Evolution and Diversification

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5.1 Introduction

The ability to obtain carbon from soil fungi is an exceptional trophic strategy in plants. The bizarre appearance of fully mycoheterotrophic plants immediately sparks questions about their evolutionary history. How and when did they originate? Are all mycoheterotrophic plant lineages closely related? What did their ancestors look like? A quick glance through the list of plant families that contain fully mycoheterotrophic species reveals that mycoheterotrophy must have evolved multiple times independently during land plant evolution and that similarities in habit and ecological interactions of many groups of mycoheterotrophs thus are the result of remarkable convergent evolution (Chap. 2). The molecular revolution in plant systematics has helped to advance our understanding of land plant evolution considerably, including the evolutionary framework behind shifts from autotrophy to full mycoheterotrophy. In combination with the substantial progress that has been made in our knowledge of the ecology of mycoheterotrophic plants and their fungal partners, we are now able to derive hypotheses of the evolutionary framework leading to mycoheterotrophy.

5.2 Reconstructing the Evolutionary History of Mycoheterotrophy

The evolutionary trajectory toward a fully mycoheterotrophic mode of life can only be studied when solid phylogenetic frameworks are available. Despite our expanding knowledge of the evolutionary relationships in most plant families, our understanding of the affinities of many mycoheterotrophic plant lineages is somewhat lagging behind. Indeed, in many cases, several obstacles cause the identification of relatives of full mycoheterotrophs to be a phylogenetic challenge. First, many mycoheterotrophic plants are rare, difficult to find, or only grow at remote localities (Chap. 2). In some extreme cases, particular species are known from a single collection only. Obtaining study material is therefore often the first difficulty to overcome when trying to unravel the evolutionary history of mycoheterotrophs. Second, the evolution of full mycoheterotrophy is often associated with the loss of morphological key characters, leaving few reliable characters with which to infer evolutionary relationships. In such cases, the use of DNA sequence data for unraveling phylogenetic history provides a promising endeavor. Unfortunately, the evolution of mycoheterotrophy is not only accompanied by

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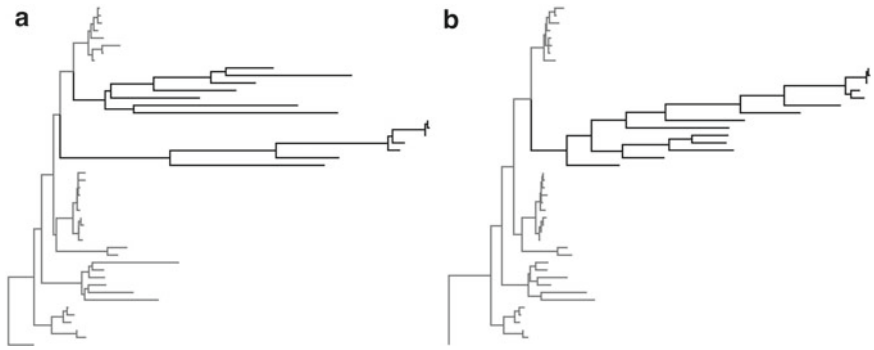

Fig. 5.1 An example of how increased substitution rates in mycoheterotrophic lineages can lead to bias in phylogenetic reconstruction. (**a**) Most optimal maximum likelihood tree of a selection of Dioscoreales species based on nuclear 18S rDNA and mitochondrial *atp1* data. Branches leading to taxa of Thismiaceae are shown in *black* and clearly display elevated substitution rates in comparison with other Dioscoreales lineages. This analysis suggests that Thismiaceae are a paraphyletic group. (**b**) One of the 1,824 most parsimonious trees obtained with maximum parsimony analysis of the same Dioscoreales dataset. In this analysis, Thismiaceae (black lineages) are monophyletic, but this was shown to be the result of a long-branch attraction artifact (Figure adapted from Merckx et al. (2009))

