree of soil fertility, which is also linked to pH variations. In the Amazon, where fertility is higher and heterogeneous, mycoheterotrophic plants avoid high NO_3 conditions while K does not vary. In contrast, in the Coast, where fertility is lower and more homogeneous among the plots, plants select for higher availability of K. While there seems to be a consistent avoidance of higher fertility patches, also reflected in the lower density of plants found with increasing NO_3 availability, it remains unclear how K influences the distribution of these plants. A possible explanation for the overall increased availability and patchiness of K in the Coast can be the effect of salt spray from sea that is close by. The uptake of K by photosynthetic plants is enhanced by the association with AM fungi, which require a minimum availability of K in the soil for the stability of the AM symbiosis (Khan *et al.* 2015).

Mycoheterotrophic plants preferred patches with lower N:K ratios compared to their paired negative plot in the Amazon (Table 1), and the positive plots in the coast had a trend of lower N:K ratios than the negative plots (Table S2, $P < 0.1$). The N:K ratio is highly correlated with NO_3 concentrations, not allowing to disentangle the effect of both predictors in explaining the occurrence of mycoheterotrophic plants in this study. We speculate that in the Amazon, the preference for a lower N:K ratio is mostly related to the avoidance of fertile conditions, while in the Coast, mycoheterotrophic plants prefer higher K availability.

Despite the known importance of N:P ratios in the occurrence and distribution of plants and AM fungi, the N:K ratio and not the N:P ratio appeared to be the relevant predictor for the local distribution of mycoheterotrophic plants. According to the trade balance model (Johnson, 2010), a stable mutualistic mycorrhizal interaction is expected at high N:P ratios. At the same time, a strong positive correlation between the accumulation of P and K has been reported during the AM symbiosis (Olsson *et al.* 2011), while the accumulation of K in AM fungi has been related to low carbon supply from the host plant (Hammer *et al.* 2011). Together, this suggests that accumulation of K may be a consequence of the accumulation of the P that AM fungi do not transfer to plants at low carbon availability conditions (Garcia & Zimmermann, 2014). Thus, it is likely that the performance of the AM symbiosis is affected in a similar manner by the N:P and N:K ratios. Following the trade balance model, our results indicate that in comparison with the negative plots, the lower N:K ratio in the positive plots could indicate that mycorrhizal networks between photosynthetic plants and mycorrhizal

fungi are potentially stronger in the negative plots, and thus mycoheterotrophic plants avoid patches with conditions that favor a strong mutualism between plants and AM fungi.

As indicated above, phosphorus did not relate to local mycoheterotrophic occurrences in this study while available phosphorus – together with soil moisture - has been suggested to be the strongest environmental predictor of plant species distributions in tropical forests (Condit *et al.* 2013), and while phosphorus is considered to be the main limiting element in tropical forests for microbial processes, including mycorrhizal fungi (Camenzind *et al.* 2017). Sheldrake et al. (2017) reported a threshold of 2 mg P Kg^{-1} beyond which mycoheterotrophic plants did not occur across a natural fertility gradient in Panama. Yet, in our study, we found mycoheterotrophic plants growing at much higher concentrations of P in the Coast ranging from 1 to 29 mg P Kg^{-1} (mean = 5.2 mg P Kg^{-1}, sd = 8.7), and in the Amazon from 4 to 5 mg P Kg^{-1} (mean = 4.8 mg P Kg^{-1}, sd = 0.2). In fact, in our study, phosphorus did not significantly vary between the positive and negative plots. This suggests that there is not a direct selection for particular concentrations of phosphorus, and that the availability of other nutrients in the soil may play an important role on the impact of phosphorus in this system, at least under the range of available phosphorus of our sampling sites, and for the species considered in our study. Instead, our results highlight that the nutrient stoichiometry rather than the actual concentration of each nutrient drives the occurrence of mycoheterotrophic plants. Particularly the balance between NO_3 and K is key for the prevalence of AM mycoheterotrophic plants in tropical rainforests. Because the influence of individual nutrient concentrations can vary due to the stoichiometry effect of other nutrients, we propose that absolute concentrations of elements may give poorer information about the probability of cheating the AM symbiosis.

Soil moisture appeared to influence mycoheterotrophic plants' occurrence, but to a lesser extent than soil fertility. Soil moisture has been hypothesized to be the main limiting factor to the occurrence of these plants, due to their sensitivity to desiccation (Leake, 1994; Klooster & Culley, 2009), but our study shows that mycoheterotrophic plants have a stronger selection for other soil conditions. The absolute values of humidity are significantly lower in the Amazon comparing to the Coast, yet our study shows a preference for patches with higher humidity only in the Coast. This finding implies that while humidity still contributes for to the occurrence probability of mycoheterotrophic plants, it is not as limiting as previously thought, or at least not at this fine scale. Possibly,

soil moisture determines the occurrence of mycoheterotrophic plants at a broader scale such as between forests (Leake, 1994), while at a finer scale of the 4 x 4 m plots that we used in this study, the heterogeneous fertility conditions within forests play a major role.

In the Amazon site, mycoheterotrophic plants were relatively rare. Three established plots only contained one individual, in one plot we found three and in another one we detected 12 specimens. At the Coast, mycoheterotrophic plants were quite abundant throughout the forest but displaying the characteristic patchy distribution and co-occurrence of distantly related species. Possibly, the lower abundance of mycoheterotrophs in the Amazon reflects less favorable environmental conditions for their occurrence in general, or in particular, at the time of sampling. The differences in success in finding these plants at both sites suggests that differences in soil properties between the two forest sites (Supporting Information Fig. S1) may explain this difference in mycoheterotrophs abundances. Even though the abundance of mycoheterotrophic plants differed between the two sites, within both sites the N:K ratio still determined their local occurrence patterns.

In conclusion, our results provide empirical support to the view that mycoheterotrophic plants avoid high fertility conditions, where fungi are prone to parasitism (high N, high P or K). We also suggest that these plants seem to avoid conditions that could favor a strong AM mutualism (high N, low P or K), according to the trade balance model (Johnson, 2010). In accordance, our findings show a negative response of the abundance of mycoheterotrophs individuals to an increase in N availability. Therefore, we propose that mycoheterotrophy is more prone to occur in conditions of weak mutualistic strength (low N, low P or K) between plants and AM fungi according to the trade balance model (Johnson, 2010). In conditions leading to commensalism between plants and AM fungi (low N, high P or K), cheating is less likely to occur, since both partners are exchanging limited resources, and therefore it is theoretically more difficult for mycoheterotrophic plants to obtain carbon from these fungi.

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Author Contribution:

All authors designed the study. S.I.F.G. and V.S.F.T. conducted fieldwork. S.I.F.G. analysed the data. S.I.F.G. wrote the manuscript. All authors contributed to discussion and to earlier versions of the manuscript.

SUPPORTING INFORMATION

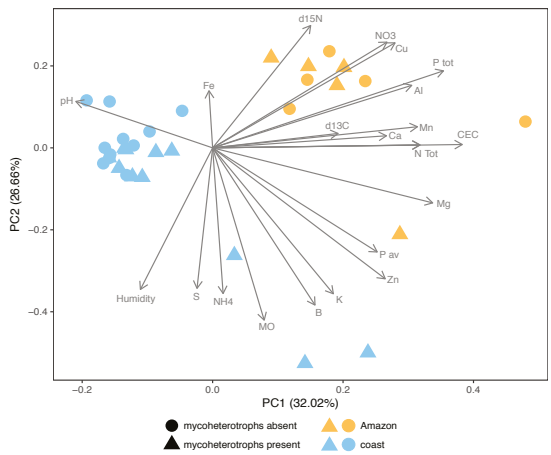

Figure S1 | Principal Components Analysis of the soil properties in the Amazon (yellow) and the Coast (blue). Positive plots (triangles) and negative plots (circles) within each site are represented. Length of the arrows represents the relative importance of predictors.

Table S1 | Mycoheterotrophic plant species present in the 16 plots. Number of individuals from each species is presented

	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	Plot 7	Plot 8	Plot 9	Plot 10	Plot 11	Plot 12	Plot 13	Plot 14	Plot 15	Plot 16
Apteria aphylla																
Gymnosiphon brachycephalus		2	1		3	7		4			3					
Gymnosiphon divaricatus		8	8	8	7	5		6	5	6	4		3			
Sciaphila sp.	11															
Sciaphila purpurea										4	8					
Soridium spruceanum				1	6		6		5	3	3					
Voyria aphylla				1		8	6		1							
Voyria chionea														1	1	2
Voyria pittieri																10
Voyria tenella		6						3				1				

Table S2 | Overall soil parameters in the negative and positive plots within the Amazon and Coast sites.

	Amazon			Coast		
	Neg plots mean	**Pos plots mean**	**P**	**Neg plots mean**	**Pos plots mean**	**P**
pH	4.26	4.03	0.106	4.49	4.31	0.156
OM	77.77	93.71	0.648	88.73	128.54	**0.038****
P_{TOTAL}	441.80	481.40	0.411	199.82	212.55	0.344
P_{AV}	6.10	4.85	**0.013***	1.38	5.21	0.175
Ca	1.68	0.97	0.319	0.31	0.69	**0.008****
Mg	0.64	0.48	0.502	0.18	0.40	**0.023****
Al	2.94	2.55	0.319	1.42	1.39	0.898
CEC	5.26	3.99	**0.077***	2.09	2.89	**0.066***
S	47.61	45.23	0.709	59.71	72.8	0.230
B	0.65	1.01	0.563	0.62	1.36	**0.071***
Zn	4.49	7.21	0.274	2.31	6.03	**0.029****
Mn	114.20	43.66	0.154	4.10	16.71	**0.094***
Fe	226.49	269.41	0.289	195.04	201.29	0.850
N_{TOTAL}	3366.48	3854.20	0.526	1841.82	2151.82	**0.058***
NH_4	34.65	25.40	0.296	38.90	48.25	0.136
NO_3	67.99	46.48	**0.035****	5.61	5.33	0.741
Humidity	409.85	445.25	0.636	521.84	654.5	**0.019****
K	0.30	0.27	0.191	0.18	0.42	**0.030****
Cu	1.80	1.87	0.829	0.55	0.59	0.507
$\delta^{13}C$	-27.07	-29.40	0.365	-29.31	-29.27	0.691
$\delta^{15}N$	5.35	5.33	0.942	4.30	3.69	**0.053***
N:P	11.78	9.72	0.217	5.07	3.48	0.170
N:K	241.93	188.01	0.157	34.98	17.82	**0.074***
K:P	0.05	0.06	0.205	0.18	0.18	0.903
K:C	0.004	0.004	0.848	0.002	0.003	**0.017****
C:P	12.74	19.30	0.396	84.94	70.97	0.270
C:N	1.36	2.80	0.383	18.46	26.57	**0.074***

The soil parameters are significantly different between the negative and positive plots within the respective site (***** $P < 0.10$; ****** $P < 0.05$).

CHAPTER 5

Global distribution patterns of mycoheterotrophy

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ABSTRACT

Mycoheterotrophy is a mode of life where plants cheat the mycorrhizal symbiosis, receiving carbon via their mycorrhizal partners. Despite being widespread, mycoheterotrophic plants are often locally rare, hampering the understanding of their global environmental drivers. Here, we explore the global environmental preferences of mycoheterotrophy, and investigate the environmental drivers of differential habitat preferences of mycoheterotrophic plants associated with arbuscular (AM) and ectomycorrhizal (EM) fungi.

We compiled the currently largest global dataset of mycoheterotrophic plant species occurrences and examined which environmental factors, including soil type, climate, vegetation type and distribution patterns of host mycorrhizal plants relate to occurrence patterns of mycoheterotrophic plant species associated with AM and EM fungi. Mycoheterotrophic plant species avoid cold and highly seasonal climates and show a strong preference for forests. AM-associated mycoheterotrophs are predominantly found in broadleaved tropical evergreen forests whereas EM-associated mycoheterotrophs occur in temperate regions and mostly in broadleaved deciduous and evergreen needleleaved forests. The abundance of AM and EM host plants was a worse predictor for mycoheterotrophs occurrences than forest type. Temperature and precipitation variables - but not edaphic factors - were the best predictors explaining the distribution patterns of AM and EM mycoheterotrophs after accounting for the effects of forest type. For individual lineages, major differences in environmental preferences (often related to edaphic factors) occurred which were significantly associated with plant evolutionary relationships, indicating that these cheater plants have limited adaptive capabilities.

The strong global geographic segregation of AM and EM mycoheterotrophs does not reflect the abundance of their green hosts, but seems to be driven by differential climate and habitat preferences. Our results highlight the non-trivial nature of mycorrhizal interactions, and indicate that identity of the partners is not enough to understand the underlying mechanisms that promote plant-mycorrhizal interactions.

Introduction

Mycoheterotrophy represents the breakdown of one of the most widespread and ecologically important mutualisms on Earth – the mycorrhizal symbiosis, where green plants exchange photosynthesized carbohydrates for mineral nutrients obtained by mycorrhizal fungi in the soil (Smith & Read 2008). In this trophic strategy, 'cheater' plants obtain carbon from their mycorrhizal partners. Mycoheterotrophic plants can use mycoheterotrophy in combination with autotrophy, or rely exclusively on their mycorrhizal fungi to obtain carbon, becoming fully mycoheterotrophic, and losing the ability to perform photosynthesis (Leake 1994; Gebauer *et al.* 2016). Mycoheterotrophy has evolved multiple times independently in flowering plants (Merckx 2013), and occurs within the two most common mycorrhizal types: the arbuscular mycorrhizal (AM) and the ectomycorrhizal (EM) fungi (Leake 1994; Smith & Read 2008).

Fully mycoheterotrophic plants occur on every continent except Antarctica (Bidartondo & Bruns, 2002; Merckx 2013) and comprise around 500 species (Leake 1994; Merckx *et al.* 2009). AM mycoheterotrophic plants occur mostly in tropical rainforests but occasionally grow in subtropical and even temperate regions, while EM mycoheterotrophs occur mostly in temperate zones but occasionally reach lower latitudes in mountain ranges (Merckx 2013). Thus, this suggests a tropical vs temperate distribution of mycoheterotrophic plants according to their mycorrhizal type, which indicates that climate plays a major role in their distribution. Nonetheless, regardless of occurring in tropical or temperate areas, all mycoheterotrophic plants seem to share a preference for humid forests with dense overstory in deep shade, with a thick layer of leaf litter on the forest floor where the occurrence of herbaceous plants is restricted (Leake 1994). Despite being widespread, mycoheterotrophic plants are often locally rare. However, when such plant is found in the field, there is a high probability to find other distantly related mycoheterotrophic species in the vicinity (Leake 1994; Merckx 2013). This suggests that mycoheterotrophic plants share environmental preferences both within and across tropical and temperate areas that still remain unexplored.

