Mycorrhizal symbioses and the evolution of trophic modes in plants

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Abstract

1. Since the early colonization of land, plants depend to various extents on mycorrhizal fungi to meet their nutrient demands. In most mycorrhizal symbioses, plants provide sugars derived from photosynthesis to the fungi, whereas the fungi provide essential minerals to the plant. However, in some plants the flow of carbon has reversed and the fungi provide carbon to the plants. These plants are called mycoheterotrophs. However, it remains unclear how and under which circumstances trophic modes change and whether transitions in trophic modes are associated with changes in mycorrhizal communities.
2. Here, we review the available literature on mycorrhizal associations and trophic modes in plants. We first outline how trophic modes can be determined and how they differ across plants. We then investigate the evolutionary context under which mycoheterotrophy originated. We also examine the mycorrhizal communities associating with autotrophic, partially mycoheterotrophic and fully mycoheterotrophic plants within different plant families and investigate whether commonalities can be observed.
3. Our overview shows that mycoheterotrophy has originated more than 40 times through evolutionary time and can be found in a wide range of plant groups, including liverworts, lycophytes, ferns, monocots and dicots. Partial mycoheterotrophy appears to be much more common than previously anticipated and represents an almost continuous gradient between autotrophy and full mycoheterotrophy.
4. Comparison of the mycorrhizal communities associating with autotrophic, partial and full mycoheterotrophic plants indicates that, although they share some commonalities, shifts from autotrophy to full mycoheterotrophy are accompanied by either losses or shifts in mycorrhizal partners, suggesting that full or partial loss of photosynthesis selects for different mycorrhizal communities.
5. *Synthesis:* Partial mycoheterotrophy appears to be much more common than previously thought and represents an almost continuous gradient from autotrophy to full mycoheterotrophy.

Evolution to full mycoheterotrophy is challenging as it requires specific adaptations and often a switch to other mycorrhizal partners. More detailed analyses of the functionality of different mycorrhizal systems co-occurring in the roots of a single plant and the costs of mycorrhizal switching are needed to understand the precise mechanisms leading to full mycoheterotrophy.

Keywords: autotrophy, mycorrhiza, partial mycoheterotrophy, stable isotopes

Introduction

Mycorrhizal symbiosis, an association between soil fungi and plant roots, has been hypothesized to be one of the critical evolutionary innovations essential for the successful colonization of land by early land plants, subsequently leading to their radiation, and providing the foundation for the majority of the extant terrestrial ecosystems (Delaux, 2017; Feijen et al., 2018; Field et al., 2015; Martin et al., 2017). Based on the morphology of the interaction and the identity of the partners, four major types of mycorrhiza have been recognized (arbuscular mycorrhiza, ectomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza), and together they include over 90% of extant land plant species and up to 10% of fungal species (Brundrett & Tedersoo, 2018; van der Heijden et al., 2015). The mycorrhizal symbiosis is mostly a mutualism, in which root-associated fungi facilitate plants with the acquisition of essential nutrients from the soil, and in return, plants generally transfer photosynthetically fixed carbon to their fungal partners (Bonfante & Genre, 2010; Merckx, 2013; van der Heijden et al., 2015).

Some plant lineages, however, evolved the ability to obtain carbon from fungi, a nutritional mode known as mycoheterotrophy (Leake, 1994). Among these, initial mycoheterotrophs depend on mycorrhizal fungi during germination and early development (e.g. many species of orchids) or during their gametophyte phase (several species of ferns and lycophytes) and become putatively autotrophic as adults or sporophytes respectively. Partial mycoheterotrophs retain the ability to obtain carbon from fungi throughout their life cycle in combination with photosynthesis. The extent of their dependency on fungal carbon may vary according to ecological factors (Preiss et al., 2010). Finally, full mycoheterotrophs are non-photosynthetic throughout their entire life cycle and solely depend on fungal carbon. Although most fully mycoheterotrophic plants are fairly small and rather inconspicuous (Fig. 1a-e), some of them can be up to 12 m tall, as illustrated by the liana *Erythrorchis altissima* from Southeast Asia (Ogura-Tsujita et al., 2018) (Fig. 1f). Because these mycorrhizal 'cheater' plants are no longer physiologically capable of trading nutrients for carbon, full

mycoheterotrophs are unequivocal examples of non-cooperative partners in the mycorrhizal symbiosis (Walder & van der Heijden, 2015) and therefore represent ideal model taxa to study the evolution of mycorrhizal interactions in relation to cooperative strategy. Nonetheless, they are routinely ignored in studies dealing with mycorrhizal cooperation, which typically focus on mycorrhizal fungi taking advantage of mycorrhizal plants rather than vice versa (e.g. Jones et al., 2015). Phylogenetic evidence suggests that the evolution of full mycoheterotrophy from autotrophy is likely to occur through intermediate initial and partial mycoheterotrophic stages (Motomura et al., 2010; Merckx et al., 2013a). This indicates that a plants' symbiosis with mycorrhizal fungi can therefore be considered as a dynamic interaction along a continuum of possible outcomes, ranging from initial mycoheterotrophy over autotrophy, partial mycoheterotrophy to full mycoheterotrophy. Shifts along the continuum can occur at a developmental, ecological, and evolutionary scale.

At present, little is known about whether and how mycorrhizal communities change during the transition from initial mycoheterotrophy to autotrophy, partial or full mycoheterotrophy. The few available studies have shown that in some cases mycorrhizal communities shift during ontogenetic development. For example, germinating seeds and the first underground stages of several *Pyrola* (Ericaceae) species have been shown to associate with multiple partners simultaneously, but the fungi that associated with seeds differed from those associating with adult plants (Hashimoto et al., 2012; Hynson et al., 2013; Johansson et al., 2017; Jacquemyn et al., 2018), suggesting that plants may serially associate with different partners rather than specializing on a single 'best' partner. Similarly, the mycorrhizal communities associating with protocorms and adult plants of the orchid *Liparis loeselii* (Orchidaceae) were diverse and varied among life cycle stages (Waud et al., 2017). During this conversion, trophic modes change from full mycoheterotrophy in protocorms to partial mycoheterotrophy in adults plants (Schweiger et al., 2018). Broad host specificity may allow seeds to germinate in a wide range of habitats with contrasting mycorrhizal communities and to provide the necessary resources for germination and subsequent growth to aboveground seedling, whereas switching of partners across life cycle stages may indicate selectivity

towards ‘high-quality’ partners or redundancy of partners that were needed to initiate germination, but are no longer needed to support adult plants. However, mycorrhizal community shifts during ontogenetic development were not observed in all cases: in the ferns and lycophytes species of *Botrychium* (Ophioglossaceae), *Huperzia* (Lycopodiaceae) and *Psilotum* (Psilotaceae) for example, autotrophic sporophytes were found to associate with the same arbuscular mycorrhizal fungi as their mycoheterotrophic gametophytes (Winther & Friedman, 2007, 2008, 2009). This evidence indicates that at least in some cases shifts in fungal communities have occurred during ontogenetic development of plants, but whether similar changes have occurred during the transition from autotrophy to initial, partial, and full mycoheterotrophy at an evolutionary time scale and what determines such shifts remain to some extent unclear.

In this paper, we review recent studies that have provided novel insights in the potential mechanisms leading to mycoheterotrophy. First, we assess to what extent autotrophy, partial mycoheterotrophy and mycoheterotrophy represent distinct classes of trophic modes or rather represent a continuum of trophic modes that gradually change from one state into another. Second, we assess the phylogenetic background of the origination of mycoheterotrophy. Finally, we investigate whether within plant genera mycorrhizal communities differ between autotrophic, partial mycoheterotrophic and mycoheterotrophic plants, and assess whether presence of particular fungi may allow or facilitate the transition from one trophic mode to another.

Trophic modes: a continuum from autotrophy to mycoheterotrophy?