physiological, anatomical, and morphological adaptations but also by high rates of molecular evolution. Chloroplast DNA data is the preferred choice for phylogenetic reconstruction of plants. However, including nonphotosynthetic full mycoheterotrophs in phylogenies based on plastid DNA data poses many difficulties. Some achlorophyllous species still contain amplifiable plastid DNA, but the resulting sequences are often problematic in alignment and analyses due to elevated substitution rates (Chase et al. 1993; Caddick et al. 2002). Furthermore, as we discuss further in this chapter, these high rates of molecular evolution are not restricted to the chloroplast genome but are often present in nuclear and mitochondrial genes as well. The occurrence of substantial heterogeneity in rates of molecular evolution hampers sequence alignment and may also cause bias in phylogenetic reconstruction. Particularly, long phylogenetic branches can positively mislead parsimony analyses, causing the wrong tree to be estimated with increasing confidence as more characters are added. This phenomenon is called long-branch attraction (Felsenstein 1978). Model-based phylogenetic reconstruction methods are less sensitive—but not immune—to long-branch attraction (e.g., Gaut and Lewis 1995; Huelsenbeck 1995). Long-branch attraction has been shown to prevent accurate phylogenetic reconstruction of Dioscoreales using parsimony analyses of nuclear and mitochondrial data due to the excessively increased substitution rates in the mycoheterotrophic lineages (Merckx et al. 2009; Fig. 5.1). To minimize potential effects of long-branch attraction in phylogenetic inference of mycoheterotrophic plant lineages, it is suggested to (1) sequence DNA regions that are less prone to rate heterogeneity (nuclear and mitochondrial DNA regions may be better candidates than chloroplast regions), (2) maximize taxon sampling so that long phylogenetic branches are "broken up," and (3) use different phylogenetic reconstruction methods, for example, results obtained with parsimony methods should be compared to results of model-based methods (maximum likelihood and Bayesian methods). When long-branch attraction is suspected to have influenced the results, its influence can be assessed by repeating the analysis without particular long branches (see Bergsten 2005) or by performing topology tests, such as the Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999) or the Swofford–Olsen–Waddell–Hillis test (Swofford et al. 1996).

Despite these drawbacks, the phylogenetic position of many mycoheterotrophic groups has been successfully inferred using DNA data, sometimes with surprising results, which in turn

urged a reevaluation of morphological characters. For example, the mycoheterotrophic family Triuridaceae was previously considered closely related to the alismatid families (Maas-van de Kamer 1995; Maas-van de Kamer and Weustenfeld 1998), but phylogenetic hypotheses based on molecular data surprisingly placed them in Pandanales (Rudall and Bateman 2006).

5.3 Evolution Toward Mycoheterotrophy

5.3.1 Origins of Mycoheterotrophy

Precise information about the closest relatives of some mycoheterotrophic plant lineages is still lacking. Nevertheless, all available evidence suggests that plants capable of mycoheterotrophy evolved from autotrophic, mycorrhizal ancestors. Indeed, taxonomic and phylogenetic evidence suggests that full mycoheterotrophs living on arbuscular mycorrhizal fungi are most closely related to chlorophyllous arbuscular mycorrhizal plants. For example, mycoheterotrophic species in the genera *Voyria*, *Voyriella*, *Exacum*, and *Exochaenium*, as well as the putative partially mycoheterotrophic species in *Obolaria* and *Bartonia* are all part of Gentianaceae, of which the majority of the species are autotrophic and form arbuscular mycorrhiza (Struwe and Albert 2002; Yuan et al. 2003; Wang and Qiu 2006; Matthews et al. 2009; Cameron and Bolin 2010). Other arbuscular mycorrhizal mycoheterotrophs are imbedded in, or most closely related to, clades of arbuscular mycorrhizal autotrophs as well, for example, Burmanniaceae (Merckx et al. 2006), Thismiaceae (Merckx et al. 2008), *Geosiris* (Goldblatt et al. 2008), and *Petrosavia* (Cameron et al. 2003).

Similar observations have been made for plants that perform mycoheterotrophic interactions through ectomycorrhizal fungi. Phylogenetic evidence shows that an ectomycorrhizal fungal association capable of simultaneously infecting the roots of neighboring plants was probably in place in the common ancestor of the mycoheterotroph liverwort *Aneura mirabilis* and its most closely related species (Kottke and Nebel 2005; Wickett and Goffinet 2008). In Ericaceae, the phylogenetic relationships remain unclear, but it is safe to infer that all monotropoid mycoheterotrophs evolved from photosynthetic plants that formed ectomycorrhizas (Bidartondo 2005). Similarly, in Pyroleae, phylogenetic hypotheses suggest the full mycoheterotroph *Pyrola aphylla* evolved from autotrophic ancestors (Liu et al. 2010).