Because mycoheterotrophic plants obtain their carbon through a belowground fungal network, and ultimately from surrounding green plants (Bidartondo *et al.* 2002; Yamato *et al.* 2016; Gomes *et al.* 2017a), the distribution of mycoheterotrophy might be limited by the abundance of green host plants that act as a carbon source for their mycorrhizal fungi. Furthermore, besides the ecological drivers, the evolutionary history of mycoheterotrophic plants may also play an important role in their global distribution

patterns since species tend to be restricted to biogeographical realms (Jonker, 1938).

Here, we explore global environmental preferences of mycoheterotrophy. Specifically, we test whether the differential distribution of mycoheterotrophic plants associated with AM and EM fungi can be better explained by soil and climate, by distinct types of vegetation, or by the distribution of their host plants that share the same mycorrhizal type, i.e. AM vs EM dominant vegetation. Moreover, we explore potential drivers for the distribution of mycoheterotrophic plant lineages within each mycorrhizal type to investigate the habitat ranges that these lineages occupy. Understanding global preferences of full mycoheterotrophy will give us new insights in the environmental conditions where mycorrhizal cheating is likely to occur and therewith will enlarge our understanding of the ecology of mycorrhizas.

Materials and Methods

Mycoheterotrophic plant species data

To study the global distribution of mycoheterotrophic plants we compiled a dataset with worldwide observations of a large majority of fully mycoheterotrophic flowering plant species known to date (Merckx 2013). We combined the records from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org) for the whole globe, the Botanical Information and Ecology Network (BIEN, http://bien.nceas.ucsb.edu/bien/) for America, the African Plant Database (http://www.ville-ge.ch) for Africa, and personal datasets (SI, Table S1). We also consulted the database of ex-Soviet Union territory (Akhmetzhanova *et al.* 2012) but there were no records of mycoheterotrophic plants available for that region. Our dataset included 21 species in Gentianaceae, 11 in Ericaceae, 5 in Polygalaceae, 15 in Liliales, 2 in Petrosaviaceae, 2 in Iridaceae, 79 in Dioscoreales, and 40 in Orchidaceae. After removing potentially incorrect occurrences, the compiled dataset contained 22,853 records. We assigned the mycorrhizal types AM and EM to the mycoheterotrophic plants in our dataset based on literature descriptions compiled in Merckx 2013, and excluded those records of species associated with saprotrophic fungi or unverified mycorrhizal type. We created a 1 km^2 worldwide grid and recorded presences and absences of these plants in each grid cell considering their mycorrhizal types. In total, 1,935 (8.5%) individuals were associated with AM fungi and 20,918 (91.5%) with EM fungi. When plants associated with AM and EM fungi were

present in a single grid cell, they were assigned to both AM and EM individual datasets in the subsequent analyses.

Global drivers of mycoheterotrophic plants distribution

We generated histograms of the distribution of mycoheterotrophic plants overlain with global patterns of climatic and edaphic conditions to highlight the environmental preferences of mycoheterotrophs in its entirety (SI, Figure S1). Mycoheterotrophic plants were shown to occur at a global scale with a clear dichotomy of tropics vs temperate regions in their distribution according to mycorrhizal type (see results, Figure 1). Therefore, we focused our analysis on the drivers underpinning the differential distribution of AM and EM-associated mycoheterotrophic plants. Given the obvious differences in temperature and precipitation regimes characteristic for tropics and temperate zones we did not examine the global environmental drivers promoting the differential distribution of AM and EM mycoheterotrophs. Instead, we examined if AM and EM mycoheterotrophs had distinct preferences for a specific type of vegetation. This would reflect the common description of mycoheterotrophic plants as understory plants in closed canopy forests (Leake 1994; Merckx 2013). Alternatively, mycoheterotrophic occurrences may be associated with preference for habitats dominated by hosts of the same mycorrhizal type. This would reflect the reliance of these plants on the belowground mycorrhizal network for carbon uptake (Trudell *et al.* 2003). To investigate these alternative hypotheses, we considered the land class categories from the CCI Land Cover maps (ESA 2015) to infer vegetation type. For the association with the host green plant featuring the same mycorrhizal type, we used the global maps of abundance of green plants associated with AM and EM fungi, respectively (Soudzilovskaia *et al.* 2018). The Land Cover maps were obtained with a spatial resolution of 300 m, which we rescaled to the 1 km^2 grid used in this study. Maps on the abundance of mycorrhizal host types were obtained at a resolution of 10 min. To reduce noise in our dataset caused by potential imprecision of coordinates, and by overlaying the vegetation and plant abundance maps, we excluded all records that were found in areas with no vegetation since mycoheterotrophs need to be associated with mycorrhizal fungi that are subsequently associated to surrounding green plants.

Climatic and edaphic factors are known to be important predictors of plant species and mycorrhizal fungi assemblages at large scales (Tedersoo *et al.* 2014; Davison *et al.* 2015). Hence, we tested the relative importance of these potential drivers for the

distribution of mycoheterotrophic plants after accounting for the effects of vegetation type or abundance of hosts. The climatic data, obtained from the WorldClim database at 1 km^2 resolution (Hijmans *et al.* 2005), describe temperature and precipitation annual trends, seasonality and extreme or limiting environmental factors worldwide. For the soil data, since these plants have generally shallow root systems (Leake 1994; Merckx 2013), we considered only edaphic variables in the top-soil layer from the Harmonized World Soil Database (Batjes *et al.* 2009), which is a set of spatial databases of derived soil properties at a global scale. Furthermore, it is often assumed that these plants are sensitive to desiccation (Leake 1994; Merckx 2013), and therefore we also considered the actual evaporation, the evaporation stress factor, the root zone soil moisture and the surface soil moisture (GLEAM maps; Martens et al. 2017) as potential drivers.

Environmental preferences of individual mycoheterotrophic lineages

Mycoheterotrophic plant species and genera are often restricted to particular biogeographical regions (Jonker, 1938; Merckx 2013; Mennes *et al.* 2015a), suggesting that evolutionary relationships may shape the distribution patterns of these plants. Therefore, we tested whether the evolutionary history of mycoheterotrophic species limits their occurrence to particular ecozones. For this purpose, we considered the 15 independent shifts towards mycoheterotrophy represented by our data (SI Table S2). Based on recent phylogenetic insights we considered four independent shifts in Gentianaceae represented by the genera *Voyria, Voyriella, Exacum* and *Exochaenium* (Merckx et al. 2013), two shifts in Ericaceae including Monotropoideae and Pyrola (Freudenstein *et al.* 2016), and a single shift in Polygalaceae: *Epirixanthes* (Mennes et al. 2015a), Liliales: Corsiaceae (Mennes et al. 2015b), Petrosaviaceae: *Petrosavia* (Cameron *et al.* 2003), Triuridaceae (Mennes *et al.* 2013) and Iridaceae: *Geosiris* (Goldblatt *et al.* 2008). In Dioscoreales we recognized three shifts: *Afrothismia*, Thismiaceae s.s., and Burmanniaceae (Merckx *et al.* 2017). The latter group also contains chlorophyllous species, but recent evidence indicates that these are partially mycoheterotrophic (Bolin *et al.* 2015), suggesting the presence of a strong predisposition for mycoheterotrophy in the most recent common ancestor of the family. Similarly, since all Orchidaceae are initially mycoheterotrophic and many are potentially partially mycoheterotrophic (Gebauer *et al.* 2016), we considered all species in this family to be part of a single lineage. To better understand the observed dichotomy in distribution of AM and EM mycoheterotrophic plant species, we explored environmental preferences separately for each type.

Statistical analyses

To test whether mycoheterotrophic plants had a stronger preference for particular forest types or for association with host green plants with the same mycorrhizal type, we examined four alternative univariate generalized linear models testing the occurrence of AM and EM mycoheterotrophs, respectively vs. (1) eight forest, and (2) the host green plant abundance. We selected the most parsimonious models based on the highest adjusted R^2 and the Bayesian Information Criteria (BIC) (Aho *et al.* 2014). Once the variance explained by the selected predictor in the most parsimonious model was accounted for, we used hierarchical partitioning on the residuals of this model to understand if mycoheterotrophic plants had further preferences for particular environmental conditions. All predictors were standardized to avoid scaling variance issues due to different measurement scales. The selection of the predictors to be included in the models was performed in two steps. First, we excluded variables with $R^2 \leq 0.05$ in univariate linear regressions to avoid spurious correlations. Then, we assessed collinearity among variables by calculating the variance inflation factors (VIF) in a stepwise manner, discarding the variable with the highest VIF at each step, until all the variables maintained in the final model had VIF < 4 (Zuur *et al.* 2010) and Pearson correlation < |0.7| (Dormann *et al.* 2013). To evaluate the importance of each predictor, we calculated the omega squared (ω^2) as an unbiased effect size estimate on the amount of variance explained by each of the individual predictors in the models (Olejnik & Algina, 2000).

We used a one-way permutational multivariate analysis of variance (perMANOVA with 99 permutations), in which Euclidean distance between the lineages was employed as dependent variable and ecozone (to reflect biogeographical preferences) as independent. The analysis was run for the AM and EM datasets separately. Subsequently, multiple testing using BH corrections (Benjamini & Hochberg, 1995) suitable for large datasets was performed as posthoc test. To visualize the environmental preferences of the mycoheterotrophic lineages, we applied principal component analysis (PCA) on the standardized environmental variables for each dataset separately, labelling the occurrence of each lineage in PCA-space.

All analyses were carried out in R 3.4.1 (R Core Team, 2016) with the LME4, LSR, RVAIDEMEMOIRE, and VEGAN packages.

RESULTS

Global distribution patterns of mycoheterotrophy

The global distribution of mycoheterotrophic occurrences showed a clear dichotomy with the AM plants mainly occurring in the tropics and EM plants in temperate areas (Figure 1). The Palearctic is the most well represented region in our study comprising 71.1% of the total number of records, followed by the Neartic with 14.7%, Neotropic with 7.3%, Australasia with 4.3%, Afrotropics with 1.4% and finally Indomalaya with 1.2% of the total records.

When comparing the distribution of mycoheterotrophic plants with patterns in global climate and soil variables, we observed that in general mycoheterotrophy has no strong preference for particular conditions except for avoiding very cold and seasonal climates (Figure 2; see the other variables in SI Figure S1). The majority of mycoheterotrophs occur in forests (Figure 3), with clear preferences for particular forest types: AM mycoheterotrophs occur mostly in broadleaved evergreen forest (Figure 4a), while EM mycoheterotrophs occur mostly in other forest types, preferring needleleaved evergreen forests, broadleaved deciduous forests, forests with mixed leaf habits, and forests with shrub cover (Figure 4b). AM and EM mycoheterotrophic plants showed clear preferences for climatic conditions coinciding with their tropical and temperate distribution, respectively, but not for particular soil conditions (SI, Figure S1). The selection of the most parsimonious models resulted in the evergreen forests (BIC: 6936; Adj R^2: 0.49 for AM; BIC: 6784; Adj R^2: 0.48 for EM) as being the best predictor among all forest types.

The global abundance of mycoheterotrophic plants seems to follow the global trend of AM hosts abundance (Figure 4c), better than the abundance of EM hosts (Figure 4d). However, models based on forest type were consistently significantly better than models including the host plants associated with AM type (BIC: 7428.2; Adj R^2: 0.45 for AM; BIC: 7026.2; Adj R^2: 0.46 for EM).

The hierarchical partitioning analyses on the residuals of the best models - which had evergreen forest as single predictor - showed that evergreen forests were the main predictors of the distribution of mycoheterotrophic plants, with climate and soil variables hardly showing any explanatory power, since only one climatic variable showed medium importance (ω^2 >0.06; Cohen 1988) for either of the mycorrhizal

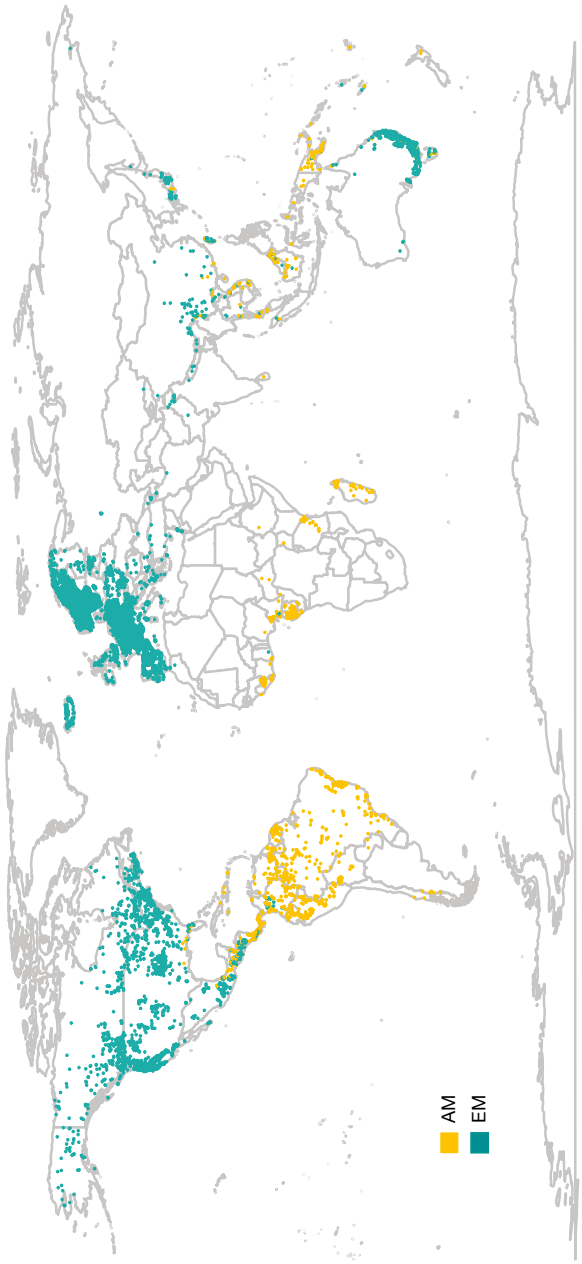


Figure 1 | Global distribution of mycoheterotrophic plants associated with arbuscular (AM) and ectomycorrhizal (EM) fungi. Records were obtained from public databases (see methods).