To be able to test the hypothesis that mycoheterotrophic plants evolved from mutualistic ancestors, possibly through an intermediate stage of partial mycoheterotrophy, it is important that the trophic mode of an individual plant can be accurately assessed. The trophic mode of an individual can be measured using stable isotopes, mostly ^{13}C and ^{15}N (Ziegler, 1996; Gebauer & Meyer, 2003), and more recently also ^{2}H and ^{18}O (Gebauer et al., 2016). The general principle behind isotope analysis is

that fungi are heterotrophic and therefore significantly enriched in ^{2}H, ^{13}C and ^{15}N compared to their substrates and to autotrophic plants (Gebauer & Dietrich, 1993; Gleixner et al., 1993; Ziegler, 1994; Trudell et al., 2003). Mycoheterotrophic plants take up fungal material through either hyphal lysis or transfer across intact membranes of fungus and plant (Kuga et al., 2014). Because the relative abundance of ^{13}C and ^{15}N systematically increases at each trophic level in the food chain (DeNiro & Epstein, 1978), tissues of mycoheterotrophic plants will therefore mirror the isotopic signature of their associated fungi (Gebauer & Meyer, 2003), whereas autotrophic plants will show no such pattern.

Box 1. The principle of stable isotope analysis

Most elements of biological interest are composed of two or more stable and/or nonstable (radioactive) isotopes, distinguished by different atomic mass units. These differences in atomic properties cause thermodynamic isotope effects, which lead to predictable changes in the isotopic composition of ecosystem compartments as elements cycle through the biosphere. Natural abundances of stable isotopes in a given material are usually expressed as the ratio of the minor (usually heavier) over the major abundant (usually lighter) isotope of a given element (e.g. $^{2}H/^{1}H$ or $^{13}C/^{12}C$). Because changes in isotope abundance are usually very small at natural abundance levels, measured isotope ratios of a given sample are usually expressed relative to a contemporaneously measured isotope ratio of a standard of known isotopic composition (Meier-Augenstein & Kemp, 2012). To make formal comparison of the resulting figures possible, the "delta notation" (δ) was proposed. The δ-value of the heavier isotope *h* of a chemical element E in a sample is defined by the following equation:

$$\delta^{h}E = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000,$$

where R_{sample} is the measured isotope ratio of the heavier isotope over the lighter (e.g., $^{13}C/^{12}C$) for the sample and $R_{standard}$ is the measured isotope ratio for the corresponding international reference material (RM) (Meier-Augenstein & Kemp, 2012). The results of this equation are referred to as per mil values (‰). A positive δ-value means that the sample has a higher abundance of the heavier isotope than the international reference standard that defines the scale for a particular isotope, while a negative δ-value indicates that the sample has a lower abundance of the heavier isotope than the international reference standard (Meier-Augenstein & Kemp, 2012).

Comparing δ-values (see Box I for explanation) across different plant species or life cycle stages allows to get insights into the trophic modes of plants or different stages within the life cycle of a plant. Usually, autotrophic plants are used as a reference and stable isotope signatures of putative mycoheterotrophic plants are compared with those of autotrophic reference plants. A δ-value of a certain element that is significantly higher than the average of δ-values of that element of reference autotrophic plants means that the plant is significantly enriched for that element. However, because stable isotopes are site-dependent and often depend on the prevailing light conditions (Preiss et al., 2010; Matsuda et al., 2012; Gonneau et al., 2014), comparison between multiple sites is not always possible. To overcome this problem, Preiss & Gebauer (2008) suggested using normalized enrichment factors (ε), which are calculated as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$ or $\delta^{15}N$ value of a mycoheterotrophic individual, a fungal sporocarp or an autotrophic reference plant, and δ_{REF} is the mean value of all autotrophic reference plants occurring at the site. Normalized enrichment factors allow comparing the trophic mode of many plant species, irrespective of the site where they were sampled, and is therefore especially useful for comparative analyses (Preiss & Gebauer, 2008).

After the pioneering work of Gebauer & Meyer (2003) and Trudell et al. (2003), many studies have applied stable isotope analyses to assess the trophic modes of plants (e.g. Bidartondo et al., 2004; Abadie et al., 2006; Tedersoo et al., 2007; Zimmer et al., 2007; Hynson et al., 2009; Johansson et al., 2015; Hynson et al., 2016; Jacquemyn et al., 2017a). Bidartondo et al. (2004), for example, analyzed isotope signatures in several orchid species inhabiting forest habitats and showed that orchids that associated primarily with rhizoctonia fungi were not significantly enriched in ^{13}C, whereas orchids associated with ectomycorrhizal fungi showed both significant enrichment in ^{13}C and ^{15}N. Similarly, Hynson et al. (2009) showed evidence for mycoheterotrophy on ectomycorrhizal fungi in several species of the tribe Pyroleae. Within Orchidaceae, mycoheterotrophy on saprotrophic fungi was investigated by Ogura-Tsujita et al. (2008), Martos et al. (2009), Dearnaley & Bougoure (2010), Sommer et al. (2012), and Lee et al. (2015). In contrast, isotopic composition on

mycoheterotrophic plants associated with arbuscular mycorrhizal fungi has been studied for very few species, including fully mycoheterotrophic Burmanniaceae and Gentianaceae (Merckx et al., 2010; Courty et al., 2011), and putative partially mycoheterotrophic species within the same families (Cameron & Bolin, 2010; Bolin et al., 2017).

Recent analyses have revealed that partial mycoheterotrophy may be more widespread than previously assumed, as plants that were initially assumed to be autotrophic based on their ^{13}C signature appeared to be significantly enriched in isotopes other than ^{13}C (Gebauer et al., 2016). Using normalized enrichment factors, Schiebold et al. (2017), for example, showed that in a large number of terrestrial orchids of the genus *Epipactis*, normalized enrichment factors for ^{15}N showed a continuous gradient from low values (<5) in so-called 'rhizoctonia' orchids (*Epipactis palustris* and *E. gigantea*) (Fig. 2) to high values for species that are assumed to form orchid mycorrhizas exclusively with ectomycorrhizal ascomycetes (*E. neglecta*, *E. muelleri*, *E. leptochila* and *E. distans*). Similarly, normalized enrichment factors for ^{13}C continuously increased from zero to 5 (Fig. 2). In another study, Schiebold et al. (2018) investigated stable isotope signatures in a range of meadow orchids associating with rhizoctonia fungi and found significant enrichment in ^{15}N and ^{2}H, although no signs of ^{13}C enrichment were found and in some cases even ^{13}C depletion. Stöckel et al. (2014) compared enrichment factors between protocorms and adult plants of both orchids associated with ectomycorrhizal fungi and rhizoctonia. As could be expected, protocorms of ectomycorrhiza-associated orchids were significantly enriched in ^{13}C and ^{15}N compared to autotrophic reference plants similar to fully mycoheterotrophic adults. In contrast, the protocorms of orchid species associated with rhizoctonia were also enriched in ^{13}C and ^{15}N, but significantly less compared to the protocorms of ectomycorrhiza-associated orchids and almost similar to that of adult plants, indicating that the trophic status of rhizoctonia associated orchids cannot be unequivocally determined based on their C and N isotope composition alone. These results were later corroborated by Schweiger et al. (2018), who used protocorms as fully mycoheterotrophic endpoints and showed that the photosynthetic orchids *Platanthera bifolia* and *Ophrys insectifera* were

significantly enriched in organic matter derived from fungi, although fungal gains were at the low end of the range reported for partially mycoheterotrophic orchids. These results therefore indicate a pattern of trophic modes that can be best summarized as a continuous gradient from fully mycoheterotrophic plants in the early stages of their development that grow into auto- or partially mycoheterotrophic plants or remain fully mycoheterotrophic (Fig. 3), and suggest that partial mycoheterotrophy may be much more widespread than previously assumed.