Also in Orchidaceae, there is ample evidence that fully mycoheterotrophic species are embedded in clades of green orchids (e.g., Molvray et al. 2000). It is well known that orchids form mycorrhizas. However, while an association with saprotrophic fungi is probably the ancestral state for Orchidaceae (Yukawa et al. 2009), many fully mycoheterotrophic species of orchids associate with ectomycorrhizal fungi (Chap. 7). Some evidence suggests that the establishment of a symbiosis with ectomycorrhizal fungi in these lineages served as preadaptation for the evolution of the full mycoheterotrophy (Motomura et al. 2010; see further).

Thus ultimately the evolutionary start point of mycoheterotrophy is mycorrhizal autotrophy, that is, an interaction in which a photosynthetic plant has a mutualistic association with mycorrhizal fungi. An evolutionary breakdown then leads to a shift from this mutualistic plant–fungus association to a situation in which the plant exploits its mycorrhizal fungi.

5.3.2 Evolutionary Stability of the Mycorrhizal Mutualism

In general, the breakdown of mutualisms is expected because they are theoretically unstable (Axelrod and Hamilton 1981; Bull and Rice 1991; Sachs et al. 2004). Given the selfish interest of individuals, why expend resources to benefit another species when resources could be redirected for one's own fitness (Kiers and van der Heijden 2006)? However, mutualisms are ubiquitous in nature, and many mutualisms have an ancient origin, which suggest that they are evolutionary stable. A prime example is the arbuscular mycorrhizal mutualism, which encompasses more

than 80% of all land plants and is believed to have played a major role in the invasion of the land by plants (Smith and Read 2008). About 3% of seed plant species have mutualistic interactions with ectomycorrhizal fungi, but while this number is relatively small, the global importance of ectomycorrhizal plants is greatly increased by their disproportionate occupancy of terrestrial habitats. Furthermore, ectomycorrhizal plant–fungus associations have a long history as well, which demonstrates its evolutionary stability (Smith and Read 2008; Hibbett and Matheny 2009).

In one-to-one plant–fungal symbiont interactions, fungal symbionts will increase their own fitness by helping plants grow, and vice versa. However, mycorrhizas are diffuse symbioses because a mycorrhizal plant typically associates simultaneously with multiple fungi and a mycorrhizal fungus often associates simultaneously with multiple plants (Giovannetti et al. 2004; Lian et al. 2006). This can select for parasitism by exploitation of the benefits provided by others while avoiding the costs of supplying resources. Plants may be able to "enforce" cooperation of mycorrhizal fungi, as has been observed in plant–pollinator (Pellmyr and Huth 1994; Goto et al. 2010; Jandér and Herre 2010) and legume–rhizobium interactions (Kiers et al. 2003, 2006; Simms et al. 2006). However, in these systems, sanction mechanisms rely on a single host interacting with multiple partners. In contrast, the mycorrhizal symbiosis involves interactions with multiple fungi and multiple host plants, and it is not clear whether sanctions could operate in the same way (Kiers et al. 2011).

In the arbuscular mycorrhizal mutualism, in vitro experiments have shown that both plants and AM fungi are able to detect variation in the resources supplied by their partners, allowing them to adjust their own resource allocation accordingly (Bever et al. 2009; Kiers et al. 2011). The system therefore seems to represents a "biological market," in which reciprocal rewards stabilize cooperation (Schwartz and Hoeksema 1998; Kiers and van der Heijden 2006; Kiers et al. 2011). In addition, spatial structure of AM fungi within the plant root system may enhance the stability of the AM mutualism, as less beneficial AM fungi proliferate in spatially well-mixed environments (Bever et al. 2009).