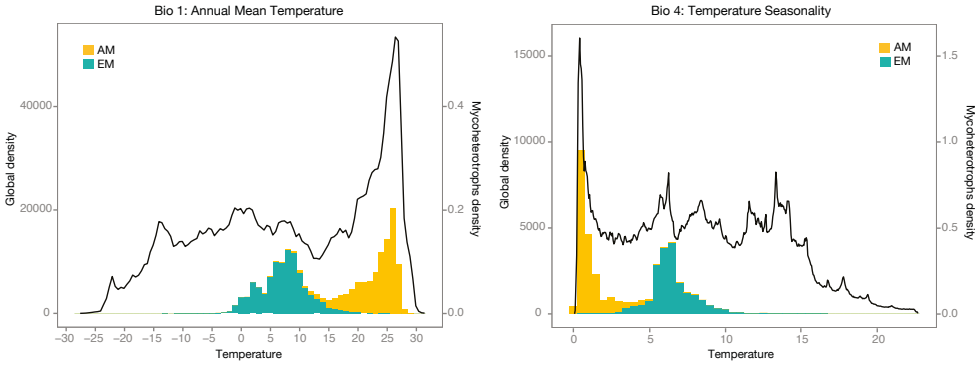
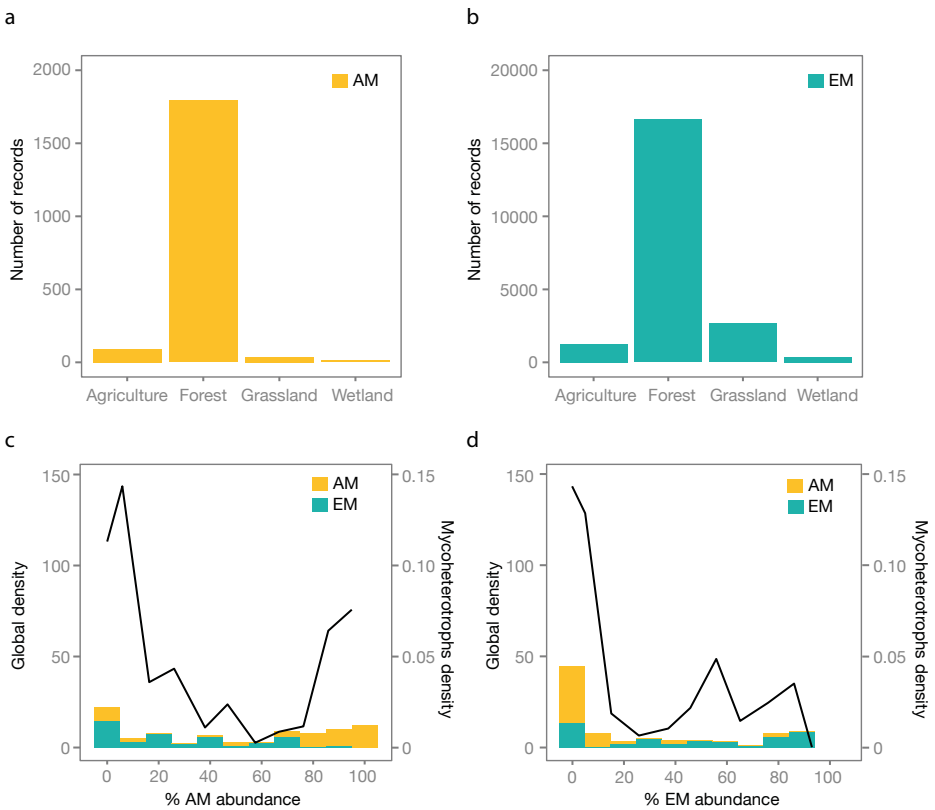


Figure 2 | Climatic preferences of mycoheterotrophic plants (histograms) and global trend of plants (solid line) for the WorldClim dataset (Hijmans et al., 2005) variables annual mean temperature (bio 1) and temperature seasonality (bio 4).



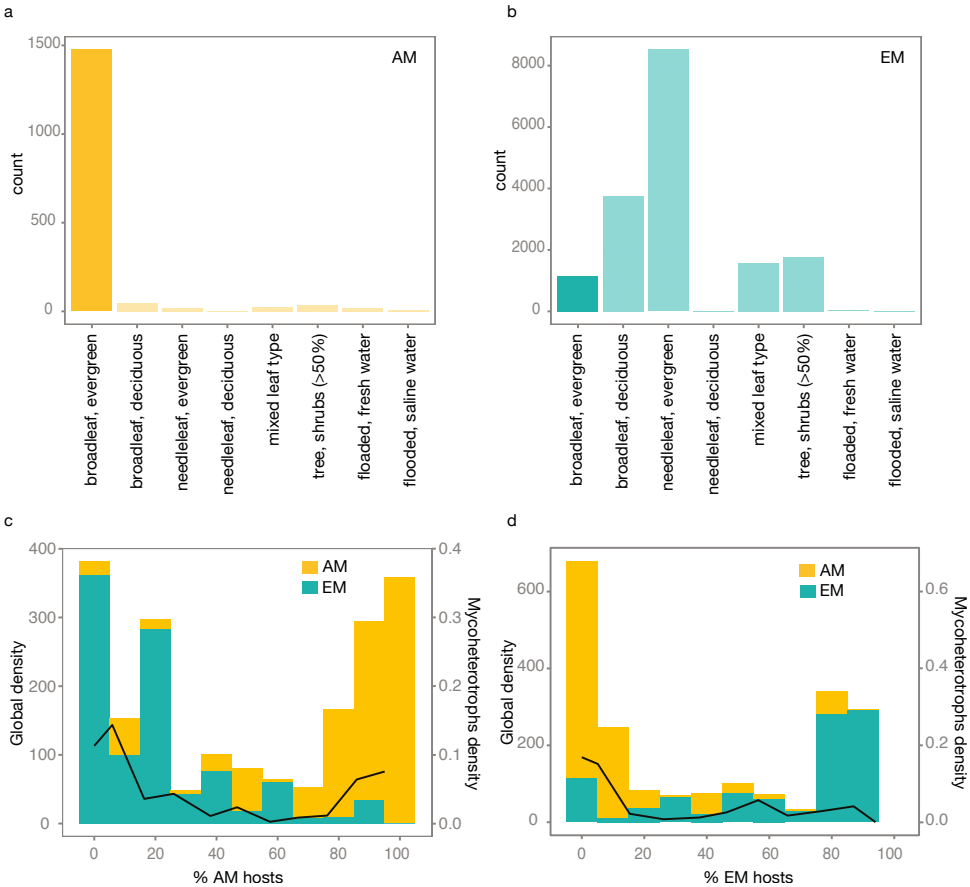


Figure 4 | Mycoheterotrophic plants habitat preference within forest types based on the categories of the CCI Land Cover maps for AM (a) and EM (b) mycoheterotrophs. Full bars highlight the forest type that best predictor of the dichotomic distribution pattern among mycorrhizal types of these plants. Global abundance of green plants (hosts) associated with AM (c) and EM fungi (d), and respective abundance of records of mycoheterotrophic plants per mycorrhizal type in our dataset.

Figure 3 (previous page) | Land cover preference of mycoheterotrophic plants. The land cover categories were obtained using the CCI Land Cover maps for AM (a) and EM (b) mycoheterotrophs.

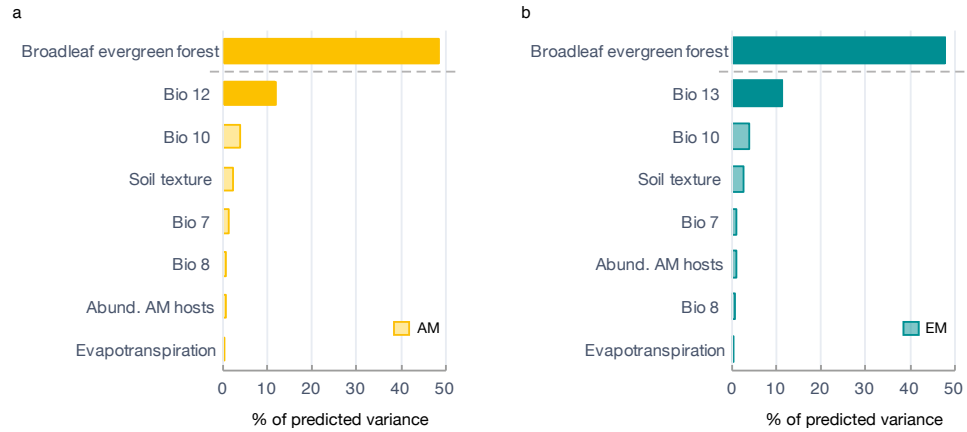


Figure 5 | Ranking of selected predictors explaining the distribution of mycoheterotrophic plants associated with AM and EM fungi as a result of the GLM (above dashed lines) and the hierarchical partitioning analyses (below dashed lines). Predictors are ranked according to the % of predicted variance reflecting their importance in the models. Important predictors are represented as full bars.

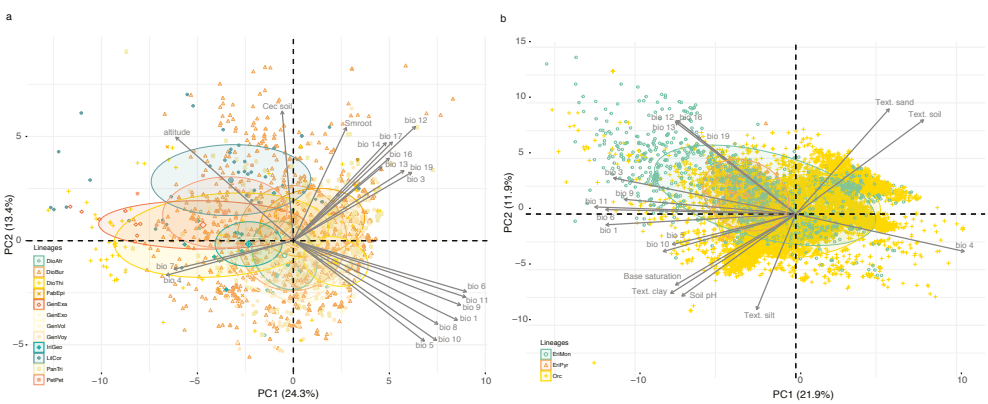


Figure 6 | Principal Components Analysis of the environmental space occupied by mycoheterotrophic plants associated with AM (a) and EM (b) fungi. Only the predictors significantly associated with the first and second principal components are shown. Length of the arrows represent the relative importance of predictors. Clouds of points are the records in the dataset. Ellipses delimitate the environmental space occupied by each lineage.

types, namely annual precipitation (model coefficient: 0.12; $\omega^2 = 0.12$) and the mean precipitation of the wettest month (model coefficient: -0.16; $\omega^2 = 0.11$) for the AM and EM mycoheterotrophs, respectively. All the other predictors in the models had $\omega^2 < 0.06$ (Figure 5).

Environmental preferences of lineages

The one-way perMANOVA indicated that mycoheterotrophic lineages showed preferences for specific ecozones, both for AM (*df* = 11, *Pseudo-F* = 9.0, $R^2 = 0.05$, *P* = 0.01) and for EM fungi (*df* = 2, *Pseudo-F* = 315.8, $R^2 = 0.03$, *P* = 0.01). Pairwise permutation comparisons of lineage indicated differences between all AM lineages (*P* = 0.013 – 0.044), except for *Exochaenium* with *Afrothismia, Burmanniaceae, Exacum, Voyria, Voyriella* (*P* = 0.054 – 0.760), and *Petrosavia* with *Exacum* (*P* = 0.213). This exception can be an artifact due to the low number of records for Exochaenium in our dataset. For the EM dataset, pairwise permutation comparisons of lineage indicated significant differences among all the three EM lineages (*P* = 0.010).

The Principal Component Analyses indicated that mycoheterotrophic lineages associated with AM and EM fungi have different habitat preferences within tropical and temperate areas, respectively. For the AM mycoheterotrophs, the first two components of the PCA explained 37.7% of the total variance, and contain mainly variables related to temperature and precipitation. The cation exchange capacity and the root zone soil moisture also showed a significant contribution (Figure 6a). The clustering per lineage within these environmental variables is significant (see perMANOVA above) but not strong (see diffuse separation in the PCA, Figure 6a). Furthermore, we checked PC3 and PC4 for their potential to explain variance not accounted for by PC1 and PC2, but their additional explanatory power was low compared to PC1 and PC2. For the EM mycoheterotrophs, the first two components of the PCA explained 33.8% of the total variance. We observed an influence of mostly temperature variables, and then precipitation ones. For mycoheterotrophs within this mycorrhizal type, edaphic variables such as soil texture, density, base saturation and pH also provide a suitable range of habitats (Figure 6b). Compared to AM, we observed that EM mycoheterotrophs showed a significant (see perMANOVA above) and stronger (see the clearer clustering between lineages in the PCA, Figure 6b) association per lineage within climatic space.

Discussion

This study is the first global study to assess the biogeography of mycoheterotrophs, taking into account both ecological and evolutionary aspects. According to our study, the trophic strategy of non-photosynthetic plants to obtain carbon from mycorrhizal networks occurs in forests worldwide, following Leake's (1994) hypothesis, without specific environmental preferences except for avoiding very cold and seasonal climates. Apart from occurring in broadleaved evergreen forests (AM mycoheterotrophs) or avoiding them (EM mycoheterotrophs), hardly any environmental predictor contributed to the segregated distribution of mycoheterotrophic plants according to their mycorrhizal type within tropical vs temperate forests. Climatic predictors such as annual precipitation and precipitation during the wettest month were the only variables that explained some variance in AM and EM mycoheterotrophic plant occurrence which had remained unexplained by forest type in the hierarchical models for AM- and EM-associated mycoheterotrophs, respectively. However, these variables had low explanatory power. Thus, humidity was revealed to be the only marginally important factor explaining the occurrence of mycoheterotrophy within evergreen (AM mycoheterotrophs) and other forests (EM mycoheterotrophs). The nearly exclusive occurrence in forests may be the result of a competitive advantage over other plants that grow in low-light conditions. Alternatively, forests in general may offer specific micro-habitats, such as favorable humidity levels, supporting the patchy distribution of these plants (Leake 1994), which are difficult to disentangle in a global scale analysis.

The evident dichotomic distribution of mycoheterotrophic plants according to mycorrhizal type between tropical and temperate forests (Figure 1) suggests a major importance of climate conditions in explaining this pattern, even though it coincides with a minimal importance of these same factors for explaining the distribution of AM and EM mycoheterotrophic plants within their preferred forest types. Hence, climatic conditions do not restrict the wide range of niches that the mycoheterotrophic life strategy occupies. Beyond climatic characteristics, our results show that AM mycoheterotrophs withstand a wide range of root zone soil moisture, being not so restricted to the most humid areas as previously predicted (Leake 1994). Also, these plants have been described to mainly occur in humus rich soils, which was not apparent from our analyses, perhaps due to the patchy character of soils at small scales that is not reflected in a global analysis.