Multiple origins of full mycoheterotrophy

Phylogenetic evidence indicates that full mycoheterotrophy evolved multiple times independently in land plants (Fig. 4a). There are c. 580 leafless achlorophyllous species that are potentially fully mycoheterotrophic. Apart from one liverwort species (*Aneura mirabilis*, Aneuraceae), and a single species in gymnosperms with a controversial status (*Parasitaxus usta,* Podocarpaceae is either a full mycoheterotroph or a holoparasite; Feild & Brodripp, 2015), all these fully mycoheterotrophic species are flowering plants. The monocots have the highest diversity of fully mycoheterotrophic species, c. 530 species within the families Orchidaceae (295 spp.), Petrosaviaceae (3 spp.), Burmanniaceae (63 spp.), Thismiaceae (83 spp.), Triuridaceae (56 spp.), Corsiaceae (27 spp.), and Iridaceae (3 spp.). Within eudicots, fully mycoheterotrophic species are present in Ericaceae (17 spp.), Gentianaceae (25 spp.), and Polygalaceae (7 spp.) (Merckx, 2013, updated based on WCSP (2018)). Collectively, these species are part of over 40 evolutionary lineages, suggesting that full mycoheterotrophy has evolved independently over 40 times within land plants (Merckx et al., 2013a). The majority of evolutionary shifts from mycorrhizal mutualism to mycoheterotrophy occurred in the plant family Orchidaceae (Merckx, 2013), which is one of the most species-rich families of land plants and contains more than 28,000 species across 736 genera (Christenhusz & Byng, 2016). The family is widely distributed, from tropical rainforest to temperate grassland, only excluding the polar regions and driest deserts (Chase et al., 2015; Givnish et al., 2015; Jacquemyn et

al., 2017b). Fully mycoheterotrophic orchid species, however, are mostly restricted to tropical, temperate and boreal forests (Merckx, 2013).

Due to its cryptic manifestation partial mycoheterotrophy has so far only been confirmed for over 20 species of Orchidaceae (Hynson et al., 2016; Schiebold et al., 2017), although it seems much more likely that many more orchid species, if not all, show evidence of partial mycoheterotrophy (Schiebold et al., 2018). Further evidence for partial mycoheterotrophy has been found in c. 8 species of Ericaceae (within *Pyrola, Orthilia,* and possibly *Chimaphila* and *Moneses,*), a single species of Burmanniaceae (*Burmannia coelestis*), and has been suggested for Gentianaceae (*Bartonia virginica* and *Obolaria virginica*) (Cameron & Bolin, 2010; but see Hynson et al., 2013). Based on their obligate symbiotic germination initial mycoheterotrophy is the likely nutritional mode for the majority of Orchidaceae species and some Ericaceae species (*Chimaphila* and *Moneses* when autotrophic as adults; Matsuda et al., 2012). Initial mycoheterotrophy, however, is potentially prevalent in families with species producing so-called 'dust seeds', i.e. minute seeds that lack a sufficient amount of resources and are dependent on mycorrhizal fungi for germination and subsequent growth to a seedling (Eriksson & Kainulainen, 2011). In addition, a number of taxa in the Lycopodiaceae, Psilotaceae, Ophioglossaceae, Schizaeaceae, and Gleicheniaceae have unambiguously mycoheterotrophic gametophytes and pre-emergent sporophytes (Boullard, 1979). Adult sporophytes are consistently photosynthetic, and many can be cultivated. It appears that many of these taxa may be initially mycoheterotrophic, although the trophic status of adult sporophytes under field conditions has yet to be investigated (Hynson et al., 2013).

While some lineages of fully mycoheterotrophic species are evolutionary 'isolated' and diverged from their most closely related photosynthetic relatives many million years ago (e.g. *Voyria;* Merckx et al., 2013b, Corsiaceae; Mennes et al., 2015), several fully mycoheterotrophic species have close photosynthetic relatives, which are confirmed as, or candidates for, partial mycoheterotrophs (e.g. Motomura et al., 2010). Within Orchideaceae, and potentially also Ericaceae (Pyroleae), these partially mycoheterotrophic clades are on their part imbedded within initially

mycoheterotrophic groups (Lallemand et al., 2016). This suggests that evolution from autotrophy to full mycoheterotrophy is a graduate process, in which initial and partial mycoheterotrophy are intermediate steps. In this respect, the evolution of initial mycoheterotrophy is the critical barrier for a departure from full autotrophy. The origin of initial mycoheterotrophy is often linked with the evolution of dust seeds, which depend on external fungal carbon for germination and early development (Ramsbottom, 1922; Eriksson & Kainulainen, 2011). Under this scenario, 'compensated trait loss' (Ellers et al., 2012), in which interactions with fungi replaced the dependence on seed endosperm, allowed for an evolutionary trajectory towards new nutritional modes in plants.

Partner selectivity and niche breadth

For some plant lineages it is assumed that autotrophic, initially, partially, and fully mycoheterotrophic plants associate with distinct sets of mycorrhizal fungi. For example, within the orchid family, a distinction has been made between so-called 'ectomycorrhiza-associated' orchid species and 'rhizoctonia-associated' species (Bidartondo et al., 2004; Schiebold et al., 2018). The latter have long been assumed to be autotrophic as adults, whereas the former usually show partial or full mycoheterotrophy. However, the assumed autotrophy of rhizoctonia-associated orchid species has recently been challenged (Gebauer et al., 2016; Schiebold et al., 2018; Schweiger et al., 2018). Moreover, detailed molecular analyses using high-throughput sequencing techniques have shown that many so-called 'rhizoctonia-associated' species also associate with ectomycorrhizal fungi and *vice versa* (Jacquemyn et al. 2014; 2015a; 2017a), indicating that a strict distinction between rhizoctonia-associated and ectomycorrhiza-associated species cannot be maintained, neither based on their stable isotope signatures nor their mycorrhizal communities. Similar results have been observed in other genera displaying autotrophy and mycoheterotrophy. The mycoheterotrophic *Pyrola aphylla* (Ericaceae), for example, associates with a wide range of endophytic and ectomycorrhizal fungi, which partly overlap with the associations of its partially mycoheterotrophic sister species *P. picta*, and other species within Pyroleae (Hynson & Bruns, 2009). The fully

mycoheterotrophic liverwort *Aneura mirabilis* (Aneuraceae) harbors similar symbiotic *Tulasnella* fungi as its close autotrophic relatives (Bidartondo & Duckett, 2010). These *Tulasnella* species are able to form ectomycorrhizas with *Betula* and *Pinus sp.* (Bidartondo et al., 2003). While only limited data are available for arbuscular mycorrhizal mycoheterotrophic lineages, observations in *Burmannia* (Burmanniaceae) and Petrosaviaceae show that fully mycoheterotrophic species tend to target Glomeraceae fungi exclusively, while related photosynthetic species grow with a wider range mycorrhizal community, which, besides Glomeraceae, includes families such as Acaulosporaceae and Diversisporaceae (Merckx et al., 2010; Suetsugu et al., 2012; Ogura-Tsujita et al., 2013; Yamato et al., 2014).