The reliance of mycoheterotrophy on specific fungal partners for carbon uptake suggests that mycoheterotrophic plants could occur everywhere where the suitable fungal partner is present. AM fungi are abundant inside and outside the tropics and constitute important components of temperate forests (Phillips *et al.* 2013). At the same time, EM fungi are widespread in the tropics besides their predominant abundance in temperate areas (Roy *et al.* 2016). However, in our study, mycoheterotrophs with AM mycoheterotrophs occur predominantly in AM-dominated forests in the tropics, while EM mycoheterotrophs avoid AM-dominated forests in temperate forests (see Figure 4). Therefore, the observed dichotomy in the distribution of AM and EM mycoheterotrophic plants does not reflect the global distribution pattern of AM and EM fungi, indicating that the distribution of particular mycorrhizal types does not constrain the global distribution of mycoheterotrophic plants. Previous studies, focusing on a finer taxonomic scale, suggested that the abundance of mycoheterotrophic plant species is related to the abundance of their specific fungal partners (Hazard *et al.* 2012; Yamato *et al.* 2016). This indicates that the mere presence of a suitable fungal partner is not sufficient to promote a mycoheterotrophic relationship of a plant with its mycorrhizal partners, even though the abundance of host green plants supports the required mycorrhizal type. The habitat preferences associated to particular forest types likely restrict the distribution of mycoheterotrophic plants to a subset of environmental conditions of their fungal partners. Other factors that may constrain the occurrence of mycoheterotrophic plants is that they are likely to grow and reproduce only if their associated fungus is able to provide enough carbon from co-associated plants (Taylor & Bruns 1997). This may be influenced by the dynamics within fungal networks, including their size, and the age, identity, and fitness of its associated green plants (Merckx 2013; Fellbaum *et al.* 2014). In addition, competition between fungal species may influence their ability to obtain photosynthesized carbohydrates as well (Bever *et al.* 2009; Kiers *et al.* 2011), and only permit the presence of cheaters under particular conditions.

Despite the ubiquitous occurrence of mycoheterotrophic plants in forests, individual lineages show clear preferences for particular environmental conditions, resulting in a significant clustering of mycoheterotrophic lineages in environmental space. EM mycoheterotrophic lineages were clustered based on preferences for particular soil texture, soil base saturation and soil pH, while AM mycoheterotrophic lineages were separated based on soil cation exchange capacity. This suggests that edaphic factors are more relevant for the local distribution of individual mycoheterotrophic species than previously expected, and should be studied more in detail.

The clustering corresponds to a pattern of phylogenetic niche conservatism (Wiens *et al.* 2010), indicating spatial aggregation of related species. The strength of this spatial aggregation should depend on dispersal ability of species (Cavender-Bares *et al.* 2009), suggesting that mycoheterotrophic plants have limited dispersal capabilities. Despite their small seed size conferring a potential advantage promoting seed dispersal (Eriksson & Kainulainen, 2011), their habitat preference for dense close-canopy forests reduces their potential to disperse over large distances via wind (Wapstra *et al.* 2005). Many lineages, particularly those endemic to a single continent, are estimated to have evolved long after the breakup of Gondwana, further reducing their chances for effective intercontinental dispersal (Merckx 2013). Thus, low dispersal capability together with the divergence history of these plants can be a viable explanation for the observed restricted distribution of certain clades to specific ecozones (Jonker 1938; Mennes et al. 2015). This suggests an intricate connection between environmental factors and evolutionary history to explain the distribution patterns of mycoheterotrophic lineages.

The temperate versus tropical distribution pattern of AM and EM mycoheterotrophs also seems to have an evolutionary component. Interestingly, in the common ancestor of the Ericaceae clade, which includes the Monotropoideae and *Pyrola* lineages, there was a shift in mycorrhizal associations from AM fungi to specialized ericoid mycorrhizas formed by Ascomycota and Basidiomycota, a feature that may have been instrumental in their niche expansion (Lallemand *et al.* 2016). Similarly, for Orchidaceae, the specialized association with orchid mycorrhiza involving members of Ascomycota and Basidiomycota was the result of a shift from the ancestral AM associations in the common ancestor of the family (Yukawa *et al.* 2009). Moreover, the orchid's ability to recruit free-living saprotrophic fungi into novel mycorrhizae may also have led to niche expansions and radiations, also into temperate habitats (Givnish *et al.* 2016). Yet remarkably, nearly all mycoheterotrophic lineages within both Ericaceae and Orchidaceae have shifted from specialized ericoid and orchid mycorrhiza respectively towards an association with EM fungi. The only exceptions are a few tropical fully mycoheterotrophic orchids in Southeast Asia (Waterman *et al.* 2013). Thus, ericoid and orchid mycorrhizas fail to support full mycoheterotrophy, despite their participation in partial mycoheterotrophic interactions (Gebauer *et al.* 2016). Furthermore, mycoheterotrophy in temperate regions evolved in lineages with pre-adaptations to form mycorrhizas with Ascomycota and Basidiomycota fungi, and nearly exclusively occurs through shifts towards EM fungi, but not AM fungi. One explanation might be that in temperate forests only EM networks provide sufficient conditions of carbon availability to sustain mycoheterotrophic plants.

We may also use this evolutionary perspective to understand the wide distribution of mycoheterotrophy across many climatic zones as their association with EM fungi could have provided an advantage to plants to expand their niche from the tropics to colder and more seasonal areas (Wang *et al.* 2017). These colder and more seasonal climatic conditions are described to have been the main limitation for land plants to adapt and migrate out of the tropics during the Tertiary, potentially generating the latitudinal diversity gradient observed nowadays at a global scale (Wiens & Donoghue 2004). This latitudinal diversity gradients also seems present for mycoheterotrophy. From the about 500 species described to date, most of the species occupy tropical areas (Merckx *et al.* 2009). This suggests that mycoheterotrophic plants may have been under similar climatic pressures as host green plants in the colonization of temperate regions.

In conclusion, our study demonstrates that the global distribution of full mycoheterotrophy is mainly determined by forest occurrence and type, while the occurrence of mycoheterotrophic plants is further limited by their evolutionary history and mycorrhizal type of their associations. Thus, cheating belowground networks is only possible under particular conditions, and the vulnerability of the mycorrhizal symbiosis to be cheated by plants differs among climatic regimes in the globe. AM networks are more prone to be cheated in the tropics, and EM networks in temperate areas, despite the distribution of both mycorrhizal types across these regions. This suggests that the mutualistic stability of mycorrhizal networks is context dependent, and thus we should not expect to find a single underlying mechanism to understand the dynamics of plant-mycorrhizal interactions.

Acknowledgements

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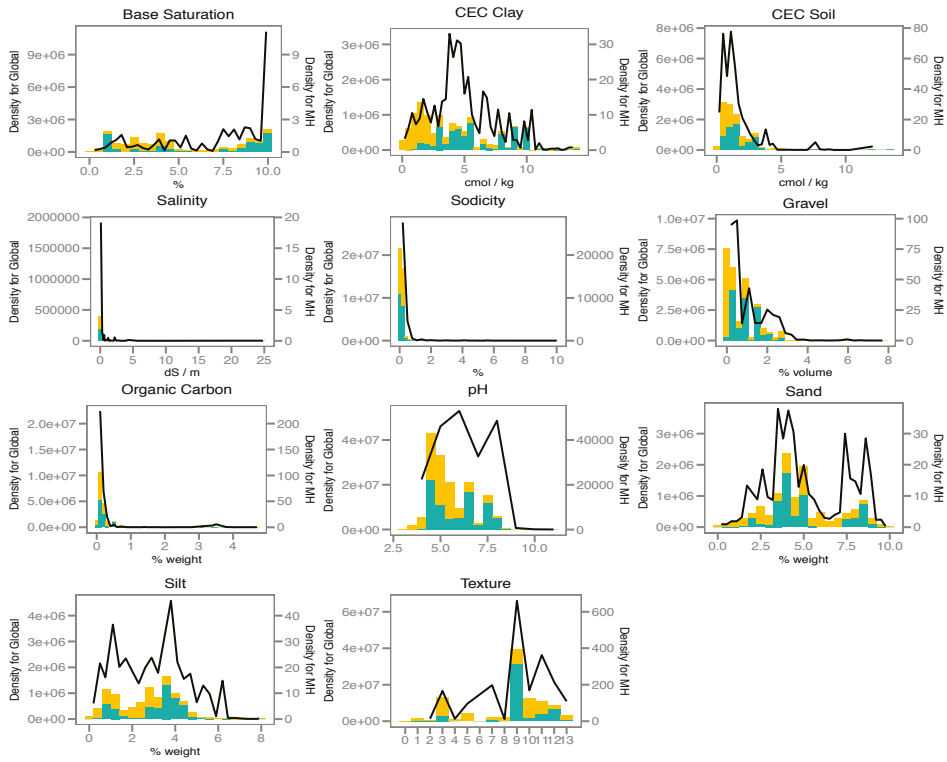
Supporting Information



5

Figure S1 | Representation of the environmental space occupied by the mycoheterotrophic plant species (histograms) and global trend of plants (solid lines), for the 19 BioClim (World Clim database) variables and the 12 top-soil variables (Harmonized World Soil Database). The colors of the histograms represent the mycorrhizal type association. The x-axis represents the range of values for each environmental variable and two y-axis represent the density of records with those conditions for the global trend of plants (left) and mycoheterotrophic plants (right).

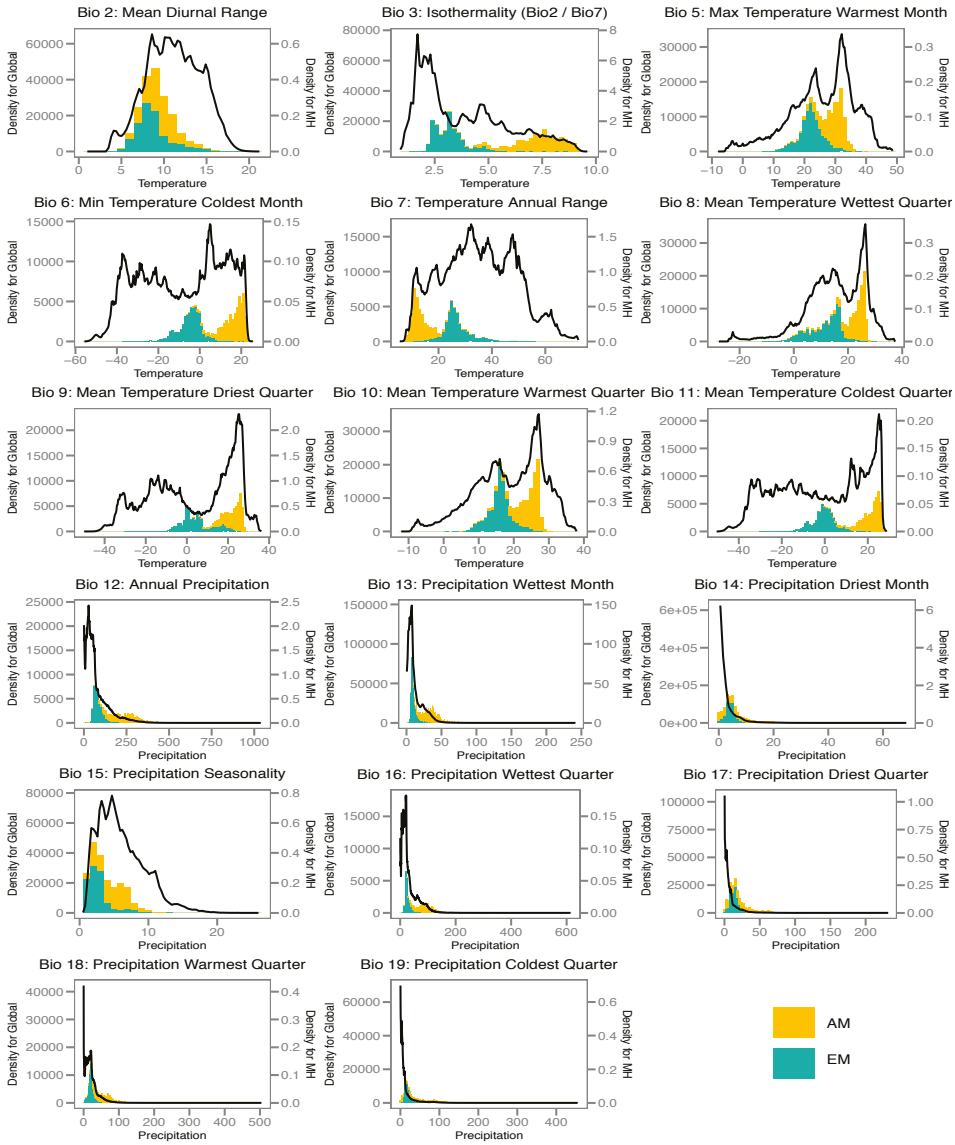

Bio 2: Mean Diurnal Range
Bio 3: Isothermality (Bio2 / Bio7)
Bio 5: Max Temperature Warmest Month
Bio 6: Min Temperature Coldest Month
Bio 7: Temperature Annual Range
Bio 8: Mean Temperature Wettest Quarter
Bio 9: Mean Temperature Driest Quarter
Bio 10: Mean Temperature Warmest Quarter
Bio 11: Mean Temperature Coldest Quarter
Bio 12: Annual Precipitation
Bio 13: Precipitation Wettest Month
Bio 14: Precipitation Driest Month
Bio 15: Precipitation Seasonality
Bio 16: Precipitation Wettest Quarter
Bio 17: Precipitation Driest Quarter
Bio 18: Precipitation Warmest Quarter
Bio 19: Precipitation Coldest Quarter
Density for Global
Density for MH
Temperature
Precipitation
AM
EM

Table S1 | MH dataset final. Dataset with the records of mycoheterotrophic plants.

fungi	Species	**family**	**lineage**
AMF	*Afrothismia amietii*	Burmanniaceae	DioAfr
AMF	*Afrothismia baerae*	Burmanniaceae	DioAfr
AMF	*Afrothismia fungiformis*	Burmanniaceae	DioAfr
AMF	*Afrothismia gesnerioides*	Burmanniaceae	DioAfr
AMF	*Afrothismia hydra*	Burmanniaceae	DioAfr
AMF	*Afrothismia insignis*	Burmanniaceae	DioAfr
AMF	*Afrothismia korupensis*	Burmanniaceae	DioAfr
AMF	*Afrothismia mhoroana*	Burmanniaceae	DioAfr
AMF	*Afrothismia mvileyi*	Burmanniaceae	DioAfr
AMF	*Afrothismia pusilla*	Burmanniaceae	DioAfr
AMF	*Afrothismia saingei*	Burmanniaceae	DioAfr
AMF	*Afrothismia sp*	Burmanniaceae	DioAfr
AMF	*Afrothismia winkleri*	Burmanniaceae	DioAfr
AMF	*Apteria aphylla*	Burmanniaceae	DioBur
AMF	*Burmannia championii*	Burmanniaceae	DioBur
AMF	*Burmannia cryptopetala*	Burmanniaceae	DioBur
AMF	*Burmannia geelvinkiana*	Burmanniaceae	DioBur
AMF	*Burmannia indica*	Burmanniaceae	DioBur
AMF	*Burmannia itoana*	Burmanniaceae	DioBur
AMF	*Burmannia kalbreyeri*	Burmanniaceae	DioBur
AMF	*Burmannia micropetala*	Burmanniaceae	DioBur
AMF	*Burmannia nepalensis*	Burmanniaceae	DioBur
AMF	*Burmannia sphagnoides*	Burmanniaceae	DioBur
AMF	*Burmannia tenella*	Burmanniaceae	DioBur
AMF	*Burmannia wallichii*	Burmanniaceae	DioBur
AMF	*Campylosiphon congestus*	Burmanniaceae	DioBur
AMF	*Campylosiphon purpurascens*	Burmanniaceae	DioBur
AMF	*Cymbocarpa refracta*	Burmanniaceae	DioBur
AMF	*Cymbocarpa saccata*	Burmanniaceae	DioBur
AMF	*Cymbocarpa sp*	Burmanniaceae	DioBur
AMF	*Dictyostega orobanchoides*	Burmanniaceae	DioBur
AMF	*Gymnosiphon sp*	Burmanniaceae	DioBur
AMF	*Gymnosiphon aphyllus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon bekensis*	Burmanniaceae	DioBur
AMF	*Gymnosiphon brachycephalus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon breviflorus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon capitatus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon constrictus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon cymosus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon dangyanus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon divaricatus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon fimbriatus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon guianensis*	Burmanniaceae	DioBur
AMF	*Gymnosiphon longistylus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon minutus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon panamensis*	Burmanniaceae	DioBur
AMF	*Gymnosiphon recurvatus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon samoritoureanus*	Burmanniaceae	DioBur