Associations with multiple mycorrhizal partners appear to be common in both autotrophic and mycoheterotrophic plants, whereas switches in mycorrhizal communities from an initial mycoheterotrophic to an autotrophic life style during ontogenetic development seem to be relatively frequent. This raises the intriguing question why plants associate with multiple fungi and why they switch from partners across life cycle stages with different trophic modes. Associating with multiple fungi simultaneously may be beneficial when partners differ in quality or functionality, because a more diverse community may be more likely to include the most beneficial partner (sampling effect) or because multiple partners provide different benefits (complementarity effect) (Batstone et al. 2018). Partners may also have complementary effects over time if the focal mutualist's needs change across ontogeny, finally resulting in a different ideal partner at different life stages (Batstone et al. 2018). Evidence for complementarity effects over time was given by Bidartondo & Read (2008), who showed that the diversity of mycorrhizal fungi associating with seedlings and adult plants in a number of orchids generally increased from the seedling stage to the adult stage, indicating that plants gradually accumulate fungi during their development. Johansson et al. (2017) investigated germination and seedling development and the diversity of fungi associated with germinating seeds and subterranean seedlings (juveniles) in partially and fully mycoheterotrophic Ericaceae species. Their results showed that fungal host specificity generally

increased during juvenile ontogeny, most pronounced in the fully mycoheterotrophic species, although a narrowing of fungal associates was also observed in two partially mycoheterotrophic species.

Besides shifts during ontogeny, associating with multiple partners may allow mutualists to overcome stressful abiotic conditions when partners exhibit different performance optima along a niche axis. McCormick et al. (2006), for example, showed that the evergreen terrestrial orchid *Goodyera pubescens* was able to switch mycorrhizal partners during periods of stress (in this case drought). Although not investigated, it is reasonable to assume that this fungus was already latently present, but became dominant when drought progressed. Further evidence for temporal turnover in mycorrhizal partners was provided by Oja et al. (2015), who showed that the mycorrhizal communities associated with the forest orchid *Neottia ovata* changed through time, whereas no such shifts were observed in the surrounding soil. The explanation for why host plants select different mycobionts from the local species pool over time may be found in the fact that different partner species exhibit different population dynamics or performance trade-offs and that associating with multiple partners will therefore lead to more consistent returns through time (so-called portfolio effect) (Batstone et al., 2018).

Mycorrhizal fungi associated with shifts in trophic modes

Arbuscular mycorrhizal lineages — Within the genus *Burmannia*, the fully mycoheterotrophic species are all associated with Glomeraceae fungi, which are a subset of the fungal associates of the autotroph *Burmannia capitata* (Merckx et al., 2010; Suetsugu et al., 2012; Ogura-Tsujita et al., 2013; Fig. 4b). A similar pattern is observed for *Petrosavia-Japonolirion* (Petrosaviaceae; Yamato et al., 2014) and *Epirixanthes-Salomonia* (Polygalaceae; Mennes et al., 2015), which suggests that the shift from autotrophy to full mycoheterotrophy in arbuscular mycorrhizal lineages occurs in parallel with an increased specificity towards fungi which were already present in the mycorrhizal communities of the autotrophic ancestor.

Orchidaceae — Together with Ericaceae, Orchidaceae is the only family with strong evidence for the occurrence of initial, partial, and full mycoheterotrophy. The evolution of initial mycoheterotrophy is hypothesized to have occurred in the most recent ancestor of Orchidaceae in parallel with a shift from arbuscular mycorrhiza towards orchid mycorrhiza (Yukawa et al., 2009; Fig. 4c), c. 90 million years ago (Chomicki et al., 2014). However, not all orchids seem to have lost the association with arbuscular mycorrhiza. For example, in at least two *Cypripedium* species the presence of *Glomus* has been observed in the roots (Shefferson et al., 2005). Due to the deep evolution of these shifts their mutual influence remains unclear. Within Orchidaceae, there have been several subsequent shifts towards partial and full mycoheterotrophy. One way to investigate how mycorrhizal communities shift from initial mycoheterotorphy to mycoheterotrophy consists of comparing mycorrhizal diversity and composition between species that display different trophic modes and that co-occur at a given site. Such situations can be found in several orchid genera (e.g. *Epipactis*, *Neottia*, *Cymbidium*) (Motomura et al., 2010; Ogura-Tsujita et al., 2012; Těšitelová et al., 2015; Yagame et al., 2016), or species within the Ericaceae. For example, comparison of the mycorrhizal fungi associating with autotrophic, partially and fully mycoheterotrophic *Cymbidium* species showed that the autotrophic species was predominantly dependent on saprobic Tulasnellaceae, the partially mycoheterotrophic species associated with fungi of the Tulasnellaceae and several ectomycorrhizal groups, including members of the Sebacinales, Russulaceae, Thelephoraceae and Clavulinaceae, whereas the two mycoheterotrophic species were mostly specialized with ectomycorrhizal Sebacinales. Within a phylogenetic perspective, evolution from initial mycoheterotrophy over partial mycoheterotrophy to full mycoheterotrophy can therefore be hypothesized to coincide with a shift from saprotrophic to ectomycorrhizal fungi via a phase of coexistence of both nutritional types of fungi in the partial mycoheterophic mode (Ogura-Tsujita et al., 2012).

A similar comparison of the mycorrhizal communities of two *Epipactis* species that co-occur in dune slacks showed that the putatively autotrophic *Epipactis palustris* mainly associated with

fungi of the genera *Tulasnella* and *Ceratobasidium*, whereas fungal communities in the partially mycoheterotrophic *E. helleborine* subsp. *neerlandica*) were primarily dominated by members of the ectomycorrhizal genera *Tuber* and *Geopara* (Jacquemyn et al., 2016, 2017a) (Fig. 5). Interestingly, several ectomycorrhizal genera were also present in *E. palustris*, albeit at much lower abundances. Similarly, in *Neottia* partially mycoheterotrophic species, such as *N. ovata* (Schiebold et al., 2017) interact with Sebacinales B fungi, while leafless species grow on ectomycorrhizal Sebacinales A fungi. However, Sebacinales Group A fungi were detected in a small portion of the sampled green species, most closely related to the leafless species (Těšitelová et al., 2015; Yagame et al., 2016). Moreover, deeper sequencing of multiple populations of *N. ovata* in Belgium showed that the species associated with a wide range of putative mycorrhizal fungi, including ectomycorrhizal fungi and representatives of both Sebacinales Group A and B (Jacquemyn et al., 2015b).

Interestingly, a few fully mycoheterotrophic orchids have been reported to associate with saprotrophic non-rhizoctonia fungi and obtain nutrients through the ability of the fungi to cause wood or litter decay (e.g. Lee et al., 2015; Ogura-Tsujita et al. 2018). Their phylogenetic relationships as well as the mycorrhizal communities and the trophic modes of their photosynthetic relatives remain to be investigated in detail. However, saprotrophic non-rhizoctonia fungi are often present in the roots of photosynthetic orchids, either as facultative biotrophic encounters or as endophytes, thus their presence may act as a predisposition for the evolution of full mycoheterotrophy (Selosse et al., 2010).

Ericaceae — In Ericaceae, two independent evolutionary origins of mycoheterotrophy were preceded by a single shift from arbuscular mycorrhizal interactions to an interaction with ectomycorrhizal fungi, in the common ancestor of the clade formed by Pyroleae, Arbutoideae, Monotropeae, and Pterosporeae (Lallemand et al., 2016; Freudenstein et al., 2016; Fig. 4d). In Pyroleae, initially, partially, and fully mycoheterotrophic species all associate with a wide range of endophytes and ectomycorrhizal fungi of which *Tomentella*, *Sebacina*, *Wilcoxina* and *Inocybe* appear

to be common (Hynson & Bruns, 2009). An extensive phylogenetic comparison is lacking, but a comparison between the mycorrhizal communities of the full mycoheterotroph *Pyrola aphylla* and its co-occurring partially mycoheterotrophic relative *P. picta* did not reveal strong differences. In contrast to the wide diversity of Pyroleae mycorrhizal communities, fully mycoheterotrophic species in Monotropoideae and Pterosporeae are each associated with a distinct narrow range of ectomycorrhizal fungi (Bidartondo & Bruns, 2002; Bidartondo, 2005). Their most closely related relatives, the putatively autotrophic species in Arbutoideae, associate with a wide range of ectomycorrhizal fungi (Kennedy et al., 2012).