Table S1 | MH dataset final. Dataset with the records of mycoheterotrophic plants (continued).

fungi	Species	**family**	**lineage**
AMF	*Gymnosiphon sp*	Burmanniaceae	DioBur
AMF	*Gymnosiphon suaveolens*	Burmanniaceae	DioBur
AMF	*Gymnosiphon tenellus*	Burmanniaceae	DioBur
AMF	*Gymnosiphon usambaricus*	Burmanniaceae	DioBur
AMF	*Hexapterella gentianoides*	Burmanniaceae	DioBur
AMF	*Hexapterella steyermarkii*	Burmanniaceae	DioBur
AMF	*Oxygyne sp*	Burmanniaceae	DioThi
AMF	*Thismia abei*	Burmanniaceae	DioThi
AMF	*Thismia alba*	Burmanniaceae	DioThi
AMF	*Thismia annamensis*	Burmanniaceae	DioThi
AMF	*Thismia arachnites*	Burmanniaceae	DioThi
AMF	*Thismia betung-kerihuensis*	Burmanniaceae	DioThi
AMF	*Thismia brunneomitra*	Burmanniaceae	DioThi
AMF	*Thismia clavarioides*	Burmanniaceae	DioThi
AMF	*Thismia clavigera*	Burmanniaceae	DioThi
AMF	*Thismia glaziovii*	Burmanniaceae	DioThi
AMF	*Thismia gongshanensis*	Burmanniaceae	DioThi
AMF	*Thismia haxagona*	Burmanniaceae	DioThi
AMF	*Thismia hillii*	Burmanniaceae	DioThi
AMF	*Thismia hongkongensis*	Burmanniaceae	DioThi
AMF	*Thismia huangii*	Burmanniaceae	DioThi
AMF	*Thismia hyalina*	Burmanniaceae	DioThi
AMF	*Thismia lauriana*	Burmanniaceae	DioThi
AMF	*Thismia megalongensis*	Burmanniaceae	DioThi
AMF	*Thismia mucronata*	Burmanniaceae	DioThi
AMF	*Thismia mullerensis*	Burmanniaceae	DioThi
AMF	*Thismia nigricans*	Burmanniaceae	DioThi
AMF	*Thismia okahensis*	Burmanniaceae	DioThi
AMF	*Thismia panamensis*	Burmanniaceae	DioThi
AMF	*Thismia puberula*	Burmanniaceae	DioThi
AMF	*Thismia rodwayi*	Burmanniaceae	DioThi
AMF	*Thismia saulensis*	Burmanniaceae	DioThi
AMF	*Thismia singeri*	Burmanniaceae	DioThi
AMF	*Thismia sp*	Burmanniaceae	DioThi
AMF	*Thismia taiwanensis*	Burmanniaceae	DioThi
AMF	*Thismia tentaculata*	Burmanniaceae	DioThi
AMF	*Tiputinia foetida*	Burmanniaceae	DioThi
ECM	*Hemitomes congestum*	Ericaceae	EriMon
ECM	*Monotropa hypopitys*	Ericaceae	EriMon
ECM	*Monotropa sp*	Ericaceae	EriMon
ECM	*Monotropa uniflora*	Ericaceae	EriMon
ECM	*Monotropastrum humile*	Ericaceae	EriMon
ECM	*Monotropastrum sciaphilum*	Ericaceae	EriMon
ECM	*Monotropastrum sp*	Ericaceae	EriMon
ECM	*Monotropsis odorata*	Ericaceae	EriMon
ECM	*Pityopus californica*	Ericaceae	EriMon
ECM	*Pleuricospora fimbriolata*	Ericaceae	EriMon
ECM	*Sarcodes sanguinea*	Ericaceae	EriMon

Table S1 | MH dataset final. Dataset with the records of mycoheterotrophic plants (continued).

fungi	Species	**family**	**lineage**
ECM	*Pyrola picta*	Ericaceae	EriPyr
AMF	*Epirixanthes cylindrica*	Polygalaceae	FabEpi
AMF	*Epirixanthes elongata*	Polygalaceae	FabEpi
AMF	*Epirixanthes kinabaluensis*	Polygalaceae	FabEpi
AMF	*Epirixanthes pallida*	Polygalaceae	FabEpi
AMF	*Epirixanthes papuana*	Polygalaceae	FabEpi
AMF	*Epirixanthes sp*	Polygalaceae	FabEpi
AMF	*Exacum paucisquamum*	Gentianaceae	GenExa
AMF	*Exacum tenue*	Gentianaceae	GenExa
AMF	*Exochaenium oliganthum*	Gentianaceae	GenExo
AMF	*Voyriella parviflora*	Gentianaceae	GenVol
AMF	*Voyria acuminata*	Gentianaceae	GenVoy
AMF	*Voyria aphylla*	Gentianaceae	GenVoy
AMF	*Voyria aurantiaca*	Gentianaceae	GenVoy
AMF	*Voyria caerulea*	Gentianaceae	GenVoy
AMF	*Voyria chionea*	Gentianaceae	GenVoy
AMF	*Voyria clavata*	Gentianaceae	GenVoy
AMF	*Voyria corymbosa*	Gentianaceae	GenVoy
AMF	*Voyria flavescens*	Gentianaceae	GenVoy
AMF	*Voyria obconica*	Gentianaceae	GenVoy
AMF	*Voyria parasitica*	Gentianaceae	GenVoy
AMF	*Voyria pittieri*	Gentianaceae	GenVoy
AMF	*Voyria primuloides*	Gentianaceae	GenVoy
AMF	*Voyria rosea*	Gentianaceae	GenVoy
AMF	*Voyria sp*	Gentianaceae	GenVoy
AMF	*Voyria spruceana*	Gentianaceae	GenVoy
AMF	*Voyria tenella*	Gentianaceae	GenVoy
AMF	*Voyria tenuiflora*	Gentianaceae	GenVoy
AMF	*Voyria truncata*	Gentianaceae	GenVoy
AMF	*Geosiris aphylla*	Iridaceae	IriGeo
AMF	*Geosiris sp*	Iridaceae	IriGeo
AMF	*Arachnitis uniflora*	Corsiaceae	LilCor
AMF	*Corsia arfakensis*	Corsiaceae	LilCor
AMF	*Corsia boridiensis*	Corsiaceae	LilCor
AMF	*Corsia brassii*	Corsiaceae	LilCor
AMF	*Corsia clypeata*	Corsiaceae	LilCor
AMF	*Corsia cornuta*	Corsiaceae	LilCor
AMF	*Corsia huonensis*	Corsiaceae	LilCor
AMF	*Corsia lamellata*	Corsiaceae	LilCor
AMF	*Corsia merimantaensis*	Corsiaceae	LilCor
AMF	*Corsia ornata*	Corsiaceae	LilCor
AMF	*Corsia papuana*	Corsiaceae	LilCor
AMF	*Corsia purpurata*	Corsiaceae	LilCor
AMF	*Corsia sp*	Corsiaceae	LilCor
AMF	*Corsia torricellensis*	Corsiaceae	LilCor
AMF	*Corsia unguiculata*	Corsiaceae	LilCor
AMF	*Corsia wubungu*	Corsiaceae	LilCor
ECM	*Aphyllorchis alpina*	Orchidaceae	Orc

Table S1 | MH dataset final. Dataset with the records of mycoheterotrophic plants (continued).

fungi	Species	**family**	**lineage**
ECM	*Aphyllorchis anomala*	Orchidaceae	Orc
ECM	*Aphyllorchis caudata*	Orchidaceae	Orc
ECM	*Aphyllorchis elata*	Orchidaceae	Orc
ECM	*Aphyllorchis montana*	Orchidaceae	Orc
ECM	*Aphyllorchis pallida*	Orchidaceae	Orc
ECM	*Aphyllorchis queenslandica*	Orchidaceae	Orc
ECM	*Aphyllorchis simplex*	Orchidaceae	Orc
ECM	*Aphyllorchis sp*	Orchidaceae	Orc
ECM	*Cephalanthera austiniae*	Orchidaceae	Orc
ECM	*Chamaegastrodia inverta*	Orchidaceae	Orc
ECM	*Chamaegastrodia sp*	Orchidaceae	Orc
ECM	*Corallorhiza bulbosa*	Orchidaceae	Orc
ECM	*Corallorhiza macrantha*	Orchidaceae	Orc
ECM	*Corallorhiza maculata*	Orchidaceae	Orc
ECM	*Corallorhiza mertensiana*	Orchidaceae	Orc
ECM	*Corallorhiza odontorhiza*	Orchidaceae	Orc
ECM	*Corallorhiza sp*	Orchidaceae	Orc
ECM	*Corallorhiza striata*	Orchidaceae	Orc
ECM	*Corallorhiza trifida*	Orchidaceae	Orc
ECM	*Corallorhiza wisteriana*	Orchidaceae	Orc
ECM	*Dipodium hamiltonianum*	Orchidaceae	Orc
ECM	*Dipodium roseum*	Orchidaceae	Orc
ECM	*Dipodium variegatum*	Orchidaceae	Orc
ECM	*Epipogium aphyllum*	Orchidaceae	Orc
ECM	*Epipogium roseum*	Orchidaceae	Orc
ECM	*Hexalectris arizonica*	Orchidaceae	Orc
ECM	*Hexalectris brevicaulis*	Orchidaceae	Orc
ECM	*Hexalectris grandiflora*	Orchidaceae	Orc
ECM	*Hexalectris nitida*	Orchidaceae	Orc
ECM	*Hexalectris parviflora*	Orchidaceae	Orc
ECM	*Hexalectris sp*	Orchidaceae	Orc
ECM	*Hexalectris spicata*	Orchidaceae	Orc
ECM	*Hexalectris warnockii*	Orchidaceae	Orc
ECM	*Limodorum abortivum*	Orchidaceae	Orc
ECM	*Limodorum sp*	Orchidaceae	Orc
ECM	*Limodorum trabutianum*	Orchidaceae	Orc
ECM	*Neottia acuminata*	Orchidaceae	Orc
ECM	*Neottia camtschatea*	Orchidaceae	Orc
ECM	*Neottia listeroides*	Orchidaceae	Orc
ECM	*Neottia nidus-avis*	Orchidaceae	Orc
ECM	*Rhizanthella gardneri*	Orchidaceae	Orc
ECM	*Rhizanthella omissa*	Orchidaceae	Orc
ECM	*Rhizanthella slateri*	Orchidaceae	Orc
AMF	*Kihansia jengiensis*	Triuridaceae	PanTri
AMF	*Kihansia lovettii*	Triuridaceae	PanTri
AMF	*Kupea jonii*	Triuridaceae	PanTri
AMF	*Kupea martinetugei*	Triuridaceae	PanTri
AMF	*Lacandonia brasiliana*	Triuridaceae	PanTri

Table S1 | MH dataset final. Dataset with the records of mycoheterotrophic plants (continued).

fungi	Species	**family**	**lineage**
AMF	*Lacandonia schismatica*	Triuridaceae	PanTri
AMF	*Peltophyllum luteum*	Triuridaceae	PanTri
AMF	*Sciaphila africana*	Triuridaceae	PanTri
AMF	*Sciaphila albescens*	Triuridaceae	PanTri
AMF	*Sciaphila arfakiana*	Triuridaceae	PanTri
AMF	*Sciaphila corallophyton*	Triuridaceae	PanTri
AMF	*Sciaphila corymbosa*	Triuridaceae	PanTri
AMF	*Sciaphila densiflora*	Triuridaceae	PanTri
AMF	*Sciaphila janthina*	Triuridaceae	PanTri
AMF	*Sciaphila japonica*	Triuridaceae	PanTri
AMF	*Sciaphila ledermannii*	Triuridaceae	PanTri
AMF	*Sciaphila oligantha*	Triuridaceae	PanTri
AMF	*Sciaphila picta*	Triuridaceae	PanTri
AMF	*Sciaphila purpurea*	Triuridaceae	PanTri
AMF	*Sciaphila quadribullifera*	Triuridaceae	PanTri
AMF	*Sciaphila rubra*	Triuridaceae	PanTri
AMF	*Sciaphila schwackeana*	Triuridaceae	PanTri
AMF	*Sciaphila secundiflora*	Triuridaceae	PanTri
AMF	*Sciaphila sp*	Triuridaceae	PanTri
AMF	*Sciaphila tenella*	Triuridaceae	PanTri
AMF	*Sciaphila thaidanica*	Triuridaceae	PanTri
AMF	*Sciaphila winkleri*	Triuridaceae	PanTri
AMF	*Seychellaria africana*	Triuridaceae	PanTri
AMF	*Seychellaria madagascariensis*	Triuridaceae	PanTri
AMF	*Seychellaria sp*	Triuridaceae	PanTri
AMF	*Soridium spruceanum*	Triuridaceae	PanTri
AMF	*Triuridopsis intermedia*	Triuridaceae	PanTri
AMF	*Triuris hexophthalma*	Triuridaceae	PanTri
AMF	*Triuris hyalina*	Triuridaceae	PanTri
AMF	*Triuris sp*	Triuridaceae	PanTri
AMF	*Petrosavia sinii*	Petrosaviaceae	PetPet
AMF	*Petrosavia sp*	Petrosaviaceae	PetPet
AMF	*Petrosavia stellaris*	Petrosaviaceae	PetPet

Table S2 | Summary of lineages of mycoheterotrophic plants included in this study and their occurrence per ecozone.