Aneuraceae —In the liverwort family Aneuraceae a shift from a non-mycorrizal ancestor (or alternatively an arbuscular mycorrhizal status) towards an association with *Tulasnella* fungi, which are either saprotrophic or ectomycorrhizal, preceded an evolutionary shift towards full mycoheterotrophy in *Aneura mirabilis* (Kottke & Nebel, 2005; Bidartondo & Duckett, 2010; Fig. 4e). Closely related *Aneura* species, which are possibly partially mycoheterotrophic (Duckett & Ligrone, 2008), grown on a similar, or a slightly larger phylogenetic range of *Tulasnella* fungi (Bidartondo & Duckett, 2010).

Evidence for mycorrhizal shifts during the evolution towards full mycoheterotrophy?

In some partially mycoheterotrophic plant species, albino plants can be observed that have lost the capacity of photosynthesis and thus potentially represent an intermediate stage between partial and full mycoheterotrophy (Renner, 1938; Salmia, 1986; Selosse et al., 2004; Julou et al., 2005; Abadie et al., 2006; Stöckel et al., 2011). As such, they represent interesting model systems to investigate how loss of photosynthesis may trigger other changes in the physiology, ecology and mycorrhizal communities associating with partially mycoheterotrophic plants, potentially leading to full mycoheterotrophy. Detailed physiological measurements and long-term observations in mixed populations of the woodland orchid *Cephalanthera damasonium* have shown that albinos are in

general less fit than their green counterparts. Albinos displayed more frequent shoot drying at fruiting, possibly due to stomatal dysfunctions, had a lower basal metabolism, showed increased sensitivity to pathogens and herbivores, had higher dormancy and showed signs of maladapted sprouting, and produced, probably due to the previous differences, fewer seeds, which in turn had a lower germination capacity (Roy et al., 2013). When all fitness costs were added, albinos were estimated to have a 10^3 x fitness reduction, compared to green plants, suggesting that a successful transition to full mycoheterotrophy is unlikely to occur from albino plants. Closer evaluation of the mycorrhizal fungi associating with both green and albino phenotypes showed that they usually associate with the same set of fungi, although the level of colonization tends to be higher in albino plants (Abadie et al., 2006). For example, in *Epipactis microphylla* both phenotypes associated with ectomycorrhizal truffles and *Cortinarius* (Selosse et al., 2004). In a Japanese population of *Epipactis helleborine,* green and albino plants also harbored similar fungi, mainly *Wilcoxina* (Suetsugu et al., 2017). Similarly, Julou et al. (2005) and Abadie et al. (2006) did not find significant differences in the mycorrhizal communities associating with albino and green plants of *Cephalanthera damasonium* and *C. longifolia*. In both species, mycorrhizal communities were dominated by ectomycorrhizal fungi of the Thelephoraceae, with additional associations with members of the Cortinariaceae and few rhizoctonias. These results indicate that a switch to fungal partners that provide more carbon had not (yet) occurred and therefore may have prevented the emergence of a fully heterotrophic lineage. On the other hand, Shefferson et al. (2016) showed that, despite the pronounced fitness reduction that was observed in albino plants, the deterministic population growth rate (λ) did not significantly differ between the two phenotypes, suggesting that albinism was neither adaptive or maladaptive and that some compensation mechanisms may open the door to the origination of full mycoheterotrophy from partially mycoheterotrophic ancestors.

Conclusion and future directions

Our overview indicates that full mycoheterotrophy evolved multiple times and only occurs on fungi that are simultaneously mycorrhizal with surrounding plants, either arbuscular or ectomycorrhiza, or on particular wood or litter decaying fungi. There is also mounting evidence that full mycoheterotrophy is one endpoint of a continuous gradient between autotrophy and full mycoheterotrophy and that partial mycoheterotrophy is much more widespread than previously assumed. Similarly, our review indicates that there is a continuous gradient in fungal community composition between autotrophic and mycoheterotrophic plants, and between initially mycoheterotrophic and autotrophic life stages. However, in most instances, a progressive change in communities has been observed between autotrophic and mycoheterotrophic plants, suggesting that in the transition from autotrophy to full mycoheterotrophy some fungi have been gradually discarded from the mycorrhizal pool present in autotrophic ancestors and replaced by others that were already latently present and became progressively more important, or that have been acquired from the soil if they were not yet present in the ancestor. Because acquisition of new fungal strains is difficult and may require alternative resource acquisition techniques (Werner et al., 2018), this may explain why the number of fully mycoheterotrophic lineages is relatively low. To fully understand the evolution of trophic modes in plants we see significant improvements in the fields of molecular quantification of fungal abundance, physiology and functionality of the fungi involved and the mechanisms leading to fungal turnover. These are briefly discussed below.

Quantifying absolute abundances of mycorrhizal fungi in the roots of autotrophic and mycoheterotrophic species

Although current advances in high-throughput sequencing have allowed to obtain more detailed insights into the mycorrhizal communities associating with autotrophic and mycoheterotrophic plants (e.g. Jacquemyn et al., 2017a; Johansson et al., 2017; Waud et al., 2017), they still are not suitable to obtain reliable estimates of fungal abundances and therefore to assess the contribution of each fungal partner to the carbon and nitrogen budget of partially and fully mycoheterotrophic

plants. Recent research has shown that differences of relative abundances generated by high-throughput techniques not necessarily reflect those of the actual taxon abundances (Zhang et al., 2018) and that relative sequence abundances may therefore lead to biased assessments of mycorrhizal abundance. Quantitative real-time PCR (qPCR) can be used to estimate absolute abundances of particular mycorrhizal strains in complex mycorrhizal communities and to relate these to normalized enrichment factors. In addition, recent advances in sequencing (e.g. whole genome shotgun sequencing) will allow for enhanced detection of fungal species, increased detection of diversity and increased prediction of genes, while the increased length, either due to longer reads or the assembly of contigs, will improve the accuracy of species detection (Ranjan et al., 2016; Laudadio et al., 2018). Further studies of trophic modes should therefore combine improved descriptions of mycorrhizal communities with the quantification of absolute abundances of the fungal communities for comparisons among autotrophic and mycoheterotrophic species or among life stages within single species.

Assessing functionality of mycorrhizal fungi

Once mycorrhizal fungi have been detected and identified, the next step would be to assess their function in autotrophic, partially and fully mycoheterotrophic plants. Recent studies have shown that in partially mycoheterotrophic orchids carbon derived from mycorrhizal fungi mostly supports young spring shoots and below-ground organs, whereas carbon originating from photosynthesis contributes most to sexual reproduction (Gonneau et al., 2014; Suetsugu et al., 2018; Lallemand et al., 2018). These results may explain why albino plants fail to produce similar levels of seeds than green plants, but show the same survival rates (Roy et al., 2013; Lallemand et al., 2018). Using fungicides, Bellino et al. (2014) were able to eliminate the mycorrhizal fungi associating with the partially mycoheterotrophic orchid *Limodorum abortivum* without impairing fruit production, supporting the idea that carbon derived from fungi contributes little to sexual reproduction. Future research could use selective fungicides to eliminate some, but not all fungi, and measure the effect

of fungicide application on below- and aboveground growth and reproduction in fully and partially mycoheterotrophic plants. However, plant-soil feedback models suggest that fungicide studies should be treated with extreme caution because the impacts on the plants and the remaining fungi can be driven by indirect effects on competition and other interactions between soil microbes. Experiments using different sets of fungi for germination and subsequent growth to adult plants may allow to compare the specific role of each fungal partner across the life cycle of autotrophic and mycoheterotrophic plants.