Fungi	Lineage	Ecozones (*)	# genera
AM	Dioscoreales, Afrothismia	Afrotropics (1)	1
AM	Dioscoreales, Burmanniaceae	Afrotropics (2), Australasia (1), Indomalaya (2), Neartic (7)	7
AM	Dioscoreales, Thismiaceae	Australasia (1), Indomalaya (2), Neartic (2), Paleotropic (1)	3
EM	Ericales, Monotropaceae	Indomalaya (1), Neartic (6), Paleotropic (2)	7
EM	Ericales, Pyrolaceae	Neartic (1)	1
AM	Fabales, Epirixanthes	Australasia (1), Indomalaya (1)	1
AM	Gentianales, Exacum	Indomalaya (1), Paleotropic (1)	1
AM	Gentianales, Exochaenium	Afrotropics (1)	1
AM	Gentianales, Voyria	Afrotropics (1), Neartic (1)	1
AM	Gentianales, Voyriella	Neartic (1)	1
AM	Iridaceae, Geosiris	Afrotropics (1)	1
AM	Liliales, Corsiaceae	Australasia (1), Neartic (1)	2
EM	Orchidaceae	Afrotropics (1), Australasia (3), Indomalaya (3), Neartic (3), Paleotropic (5)	10
AM	Pandanales, Triuridaceae	Afrotropics (3), Australasia (1), Indomalaya (1), Neartic (6), Paleotropic (1)	9
AM	Petrosaviales, Petrosavia	Indomalaya (1)	1

CHAPTER 6

General discussion and synthesis

Sofia I.F. Gomes

GENERAL DISCUSSION

Our knowledge on the mycorrhizal symbiosis has drastically increased in the past decades (van der Heijden *et al.* 2015). Yet, I believe that we are still only scratching the surface in discerning all the aspects of this intricate interaction. We struggle to disentangle a myriad of factors, biotic and abiotic, to broaden our understanding of the mycorrhizal symbiosis. In addition, there are still important technological challenges to overcome to allow us to grasp what is really going on at both sides of the interaction. Adding to this complexity is finding an objective evaluation of the outcomes of the interaction for each partner under several natural circumstances. However, despite these challenges, enormous progress has been made to comprehend the mechanisms underlying the dynamics of the mycorrhizal symbiosis, either using laboratory conditions (e.g. Bever *et al.* 2009; Kiers *et al.* 2011; Fellbaum *et al.* 2014), or under natural conditions (e.g. Simard *et al.* 1997).

The studies included in this thesis focused on field assessments of mycoheterotrophic plants. Because these plants are effectively cheating the mycorrhizal symbiosis, investigating the mechanisms that allow for this mode of life to exist and persist is particularly important for our understanding of the stability of this symbiosis. Mycoheterotrophy is locally rare, but has a worldwide distribution, and represents an extreme stage within the mutualism-parasitism continuum that spans mycorrhizal interactions (Bronstein 1994; Johnson *et al.* 1997; Egger & Hibbett 2004). Since mycoheterotrophic plants exploit mycorrhizal fungi, knowledge we derive from studying these plants, can be directly applicable to understand how the stability of the mutualistic interaction between plants and mycorrhizal fungi can be subverted.

My primary aim with this thesis was to expand our knowledge on the conditions under which mycoheterotrophy can occur (Figure 1). I investigated four aspects that proved promising to advance our comprehension on cheating within mycorrhizal interactions, specifically: fungal specificity, resource availability, local and global environmental drivers of mycoheterotrophy. To better understand this fascinating mode of life, I used the perspective of both the plant and fungal partners to investigate the biotic (chapters 2 and 3) and abiotic (chapters 4 and 5) factors that support mycoheterotrophy. In **chapter 2**, I showed that among five mycoheterotrophic *Thismia* species, and throughout their geographic range, the association with fungi is more specialized than those of their surrounding autotrophic plants. Also, my findings support the high fidelity towards fungal partners generally found in mycoheterotrophic plants, and highlight their ability

of partner choice by picking specific fungi from a broader pool of species. In **chapter 3**, I propose that species coexistence mechanisms among mycoheterotrophic plants can be explained in the light of the niche theory. By considering their fungal associates as resources, and based on the identity of co-occurring mycoheterotrophic species in communities in the field and simulated communities, I found that a possible strategy for mycoheterotrophic plants to maximize the chances of coexistence is the proportional increase of their fungal-host diversity with an increase in their fungal-host overlap. Because the occurrence of mycoheterotrophic plants is not restricted solely by their fungal-hosts, **chapter 4** investigates the importance of soil nutrient stoichiometry to the patchy occurrence of these plants. In that chapter, I found that the balance between nitrogen and potassium, and not phosphorus which is usually considered as the main driver of species diversity in tropical forests, plays an important role in understanding the local-scale conditions that allow the arbuscular mycorrhizal symbiosis to be cheated. These findings stress that the local stability of arbuscular mycorrhizal networks is probably influenced by the heterogeneity of soil characteristics at the local scale. Finally, in **chapter 5**, I investigated the global drivers for the occurrence of mycoheterotrophic plants. I found that these plants have a stronger preference to occur in forests than to follow the abundance of autotrophic plants associated with the same mycorrhizal type.

In the remainder of this final chapter, I will explore more widely the main findings of the previous chapters, considering their implications for the understanding of mycoheterotrophy, and more generally, of the mycorrhizal symbiosis, including the exploration of future steps to be taken towards a more complete view on this system.

Specificity of mycoheterotrophic interactions

As it is expected from a parasitic interaction, mycoheterotrophic plants show high specificity towards their fungal partners (but see exceptions in Hynson & Bruns, 2009; Roy *et al.* 2009). In **chapter 2**, I focused on the clade of *Thismia* species occurring in temperate climates to compare their fungal interactions with those of surrounding green plants, and the soil fungal pool, over their distribution range. This is the first study that evaluates mycorrhizal specificity of mycoheterotrophic plants considering ecological, evolutionary and geographic constraints. At least for this group of mycoheterotrophic species, their fungal interactions consistently presented a higher specificity level than the majority of the autotrophic plants in the surroundings, and both mycoheterotrophs and autotrophs associate with a subset of fungal partners from the larger available fungal

pool. However, availability of AM fungi targeted by these mycoheterotrophic plants alone were not sufficient to lead to occurrence of mycoheterotrophic plants (Merckx *et al.* 2017), suggesting that other unstudied ecological factors determine the limited occurrences of these specialized mycoheterotrophic plants, which was dealt with in more detail in **chapter 4**.

In **chapter 3**, the fungal diversity associated with mycoheterotrophic plants was also evaluated but spanning the sampling of plants to a wider taxonomical range, and targetting a different biome. As a general characteristic of the species included in

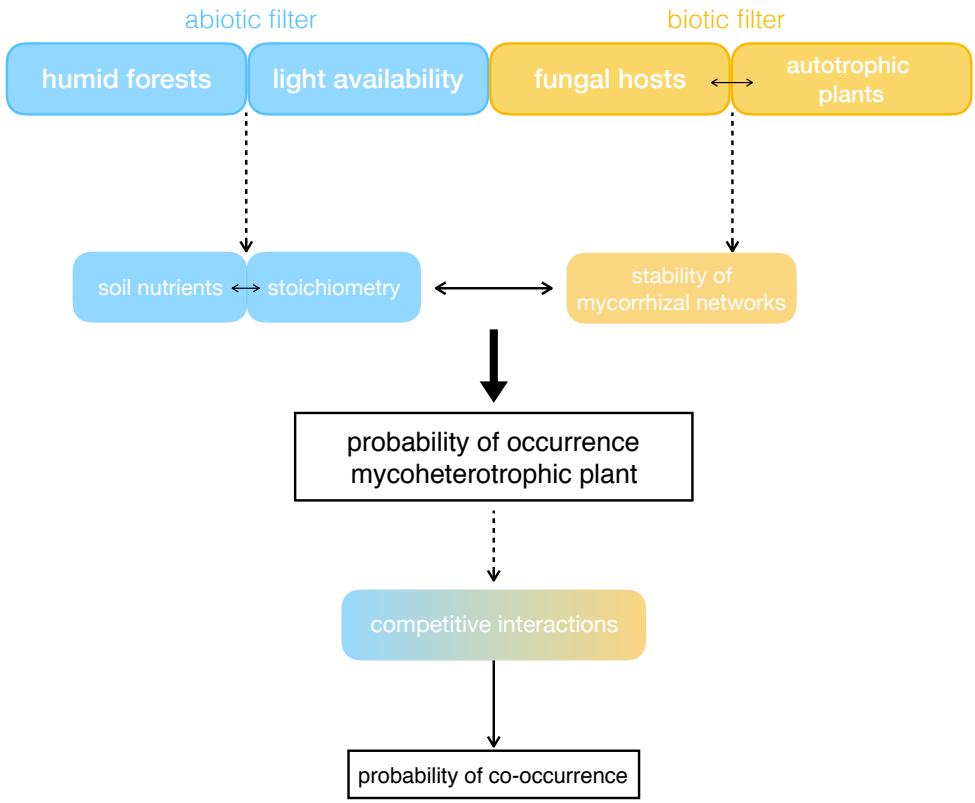


Figure 1 | Schematic representation of the factors that drive the occurrence of mycoheterotrophic plants (MH) as a summary of the chapters of this thesis. Mycoheterotrophic plants can be found inside forests (habitat; chapter 5), where their specific fungal partners are present (fungi; chapter 2) combined with edaphic factors favor conditions of weak mutualistic mycorrhizal symbiosis, at least in AM networks (edaphic factors; chapter 4), and which stable coexistence I determined by their competitive interactions (chapter 3).

that study, I showed that the 20 tropical mycoheterotrophic species collected tended to exploit more distantly related fungi than expected by chance. This finding may seem a priori contradictory with the high fungal specificity presented in **chapter 2**. However, from my point of view, this is a matter of the scale at which we look at these interactions, and the diversity of fungal pool considered in each chapter. A total of 60 individuals from five species were studied for **chapter 2**, while I used 140 individuals from 20 species for **chapter 3**. In addition, in **chapter 2** the fungal diversity of the mycoheterotrophic plants was compared in the context of total available local diversity, while in **chapter 3** the comparison was made among mycoheterotrophs, whose total diversity is more restricted. Thus, this means that among mycoheterotrophs, plants have the tendency to increase the diversity of their fungal interactions at the species level, but in comparison to the surrounding autotrophs and local fungal pool in the soil, these plants are more specialized. Furthermore, exclusively among mycoheterotrophic plants, there is a varying degree of specificity in their fungal interactions, as shown in **chapter 3**.

It is important to realize that the ITS2 region of arbuscular mycorrhizal fungi used in this study is hyper diverse, and the resolution for species delimitation is limited. This means that the high number of closely related sequences may reflect multiple genotypes within the same species or even different fungal individuals (Sanders *et al.* 1995). Thus, despite the general observation that most lineages of AM fungi can be part of a mycoheterotrophic interaction (Merckx *et al.* 2012), it may very well be that within the several distantly related lineages, only certain taxa, or genotypes within fungal species, are indeed being cheated. This remains to be confirmed.

Co-occurrence patterns of mycoheterotrophic plants

Since fully mycoheterotrophic plants entirely depend on their fungal partners for growth, these fungi are their primary nutritional resources. Therefore, the presence of specific fungal partners for a mycoheterotrophic plant is vital for its occurrence, and potentially mycoheterotrophic plants of different species may compete for the same fungal resources. According to the niche theory, species with high potential niche overlap are more prone to competitive exclusion (Macarthur & Levins 1967). Hence, even when conditions other than fungal availability are matched, competition with other mycoheterotrophs may limit the geographic expansion of certain species. In **chapter 3** I provided evidence that species co-occurrence of mycoheterotrophic plants can

contribute to shape the fungal diversity structure associated with each species, and can possibly act as drivers of the fungal community structure among mycoheterotrophs. Likewise, based on niche theory, it is possible that despite their restricted fungal associations, mycoheterotrophic plants benefit from associating with a higher diversity of fungal-hosts than expected by chance in order to increase their niche width, and therefore increase their chances to establish in new habitats. These results highlight the nutritional dependency of mycoheterotrophic plants on their fungal partners, and indicate that their mycorrhizal niche may be crucial for the understanding of their coexistence.

Ecological drivers of mycoheterotrophy

Even though the presence of specific fungal partners is essential for the occurrence of mycoheterotrophic plant species, the distribution of the host fungi alone is not likely to be sufficient to explain the occurrence of mycoheterotrophs (Yamato *et al.* 2016; Sheldrake *et al.* 2017; Merckx *et al.* 2017). Plants and fungi search for soil nutrients to satisfy their nutritional needs, thus edaphic factors can also contribute to the availability of the resources required determining specific niches to be occupied by mycoheterotrophic plants. In **chapter 4**, an interaction between potassium and nitrate availability, together with the effect of nitrate and pH were found to be the main drivers for the occurrence of mycoheterotrophy. This relationship between potassium and nitrate availability is well known to impact the effectiveness of the AM symbiosis (Ranade-Malvi, 2011). Furthermore, mycoheterotrophic plants avoided high fertility conditions, which coincide with a potential weak mutualistic mycorrhizal interaction, according to the trade balance model (Johnson 2010). Nonetheless, humidity is also an important predictor but not as much as soil nutrients. This suggests that the limiting factor for the occurrence of mycoheterotrophy is not their sensitivity to desiccation, as determined by water availability as extensively suggested (Maas *et al.* 1986; Leake 1994; Merckx 2013), but may be explained by soil nutrient stoichiometry and the stability of mycorrhizal networks. Mycoheterotrophs may indeed be sensitive to humidity levels, yet this can have a higher importance at a larger scale, since the forests where these plants have been collected are always characterized by high humidity levels (**chapter 5**). Moreover, the incidence of summers being drier than usual has been reported to reduce the reproduction levels of mycoheterotrophs (Leake 1994; Klooster & Culley 2009). This may indicate that the overall humidity conditions of certain forests, or even yearly drought events, already determine if any mycoheterotrophic plant can be found

in a certain forest. Then, for the patchy pattern at a fine scale, which is the main focus of this chapter, soil fertility plays a more crucial role, outweighing local differences in humidity.

I hypothesize that these edaphic factors do not solely represent preferences of the mycorrhizal fungi nor the mycoheterotrophic plants alone, but provide the necessary conditions under which cheating AM networks is possible. However, due to the intricate relationship between these plants and their fungi, it is hard to disentangle the abiotic factors that individually drive each partner within the interaction. Future studies should focus on measuring carbon and nutrient transfer between autotrophic plants and mycoheterotrophs, and compare the stability of the mycorrhizal networks considering the heterogeneity of soil properties. Furthermore, the edaphic drivers of EM mycoheterotrophic plants still remain to be studied, and are probably different from the ones described for the AM mycoheterotrophs, because they mostly occur in temperate forests (Phillips *et al.* 2013), which have different soil dynamics and nutrient economies compared to tropical forests (Vitousek & Sanford 1986).

Global preferences of mycoheterotrophic plants

Tailing the same rationale, mycoheterotrophic plants are expected to follow the distribution and / or abundance of autotrophic plants that associate with similar mycorrhizal types. Previous studies that found shared mycorrhizal fungi between mycoheterotrophs and surrounding autotrophs support such hypothesis (McKendrick *et al.* 2000; Waterman *et al.* 2013; **chapter 2**). However, other studies have shown that the distribution of species of *Thismia* (Merckx *et al.* 2017) and *Petrosavia* (Yamato *et al.* 2016) are limited by other factors than only the distribution or abundance of the associated AM fungi, since these fungi had more widespread distributions than the respective mycoheterotrophic plants. Considering the high specificity found in most of these plants, I hypothesized in **chapter 5** that the abundance of autotrophic plants associated with mycorrhizal fungi of the same type contributes to predict the distribution of mycoheterotrophic plants. I detected that it is definitely the case, yet it is not the main driving force at a global scale. Instead, the results showed that mycoheterotrophic plants preferably occur in forests worldwide, and in specific forests according to the mycorrhizal type they are associated with, suggesting that the environmental preferences of these plants are well represented in the abiotic conditions that characterize the respective forests. It is, thus, likely to find a considerable overlap in climatic conditions that drive the existence of

such forests, and the abundance of plants associated with each mycorrhizal type within these same forests. Nevertheless, these two mycorrhizal types are also abundant outside these forests. This may indicate why the distribution of mycoheterotrophic plants is better predicted by the occurrence of the specific forest type, and not necessarily by following the abundance patterns of the corresponding mycorrhizal types at a global scale. Furthermore, to a lesser extent, AM and EM mycoheterotrophs are also present in temperate and tropical regions, respectively, which do not follow the typical abundance of their mycorrhizal hosts. For both mycorrhizal types of mycoheterotrophic plants, hardly any other climatic or edaphic predictors were found to impact their distribution at a global scale, except the annual precipitation and the mean precipitation of the wettest month for the AM and EM mycoheterotrophs, respectively. This result further supports the explanation provided in **chapter 4** that different predictors may impact the occurrence of these plants at different scales. In this global scale analysis, the presence of certain types of forest was the main predictor, but climatic variables related to higher humidity levels were revealed to be also important for both mycorrhizal types, both in tropical and temperate regions (**chapter 5**). These achlorophyllous plants are well adapted to the low light conditions of forest floors, where they are able to avoid competition from autotrophic plants, which may also explain why they generally do not occur outside forests.

Implications

Understanding the mechanisms that underlie the maintenance of mutualistic interactions is still a major challenge for biologists. The mycorrhizal mutualism occurs in nearly all terrestrial ecosystems on Earth, and has been suggested to consist in an exchange of surplus resources (Brundrett 2002). Evolutionary theory considers mutualisms as reciprocal exploitations (Herre *et al.* 1999) in which each partner attempts to maximize the uptake of resources while minimizing their own costs, thus the mycorrhizal mutualism is expected to breakdown into cheating at the expense of cooperative partners (Kiers & Van Der Heijden 2006). Evaluating the symbiotic costs for the fungal partners is more difficult than for the plant hosts. Yet, mycoheterotrophic plants represent an ideal system to investigate the impacts of plants on their fungal partners, in which the fungal partners become the hosts for these cheater plants. It was not the goal of this thesis to measure such impacts of plants on their mycorrhizal partners, however. Instead I focused on what the interactions and the occurrence of mycoheterotrophic plants can teach us about the mycorrhizal symbiosis.

Based on the results presented in this thesis, I conclude that the occurrence of mycoheterotrophy, and thus cheating of the mycorrhizal mutualism, is influenced by an interplay of many factors, which have specific roles at different scales (Figure 1). Since mycoheterotrophic plants are completely dependent on particular fungal taxa, the presence of these fungi is the first and foremost limiting factor for their occurrence. These plants show generally high specificity towards their fungal interactions, and select their fungal partners from the local pool of taxa (**chapter 2**). Thus, either plants are highly picky and partner choice is key to determine the identity of their fungi, or ancestral lineages got stuck on specific fungi compatible with this mode of life, and fine-tuned their genetic machinery to exploit them, making host jumps a difficult step (Bidartondo & Bruns 2002).

Once mycoheterotrophic plants encounter the suitable environmental conditions, competitive interactions represent an important filter in determining their patters of co-occurrence. The ability of mycoheterotrophic plants to associate with higher or lower diversity of AM fungi gives them different capabilities to compete with other mycoheterotrophic plants that rely on similar fungi. This differential ability provide the possibility to stably coexist and can be explained in light of the niche theory (**chapter 3**). This result also suggests that mycorrhizal fungi act as nutritional resources for mycoheterotrophic plants, which supports their status as exploitative cheaters, although direct physiological evidence is still lacking. Also, the general tendency, even if only at genotype level, to harbor a diverse fungal community in their roots, can lead to an increase in likelihood to find more efficient fungal partners to which, from the mycoheterotrophic plants point of view, they should remain engaged in a mycorrhizal association.

While the presence of their mycorrhizal partners acts as a primary biotic filter for the presence of mycoheterotrophic plants, ecological settings further narrow their occurrences. In **chapter 5**, I indicate that forests are the main habitats in which the subversion of the mycorrhizal mutualism is more likely to occur, but the reason for that preference still remains unknown. The photosynthetic capacity in forests increases with canopy height (Carswell *et al.* 2000), suggesting that forests may have large carbon availability enhancing mycorrhizal abundance (Treseder 2004), and potentially support larger fungal networks. Hence, forests represent habitats where there is a surplus of carbon supply, favoring a stable mutualistic interaction between plants and mycorrhizal fungi. Moreover, these achlorophyllous plants are well adapted to the very low light

conditions of forest floors, where they are able to avoid competition from autotrophic plants, which may also explain why they rarely occur outside forests (**chapter 5**). The assumption that mycoheterotrophy represents rare events within ecosystems should be treated with care, since I demonstrated that mycoheterotrophic plants have a worldwide distribution with clear preferences for particular forests according to their mycorrhizal type. This suggests that cheating mycorrhizal networks is a much more widespread phenomenon that previously thought (**chapter 5**). This phenomenon is

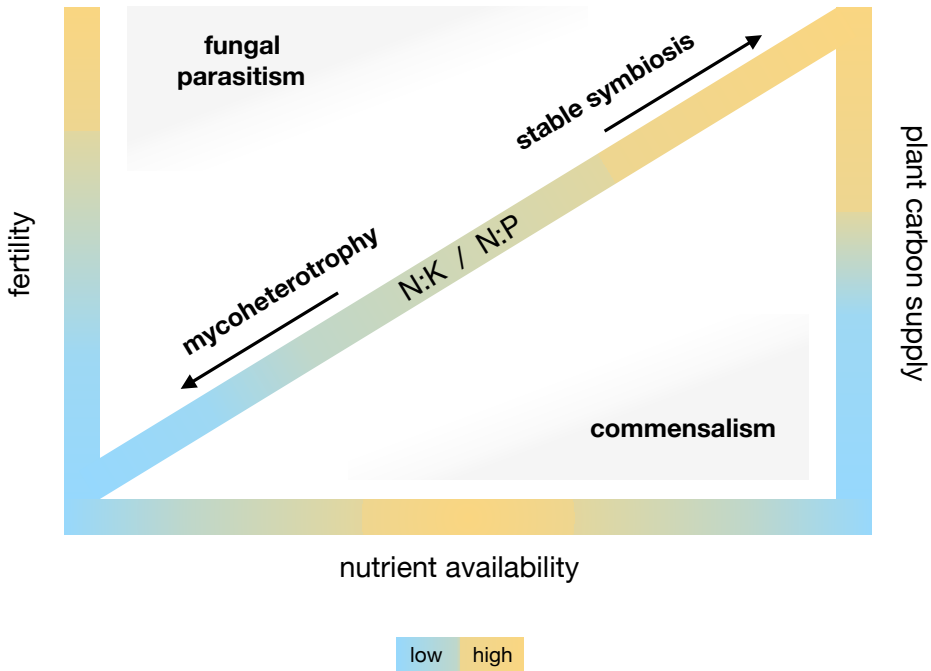


Figure 2 | Predicted outcome of mycorrhizal symbiosis according to soil fertility, nutrient availability, N:P and N:K ratios and, consequent, plant carbon supply to their arbuscular mycorrhizal partners. This model combines and updates previous predictions from Kiers & van der Heijden 2006 and Johnson 2010, based on the results obtained in chapter 4 of this thesis. At high N:P / N:K ratios and low nutrient availability, mycorrhizal symbiosis is expected to result in a strong mutualism with high C-for-P trade benefit. If nutrients become highly available, the C-for-P benefit decreases, and antagonistic fungal interactions are expected to occur. Plants are predicted to cheat the mycorrhizal symbiosis at lower N:P / N:K ratios and low fertility conditions. At low N:P / N:K ratios and low fertility, if N becomes limiting, C is the main limiting resource, favoring commensalistic interactions.

probably spread even wider, because this thesis did not include initial and partial states of mycoheterotrophy (Selosse *et al.* 2017).

Due to the soil nutrient heterogeneity, it seems that the performance of the mycorrhizal mutualism varies even at a local-scale, creating spatially constrained patches favorable for the occurrence of cheating, presenting the final filter. How mycoheterotrophic plants manage to either remain under the radar of the policy mechanisms that control cooperation in the mycorrhizal networks, or evolved ways to avoid the fungus to quit the exploitative interaction still remains to be comprehended. The stability of mycorrhizal networks is described to be influenced by resource availability of nutrients in the soil according to the trade balance model (Johnson 2010). My thesis highlights the importance of resource stoichiometry for the stability of these networks and places arbuscular mycorrhizal mycoheterotrophy in conditions of low nitrogen and potassium availability, which coincide with the predicted conditions for a weak mutualism between plants and mycorrhizal fungi (**chapter 4**). Based on this result, I propose a hypothetical model (Figure 2) where mycoheterotrophic plants could integrate the current models of mycorrhizal symbiosis outcome in the light of the plant assimilate availability as a function of nutrient availability (Kiers & van der Heijden 2006) and the trade balance model (Johnson 2010). This narrows down the hypothesis of whether mycoheterotrophic plants remain "unnoticed" or actively "trick" their mycorrhizal partners to remain engaged in this interaction to the latter. The former could suggest that mycoheterotrophic plants tap into mycorrhizal networks where there is a strong mutualistic interactions between partners, and therefore the exchange of carbon for nutrients at its maximum performance (Johnson 2010; Kiers & Van Der Heijden 2006), and they could be living on the carbon excess of these systems. The latter option suggests that mycoheterotrophic plants play an active role in keeping the mycorrhizal association, and future research should definitely address this topic further (see potential genetic mechanisms in Yuan *et al.* 2018). Understanding the circumstances under which mycoheterotrophic plants infiltrate mycorrhizal networks gives us valuable insights about the functioning of the mycorrhizal symbiosis. Despite representing an exception in respect to their relative abundance compared to autotrophic plants at the ecosystem level, mycoheterotrophic plants are prime examples of the most extreme outcome of mycorrhizal interactions within the mutualism-parasitism continuum (Bronstein 1994; Egger & Hibbett 2004), and their mere existence exposes the natural width of this symbiotic continuum. Because of the lack of a reciprocal rewarding

system, and their narrow diversity of associated fungal partners, mycoheterotrophic plants represent a system with decreased complexity compared to autotrophic plants ideal for the study of mycorrhizal symbiosis.

Taken together, the individual chapters of my thesis advance our understanding of why mycoheterotrophy does not occur in all forests worldwide, nor everywhere where the targeted fungal partners are available. At different scales, different drivers shape the probability of occurrence of mycoheterotrophic plants (Figure 1). A first filter that is imposed requires certain levels of humidity within forests to be able to harbor mycoheterotrophic plants, at the same time as their associated mycorrhizal partners are present within those forests. When this requirement is satisfied, conditions such as humidity levels or abundance of their mycorrhizal partners should not be a factor of importance. Instead, the patchy character of nutrient stoichiometry in the soil, which subsequently determines the performance of mycorrhizal networks at a local scale should determine the occurrence of mycoheterotrophic plants within the forests previously selected. This means that even if we find the local-scale conditions here described as suitable for the occurrence of mycoheterotrophic plants in any place in the world, it only makes sense to look for these plants if initially we selected forests that passed the first filter.

If we look inside particular forests where the specific fungal partners characteristic of the mycoheterotrophic plant species occur, we can potentially encounter a mycoheterotrophic plant in patches where soil conditions favor a weak AM mutualistic symbiosis. And on top of all these factors, and others that remain to be uncovered, we need to be in the right flowering season which varies among species and geography. So, finding a mycoheterotrophic plant is not about luck, but a complex interplay of biotic and abiotic factors.

Future perspectives

Mycoheterotrophic plants evolved from autotrophic mutualistic ancestors to exploit the same groups of mycorrhizal fungi that are mutualistic with green plants (Bidartondo *et al.* 2002). In this evolutionary journey, many phenotypic changes have culminated in the peculiar habits of these plants. Mycoheterotrophic plants are characterized by an obvious reduction of vegetative structures, absence of leaves and loss of photosynthesis, and of particular morphological features in the interface between plant and fungus in the roots (Imhof *et al.* 2013; Merckx 2013). Currently, considerable progress is being made

in uncovering the genetic background and consequences of the mycoheterotrophic mode of life. Sequencing full plastomes of several taxa have shown a considerable reduction of essential genes that participate in the photosynthetic apparatus, leading to the discovery of the smallest plastomes in land plants (Graham *et al.* 2017). Nonetheless, genome expansions have been also observed, suggesting a set of potential genes involved in sustaining this mode of life, and a recent study in a mycoheterotrophic orchid even revealed the largest mitochondrial genome within flowering plants (Yuan *et al.* 2018). Very soon whole genome assemblies of mycoheterotrophic plants will be available and further knowledge will be integrated at multiple levels to understand the evolution, persistence and functioning of mycoheterotrophy. A very hot topic in this area of research is to understand the dawn and persistence of cheating mycorrhizal interactions. Understanding cooperation within the mycorrhizal symbiosis is still quite challenging. Whole genome data will give important insights on the genetic machinery that was lost, gained, or modified, to allow for a cheating association with mycorrhizal fungi. These kind of studies will greatly contribute to explore the unique features that mycoheterotrophic plants display that distinguish them from autotrophic plants, in terms of how mycoheterotrophy impacts the relationship with their fungal partners, including exploring the genetic background responsible for the regulation of resource exchange. Also, knowing the genetic machinery that allows these plants to have a mycoheterotrophic nutrition will lead to more precise investigations on the actual extent of plant groups that can partially or fully rely on fungi for carbon supply, and maybe reveal that this phenomenon is taxonomically, and consequently geographically, more widespread than previously assumed.

Last, but not least, and perhaps even more defiant, is the perspective on mycoheterotrophy from the fungal point of view. The successful cultivation of these plants with their respective fungal partners, including the manipulation of environmental conditions, will be vital to understand the functionality of plants becoming mycoheterotrophic. Yet, it seems that the progress in this regard lacks behind in relation to our understanding of mycoheterotrophy from the plant's perspective. Due to the complexity of mycorrhizal networks in nature, mostly because of the intricate contribution of partners' identity and environmental conditions, it is necessary to disentangle the effect of the different players in the system. By comprehending when cheating is prone to occur, we will be able to grasp the constraints of at least one end of the mutualism-parasitism continuum that mycorrhizal symbiosis represents. This type of knowledge will greatly increase our understanding on the stability of one of the most

widespread mutualism on Earth. Besides, the occurrence of mycoheterotrophic plants at a global scale offers unique opportunities to have such perspective on mycorrhizal symbiosis in many different ecosystems, and involving an innumerous amount of plant and fungal species.