Assessing the mechanisms leading to fungal turnover and partner selectivity

The establishment and maintenance of mycorrhizal symbiosis is mediated by the complex molecular cross-talk between symbiotic partners (Floss et al., 2013; Garcia et al., 2015; Oldroyd, 2013). In general, the evolutionary processes that determine fungal specificity and partner selectivity are not clear. Our overview has indicated that in some instances partner switching and/or abandonment have accompanied the transition from autotrophy to mycoheterotrophy, suggesting that the autotrophy-mycoheterotrophy continuum in plants is supported by distinct fungal partners, and that plants may select the best partner that is available in the local community according to their trophic mode. Interestingly, fungal partners that sustain mycoheterotrophy in plants are sometimes also present in autotrophic relatives alongside the principal fungal symbionts of autotrophs, in which case their role is unknown. We hypothesize that mycorrhizal communities of autotrophic plants may routinely contain 'ineffective' mycorrhizal fungi. An emerging framework for plant-associated symbioses predicts key roles for spatio-temporal variation and host community dynamics in the maintenance of these ineffective symbionts (Thompson, 2005; Bever, 2015; Pahua et al., 2018), and ultimately their presence may increase a plant's niche breath (Batstone et al., 2018). Ecological pressures, such as intense competition for light, may subsequently change the role of ineffective symbionts to beneficial symbionts. To understand the precise circumstances under which partner switching has occurred, experimental systems should be developed to investigate the relative fitness

of different plant-mycorrhizal fungi combinations, similar to those used for legume-rhizobia associations (e.g. Pahua et al., 2018). In addition, symbiotic gene expression patterns in mycorrhizae could be compared between autotrophic, partially mycoheterotophic and fully mycoheterotrophic plants associating with different mycorrhizal partners. This will allow getting insights into the role of key genes in the symbiotic toolkit encoding crucial root mutualism effectors (Werner et al., 2018). Comparison of gene expression profiles between albino and green plants of the terrestrial orchid *Epipactis helleborine* has for example shown that genes involved in the antioxidant metabolism were upregulated in albino plants, possibly reflecting increased peloton digestion in albino plants (Suetsugu et al., 2017). Interestingly, their analysis also showed that genes related to arbuscular mycorrhizal symbiosis were upregulated in the albino variants, possibly as a mechanism to promote fungal colonization and opening doors for the establishment of new partnerships. Similar analyses could be performed between closely related autotrophic, partially and fully mycoheterotrophic plants. This experimental and genetic research will further elucidate the mechanisms of host switching in relation to shifts in trophic modes and uncover the stepwise process by which these mechanisms evolve.

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Authors contributions

HJ and VSFTM developed the ideas of the manuscript and wrote the paper.

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List of Figures

Fig. 1 Representative examples of mycoheterotrophic plants across the flowering plants. (a-f) Fully mycoheterotrophic species: (a) *Sciaphila densiflora* (Triuridaceae); (b) *Epirixanthes cylindrica* (Polygalaceae); (c) *Thismia tentaculata* (Thismiaceae); (d) *Neottia nidus-avis* (Orchidaceae); (e) *Sarcodes sanguinea* (Ericaceae); (f) *Erythrorchis altissima* (Orchidaceae); (g-h) Partially mycoheterotrophic species: (g) *Ophrys insectifera* (Orchidaceae); (h) *Platanthera bifolia* (Orchidaceae). All pictures by V. Merckx, except (f) by Y. Ogura-Tsujita.

Fig. 2 Normalized enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ for a large number of *Epipactis* species showing a continuous gradient of ^{13}C and ^{15}N enrichment. Species codes: Eat = *Epipactis atrorubens*; Edi = *E. distans*; Efi = *E. fibri*; Egi: *E. gigantea*; Ehe: *E. helleborine*, Ehn: *E. helleborine* spp. *neerlandica*; Ele = *E. leptochila*; Emi = *E. microphylla*; Emu: *E. muelleri*; Ene = *E. neglecta*, Epa: *E. palustris*; Epu: *E. purpurata*. The green box represents the mean enrichment factors (± 1 s.d.) for autotrophic reference plants that were sampled together with the *Epipactis* species. The red box represents mean enrichment factors (± 1 s.d.) of all partially mycoheterotrophic orchid species that associate with ectomycorrhizal fungi that were reviewed by Hynson et al. (2013) before publication of Schiebold et al. (2017). (Figure reproduced from Schiebold et al. (2017), with permission).

Fig. 3 Proposed continuum of trophic modes in plants. Autotrophy, partial mycoheterotrophy and full mycoheterotrophy should not be seen as three distinct stages in trophic modes, but rather as a gradual transition from one stage to the next.

Fig. 4 Evolution of trophic modes and mycorrhizal associations. (a) Phylogenetic distribution of mycoheterotrophy in land plants. Size of major land plant clades are according to species numbers reported in Christenhusz & Byng (2016). Phylogenetic relationships and divergence times follow Puttick et al. (2018) and Morris et al. (2018). (b) Evolution of trophic modes and mycorrhizal associations in *Burmannia* (Burmanniaceae). Initial mycoheterotrophy is not described for this group. Mycorrhizal associates of *Burmannia coelestis,* a species capable of partial mycoheterotrophy (Bolin et al., 2017) have not been characterized yet. Other lineages, such as Petrosaviaceae, Polygalaceae show a similar trend (Yamato et al., 2014; Mennes et al., 2015). (c) Initial mycoheterotrophy likely evolved in the common ancestor of Orchidaceae, in parallel with a shift from arbuscular mycorrhizas to orchid mycorrhizas (Yukawa et al., 2009). Two scenarios towards full mycoheterotrophy are illustrated: (1) In *Cymbidium* evolution from initial mycoheterotrophy over partial mycoheterotrophy to full mycoheterotrophy is associated with a gradual shift from rhizoctonia orchid mycorrhiza to ectomycorrhizal Sebacinales (Ogura-Tsujita et al. 2012). A similar pattern is found for *Neottia* (Yagame et al., 2016); (2) Some fully mycoheterotrophic orchid species exploit non-rhizoctonia wood and litter decaying fungi, such as *Mycena* in *Gastrodium confusa* (Ogura-Tsujita et al., 2008). Existence of partial mycoheterotrophy of species closely related to these mycoheterotrophs remains to be tested. (d) *Enkianthus,* the sister group of all other Ericaceae, associates with arbuscular mycorrhizal fungi (Obase et al., 2013), although ericoid forming fungi of Helotiales are also present (Obase & Matsuda, 2014). (1) In Pyroleae, initially, partially, and fully mycoheterotrophic taxa all associate with a broad range of ectomycorrhizal fungi, such as *Russula, Tricholoma,* and *Rhizopogon* (Hynson & Bruns, 2009). (2) Putatively autotrophic species of Arbutoideae associate with a wide range of ectomycorrhizal fungi (Kennedy et al., 2012), while fully mycoheterotrophic Monotropoideae grow on narrow ranges of ectomycorrhizal fungi (Bidartondo, 2005). (e) In Aneuraceae, mycorrhizal association with *Tulasnella* fungi were acquired from a non-mycorrhizal ancestor (Kottke & Nebel, 2005). These *Tulasnella* species may be saprotrophs or form ectomycorrhizal associations with surrounding trees, which is the case for the sole fully

mycoheterotrophic species *A. mirabilis* (Bidartondo et al., 2003). Some *Aneura* species maybe partially mycoheterotrophic (Duckett & Ligrone, 2008).

Fig. 5 Differences in mycorrhizal communities between the putatively autotrophic *Epipactis palustris* and the partially mycoheterotrophic *E. helleborine* subsp. *neerlandica* sampled in dune slacks in Belgium where the two species co-occur. Both species associate with a large number of fungal strains, but the relative proportion of sequences belonging to fungal genera differed between the two species. *Epipactis palustris* showed a high preference for fungi of the genera *Tulasnella* and *Ceratobasidium*, which were largely lacking or completely absent in *E. helleborine* subsp. *neerlandica*. In the latter, fungal communities were primarily dominated by members of the ectomycorrhizal genera *Tuber* and *Geopara* (data from Jacquemyn et al. 2016, 2017a).

Fig. 1

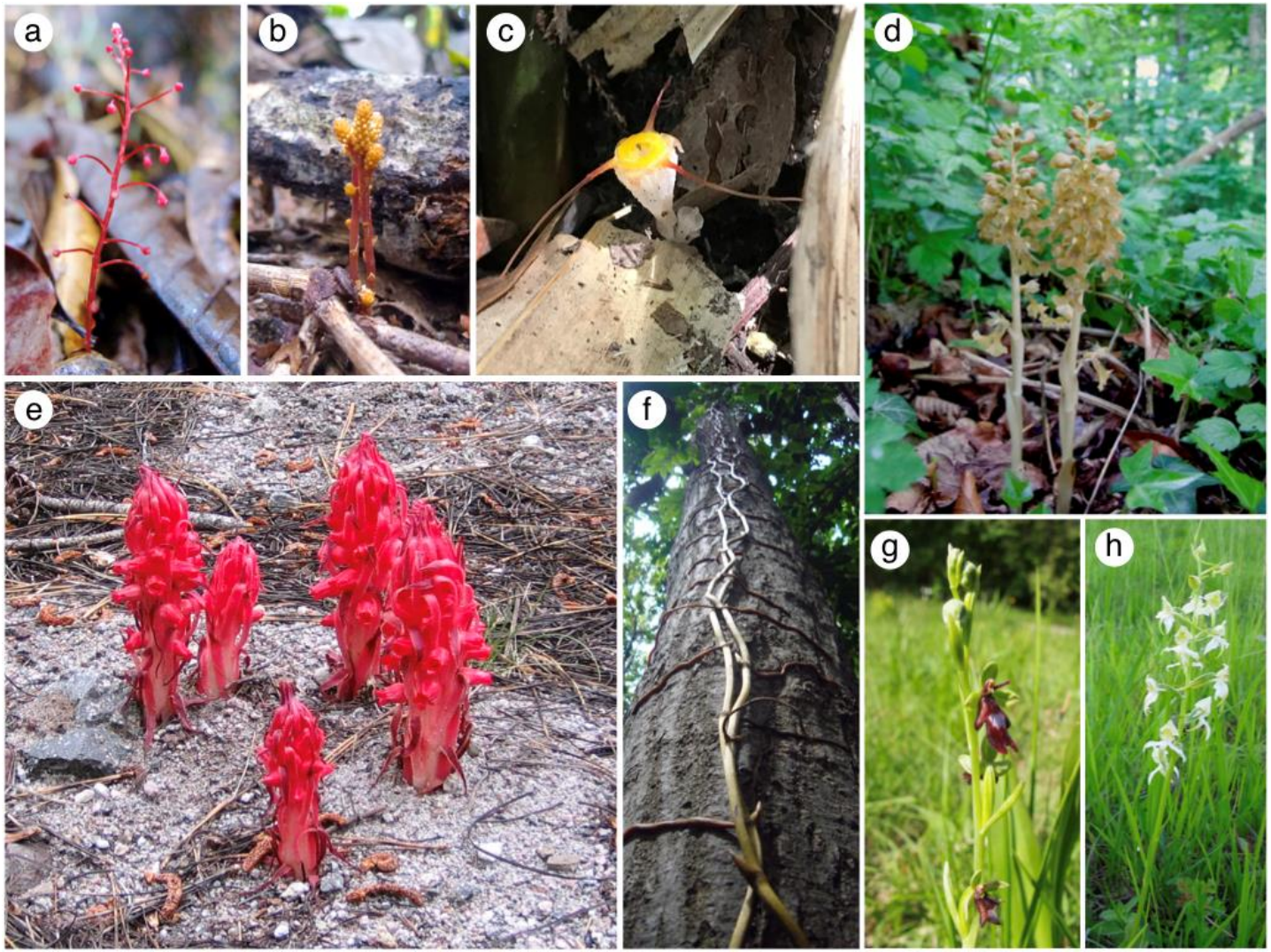

Fig. 2

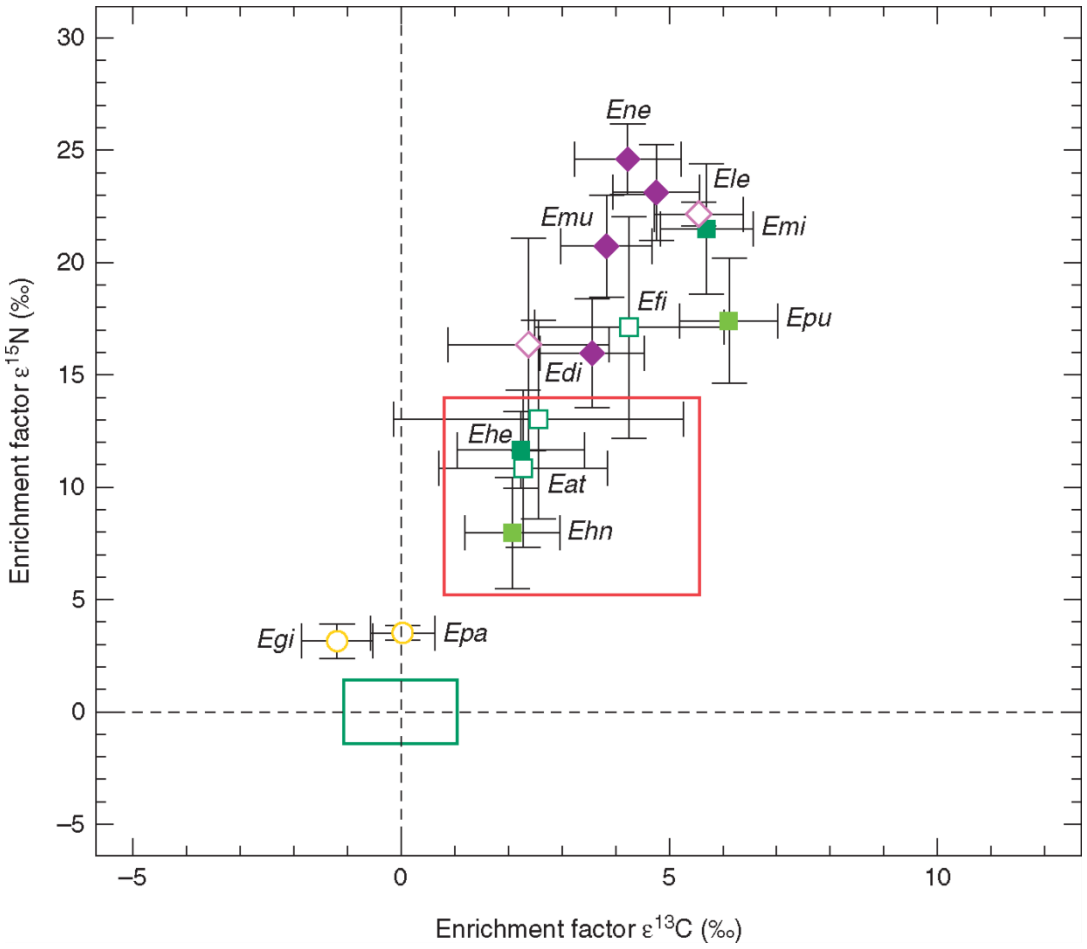
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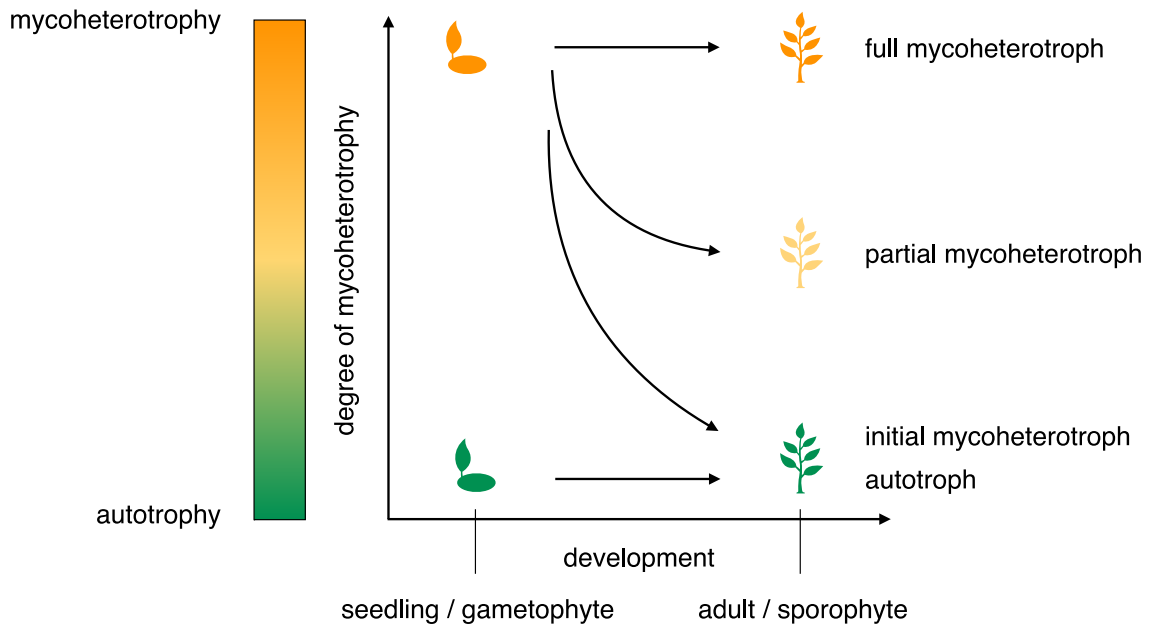
Fig. 3