Hopefully, in the years to come, substantial progress will be made both in molecular methods and cultivation techniques, allowing to discover the secrets behind the mysterious relationship of plants cheating their mycorrhizal partners.

Final remarks

As with everything in life, every single-sided perspective will be incomplete. Times in science are gone that each discipline is restricted to specific subjects, disregarding the entirety of complex systems. To understand the nature of mycorrhizal symbiosis, or more specifically - as the aim of this thesis – of mycoheterotrophy, one needs to recognize the value of each of the individual parts and put the pieces together of the big puzzle that nature represents to mankind. Said so, there is not a single driver for the distribution of mycoheterotrophic plants. Besides, the combination of the drivers of each individual partner are not enough either to grasp the whole functioning of the system. In my opinion, to approach these kind of topics, besides from looking at its ecological and evolutionary aspects, one should consider as much as possible the interactions each organism entails in the complex web of life in space and in time.

THE EARTH HAS ITS MUSIC FOR THOSE WHO WILL LISTEN.

— George Santayana

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SUMMARY

Mycoheterotrophy is a particular mode of life in which plants obtain carbohydrates from their associated fungal partners, instead of by using photosynthesis. It evolved multiple times independently within different land plant lineages, giving rise to over 500 species of achlorophyllous mycoheterotrophic plants. The majority of mycoheterotrophic plants exploit mycorrhizal fungi and thus, represent clear examples of cheating shifts from mutualistic interactions in the mycorrhizal symbiosis. Due to the complexity of mycorrhizal interactions and challenges in assessing the outcomes of this symbiosis – both for plants and fungi – mycorrhizal cheating has remained a poorly researched topic in plant ecology. This thesis aimed to shed light on the diversity, ecology and distribution of mycoheterotrophic interactions. In my approach, I considered that different drivers may be important at different scales and studied four levels of ecological complexity.

In chapter 2, the specificity of mycoheterotrophic interactions within the arbuscular mycorrhizal symbiosis was assessed at the organism level. Through the comparison of arbuscular mycorrhizal fungal communities of mycoheterotrophic Thismia species, surrounding plant species, and soil samples from different geographic locations, I was able to show that the mycoheterotrophic species consistently associate with a fungal community that is phylogenetically more restricted than autotrophic plants. These results support the view that mycoheterotrophic mycorrhizal interactions are highly specialized, and that this specificity is not caused by a limited local availability of arbuscular mycorrhizal fungi.

In chapter 3, biotic interactions between plants and fungi were studied in the framework of mycoheterotrophic plant coexistence scenarios at the population level. Since arbuscular mycorrhizal fungi are the main resources of tropical mycoheterotrophic plants, I tested the hypothesis that coexistence of arbuscular mycorrhizal mycoheterotrophic plants may be favored under symmetric patterns of fungal-host

overlap and diversity. Indeed, in communities of co-occurring mycoheterotrophic plant species in the field, and among the artificially-generated groups of mycoheterotrophic plants, we observe a trend towards increased phylogenetic diversity of fungal hosts among mycoheterotrophic plants with increasing overlap in their fungal hosts. These results indicate that fungus-mycoheterotrophic plant interactions can be better explained by understanding plant-plant interactions generated by sharing resources or fungal hosts.

In addition, edaphic abiotic factors potentially influencing the occurrence of mycoheterotrophic plants were assessed at the community level in chapter 4, through a comparison of soil chemistry and nutrients in plots where mycoheterotrophic plants were present with those lacking these plants. I found that soil pH, soil nitrate, and the interaction between soil potassium and nitrate concentrations were the best predictors for the occurrence of mycoheterotrophic plants in two lowland rainforests in South America. Mycoheterotrophic plant abundances decreased with an increase of nitrate, which suggests that that these plants avoid high fertility patches. The trade balance model predicts that similar low-fertility conditions potentially favor a weak mutualism between plants and arbuscular mycorrhizal fungi. Therefore, I suggest that arbuscular mycorrhizal mycoheterotrophic plants potentially prefer conditions where local-scale mutualism is weak.

Finally, in chapter 5 I derived the global drivers for the distribution of mycoheterotrophy for both the arbuscular and the ectomycorrhizal symbiosis from species occurrence data of these plants. The results show that while mycoheterotrophy is globally distributed, mycoheterotrophic plants – regardless of their mycorrhizal status – avoid cold and highly seasonal climates, and show a strong preference for forests. However, arbuscular and ectomycorrhizal mycoheterotrophs show a strong global geographic segregation: arbuscular mycorrhizal mycoheterotrophs predominantly prefer broadleaved tropical evergreen forests, whereas ectomycorrhizal mycoheterotrophs are mainly found in broadleaved deciduous and evergreen needle-leaved forests in temperate regions. Temperature and precipitation variables – but not edaphic factors – are the best predictors explaining the distribution patterns of arbuscular and ectomycorrhizal mycoheterotrophs after accounting for the effects of forest type. Therefore I demonstrated that the global distribution of mycoheterotrophy is mainly determined by forest occurrence and type, while the occurrence of mycoheterotrophic plants is further limited by their evolutionary history and mycorrhizal type of their associations.

Together, the chapters of this thesis highlight the scale-dependent factors that explain the occurrence of mycoheterotrophy. Primarily, mycoheterotrophic plants require at least the presence of their associated fungal partners to persist, which should occur predominantly within humid forests. When these conditions are fulfilled, the balance between soil nutrients, instead of solely absolute concentrations of nutrients, influence the conditions that favour the occurrence of these plants, which also affects the stability of mycorrhizal networks. Lastly, resource competition may contribute to shape the specificity of fungal interactions of mycoheterotrophic plants, which can vary in degree of specialization respectively to each species. The broad approach taken in this thesis highlights many intriguing aspects about mycoheterotrophy that remain to be studied. Yet, it also shows how the study of mycoheterotrophy is important in the understanding of mycorrhizal symbiosis in general.

SAMENVATTING

Mycoheterotrofie is een levenswijze waarbij planten koolhydraten verkrijgen van geassocieerde schimmelpartners, in plaats van via fotosynthese. Deze levenswijze is vele malen onafhankelijk van elkaar geëvolueerd en komt in verschillende plantengroepen voor. In totaal zijn er meer dan 500 verschillende soorten chlorofylloze mycoheterotrofe planten. De meerderheid van deze planten groeit op mycorrhizaschimmels en zijn duidelijke voorbeelden voor de evolutie van bedrog vanuit voorouderlijke mutualistische interacties in de mycorrhiza symbiose. Omdat mycorrhiza interacties vaak erg complex zijn en het moeilijk is om de effecten van de symbiose – zowel op de plant als de schimmel – te achterhalen, is bedrog in dit systeem een relatief weinig bestudeerd fenomeen. Deze thesis had als doel opheldering te verkrijgen in de diversiteit, ecologie, en verspreiding van mycoheterotrofe interacties. Hierbij veronderstelde ik dat verschillende drijfveren van belang kunnen zijn op verschillende schaalgroottes en bestudeerde ik mycoheterotrofie op vier verschillende niveaus van ecologische complexiteit.

Hoofdstuk 2 behandelt onderzoek naar de specificiteit van mycoheterotrofe interacties in de arbusculaire mycorrhizasymbiose op het niveau van het organisme. Door arbusculaire mycorrhiza schimmelgemeenschappen van mycoheterotrofe *Thismia* soorten, omringende groene planten en bodemmonsters van verschillende locaties te vergelijken, toonde ik aan dat mycoheterotrofe soorten altijd leven op schimmelgemeenschappen die fylogenetisch meer gespecialiseerd zijn dan omringende autotrofe planten. Deze resultaten ondersteunen de veronderstelling dat mycoheterotrofe mycorrhizainteracties erg gespecialiseerd zijn en dat deze specialisatie niet veroorzaakt wordt door een beperkte lokale beschikbaarheid van arbusculaire mycorrhizaschimmels.

In hoofdstuk 3 werden de biotische interacties tussen planten en schimmels bestudeerd in het kader van het samen voorkomen van mycoheterotrofe planten op populatieniveau. Omdat arbusculaire mycorrhizaschimmels de voornaamste voedselbron zijn van tropische mycoheterotrofe planten, testte ik de hypothese dat

het samen voorkomen van deze planten bevorderd wordt door een symmetrisch patroon van schimmel-gastheer overlap en diversiteit. Zowel in gemeenschappen van samengroeiende mycoheterotrofe planten in de natuur als in artificieel samengestelde groepen van mycoheterotrofe planten, observeerden we een trend waarbij een toenemende fylogenetische diversiteit van schimmelgemeenschappen samengaat met een toenemende overlap van de schimmelgemeenschappen. Deze resultaten suggereren dat de interactie tussen schimmels en mycoheterotrofe planten beter begrepen kan worden met kennis over hoe plant-plant interacties beïnvloed worden door de beschikbaarheid van schimmelgastheren.

Edafische abiotische factoren die potentieel het voorkomen van mycoheterotrofe planten beïnvloeden op het niveau van de gemeenschap werden onderzocht in een studie beschreven in hoofdstuk 4, waarbij kwadranten met en zonder mycoheterotrofe planten vergeleken werden op basis van chemische bodem karakteristieken en nutriënten. Hieruit blijkt dat bodem pH, nitraat en de interactie tussen kalium- en nitraatconcentraties de hoogste voorspellende waarde hebben voor het voorkomen van mycoheterotrofe planten in twee bestudeerde regenwouden in Zuid-Amerika. Aangezien de abundantie van mycoheterotrofe planten daalt met een toename van nitraat in de bodem, lijkt het erop dat deze planten niet goed gedijen op plaatsen met veel voedingstoffen in de bodem. Het model dat uitgaat van een gebalanceerde handel tussen planten en mycorrhizaschimmels voorspelt dat bodemomstandigheden met een geringe hoeveelheid voedingstoffen potentieel aanleiding geven tot een zwak mutualistische interactie tussen planten en arbusculaire mycorrhizaschimmels. Dit suggereert dan arbusculaire mycoheterotrofe planten een voorkeur hebben aan lokale omstandigheden waarbij het mutualisme tussen planten en schimmels zwak is.

Tenslotte onderzocht ik in hoofdstuk 5 de globale drijfveren voor de verspreiding van mycoheterotrofie in de arbusculaire en ectomycorrhizasymbiose, op basis van de verspreidingsgegevens van de verschillende plantensoorten. De resultaten laten zien dat hoewel mycoheterotrofe planten wereldwijd verspreid zijn, ze koude klimaten en klimaten met extreme seizoenen vermijden – hierbij geen rekening houdend met de identiteit van hun mycorrhizaschimmels. Ook hebben mycoheterotrofe planten een duidelijke voorkeur voor bosrijke gebieden. Arbusculaire en ectomycorrhiza mycoheterotrofe planten hebben echter afgetekende verschillen in hun verspreiding: mycoheterotrofe planten die op arbusculaire mycorrhiza leven, komen voornamelijk voor in eeuwig groene tropische loofbossen, terwijl mycoheterotrofe planten die op

ectomycorrhiza leven hoofdzakelijk voorkomen in gematigde bladverliezende loofbossen en gematigde eeuwig groene naaldbossen. Na bostypes hebben temperatuurs- en neerslagvariabelen, maar geen edafische factoren, de hoogst voorspellende waarde voor de verspreidingspatronen van arbusculaire en ectomycorrhiza mycoheterotrofe planten. Daarmee werd aangetoond dat de wereldwijde verspreiding van mycoheterotrofie voornamelijk beïnvloed wordt door de aanwezigheid van bos en het type bos, terwijl het voorkomen van mycoheterotrofe planten verder bepaald wordt door de evolutionaire geschiedenis van de planten en het type mycorrhiza associaties waarop ze leven.

Bij elkaar genomen bieden de hoofstukken uit deze thesis een overzicht van de schaalafhankelijke factoren die het voorkomen van mycoheterotrofie bepalen. In de eerste plaats hebben mycoheterotrofe planten hun specifieke schimmelpartners nodig om te kunnen groeien. Deze komen wellicht voornamelijk in vochtige bossen voor. Wanneer deze voorwaarde is vervuld, bepalen de relatieve proporties tussen bodemnutriënten, en niet hun absolute concentratie, het voorkomen van mycoheterotrofe planten, en dit beïnvloed wellicht ook de stabiliteit van de mycorrhizanetwerken in het algemeen. Ten slotte kan ook de competitie voor voedingstoffen bijdragen aan de specificiteit in schimmelinteracties bij mycoheterotrofe planten en hun voorkomen bepalen. Het effect hiervan kan verschillend zijn voor elke plantensoort. De brede aanpak van het onderzoek in deze thesis laat zien dat veel intrigerende aspecten van mycoheterotrofie nog weinig bestudeerd en begrepen blijven. Maar de behaalde resultaten onderstrepen ook het belang van de studie van mycoheterotrofie voor het begrijpen van de mycorrhiza symbiose in het algemeen.

CURRICULUM VITAE

Sofia Gomes was born on April 18th, 1988, in Lisbon, Portugal. In 2006, she finished her high school education in Liceu Camões in Lisbon. She obtained her BSc degree in Biology in 2009, in the branch of molecular biology and genetics at the Faculty of Sciences of the University of Lisbon, Portugal. There, she pursued her Master's degree in Applied Microbiology (2009-2011), where she obtained a scholarship from a local funding agency (Amadeus Dias) to participate in the project SEAVENTzymes with particular focus on the screening for chitinase enzymatic activity from extremophile microbes. Her MSc thesis focused on the morphological and genetic classification of the edible mushroom within the species-complex of Tricholoma equestre (a.k.a. yellow knight). Simultaneously, Sofia worked at Parques de Sintra – Monte da Lua, S.A., a nature management company, where she undertook a two years inventory (2010-2012) of the mushrooms growing in the managed natural area. After that, she obtained a Leonardo da Vinci scholarship (European Commission) to learn electron microscopy for the identification of Russula fungi with Dr. Jaume Llistosella at the Instituto de Recerca de la Biodiversidad (IRCBio), University of Barcelona, Spain. In 2013, she worked as a researcher in a project that captured her curiosity to pursue the topic of her PhD thesis (2014-2018), funded by Naturalis Biodiversity Center and the Institute of Environmental Sciences (CML) at Leiden University, the Netherlands. She obtained a KNAW grant from the Koninklijke Nederlandse Akademie van Wetenschappen to conduct field work for her thesis. After completing her PhD, Sofia will start a postdoc at CML to study the potential of mycorrhizal inoculation for pest protection in bulb plants, and to understand the impact of agricultural practices on the microbiota of dairy farms.

List of Publications

In peer reviewed journals

Gomes SIF, Merckx VSFT, Hynson NA (2018) Biological invasions increase the richness of arbuscular mycorrhizal fungi from a Hawaiian subtropical ecosystem. *Biological Invasions* 20: 2421-2437.

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