mycoheterotrophy
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degree of mycoheterotrophy
full mycoheterotroph
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initial mycoheterotroph
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development
seedling / gametophyte
adult / sporophyte

Fig. 4

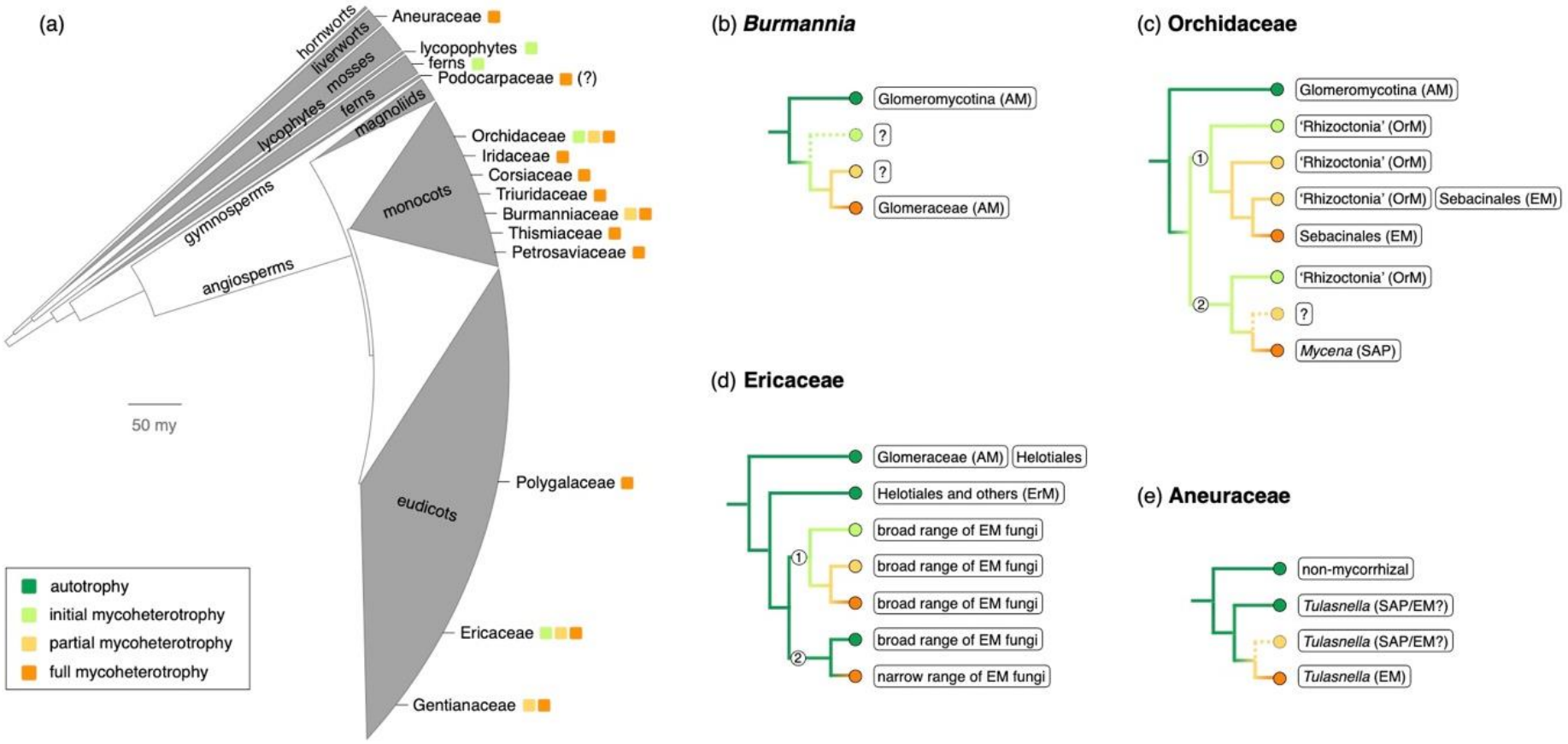
(a)
hornworts
liverworts
mosses
lycophytes
ferns
magnoliids
gymnosperms
angiosperms
monocots
eudicots
50 my
Aneuraceae
lycopophytes
ferns
Podocarpaceae (?)
Orchidaceae
Iridaceae
Corsiaceae
Triuridaceae
Burmanniaceae
Thismiaceae
Petrosaviaceae
Polygalaceae
Ericaceae
Gentianaceae
autotrophy
initial mycoheterotrophy
partial mycoheterotrophy
full mycoheterotrophy
(b) Burmannia
Glomeromycotina (AM)
?
?
Glomeraceae (AM)
(c) Orchidaceae
Glomeromycotina (AM)
'Rhizoctonia' (OrM)
'Rhizoctonia' (OrM)
'Rhizoctonia' (OrM)
Sebacinales (EM)
Sebacinales (EM)
'Rhizoctonia' (OrM)
?
Mycena (SAP)
(d) Ericaceae
Glomeraceae (AM)
Helotiales
Helotiales and others (ErM)
broad range of EM fungi
broad range of EM fungi
broad range of EM fungi
broad range of EM fungi
narrow range of EM fungi
(e) Aneuraceae
non-mycorrhizal
Tulasnella (SAP/EM?)
Tulasnella (SAP/EM?)
Tulasnella (EM)

Fig. 5

Epipactis palustris

$\delta^{13}C$ = -30.4‰; $\delta^{15}N$ = -4.2‰; %N = 2.1

- *Tulasnella*
- *Sebacina*
- *Ceratobasidium*
- *Thelephora*
- *Inocybe*
- *Tuber*
- *Geopora*
- *Cortinarius*
- *Leptodontidium*
- *Exophiala*
- *Hebeloma*
- *Trichophaea*

Epipactis helleborine subsp. *neerlandica*

$\delta^{13}C$ = -26.9‰; $\delta^{15}N$ = 6.2‰; %N = 3.1

- > 20%
- 10 – 20%
- 5 – 10%
- < 5%