5.3.3 Breakdown of the Mycorrhizal Symbiosis

Given the high costs for both partners in the mycorrhizal mutualism, the diffuse character of the partnership and the inability to assess the costs of particular associations at the time of the initial infection, a breakdown of the mycorrhizal symbiosis seems inevitable (Bruns et al. 2002; Kiers and van der Heijden 2006). Theoretically breakdown of mutualisms leads to extinction, abandonment, or exploitation (Sachs and Simms 2006). There are no straightforward examples of extinction in the mycorrhizal symbiosis. Evidence of mycorrhizal mutualists reverting to autonomy appears in the phylogenetic records of plants, while whether or not such reversals occurred in fungi is still debated. Multiple lineages of angiosperm plants, presumed to have arbuscular mycorrhizal ancestors, have switched to a non-mycorrhizal condition (e.g., species of the families Brassicaceae, Caryophyllaceae, Proteaceae, and Cyperaceae; Smith and Read 2008). In fungi, early phylogenetic hypotheses suggested that diverse lineages of free-living (saprotrophic) fungi were nested within ancestrally ectomycorrhizal clades (Hibbett et al. 2000). Hibbett et al. (2000) used parsimony reconstructions of trophic traits and tested internal node reconstructions with a likelihood model for evolution of the mycorrhizal trait. The two models the authors compared were a nonreversible model (only gain of ectomycorrhizal habit, no loss) and a model in which gains and losses were equally probable. The nonreversible model was significantly worse, and the authors estimated that nine losses of the ectomycorrhizal habit have occurred in the lineages sampled. However, the same original dataset was reanalyzed by Bruns and Shefferson (2004), and they found that all except one reversals were converted to gains when assuming that acquiring ectomycorrhizal habit was twice as probable as losing it. Indeed, while still arbitrary, assuming similar probabilities of the different switches may be biologically more justifiable, given that reverting to free-living lifestyle requires the activation of numerous metabolic pathways involved in obtaining carbon and may be, therefore, more difficult to achieve. The remaining one

reversal might have been caused by questionable state assignment for the ambiguous taxon involved (*Lentaria byssoides*) and/or may be an artifact of limited taxon sampling and too little genetic variation in the gomphoid lineage the taxon belongs to. Free-living Glomeromycota fungi are not known.

Breakdown of a mutualism into exploitation occurs when individuals obtain commodities offered by mutualists but that provide fewer commodities in return or even, in the case of pure cheaters, no commodities at all (Ferrière et al. 2007). While it remains to be demonstrated that mycoheterotrophic plants provide no commodities to their associated fungi, they seem to qualify as the result of an evolutionary breakdown of the mycorrhizal mutualism. An important aspect of this evolutionary trajectory is the ability of a mycorrhizal fungus to associate simultaneously with multiple host plants. In general, multi-partner interactions appear to help maintain newly evolved exploiters (Sachs and Simms 2006). For example, in the fig–fig wasp symbiosis, all non-pollinating wasps coexist with pollinating species, and coexisting pairs on a host tree species are not sister taxa (Pellmyr and Huth 1994; Pellmyr et al. 1996; Machado et al. 2001). In the mycorrhizal symbiosis, multi-partner interactions are of vital importance for the evolution of mycoheterotrophs. Since a mycorrhizal fungus needs an association with an autotrophic plant to obtain carbohydrates, an exclusive one-to-one interaction between a mycorrhizal fungus and a fully mycoheterotrophic plant cannot exist. An important exception to this multipartite obligation for the maintenance of mycoheterotrophic interactions is mycoheterotrophic orchids that associate with saprotrophic fungi. Although in this case, the persistence of the system relies on ability of the fungus to access dead or decaying organic matter.

5.3.4 Intermediate Evolutionary Steps Between Autotrophy and Full Mycoheterotrophy

Some groups of fully mycoheterotrophic plants consist of evolutionary isolated lineages that diverged from their most closely related extant autotrophic relatives tens of million years ago. In these cases, the large evolutionary gap between autotrophic and fully mycoheterotrophic species prevents us to study the putative transitional steps that occurred in the evolution toward full mycoheterotrophy. Examples are the families Thismiaceae and Triuridaceae that exclusively contain fully mycoheterotrophic species and of which no closely related autotrophic or partially mycoheterotrophic relatives are known. Other fully mycoheterotrophic species, particularly in Orchidaceae, do have closely related green relatives. The fact that some of these green relatives are able to obtain carbon from root-associated fungi as well (partial mycoheterotrophy) provides an interesting evolutionary perspective on the shift to full mycoheterotrophy. In particular, Motomura et al. (2010) reported that full mycoheterotrophic species of *Cymbidium* probably evolved from partially mycoheterotrophic ancestors, which suggests that in this case, full mycoheterotrophy evolved gradually rather than through a direct shift from autotrophy to full mycoheterotrophy. Partial mycoheterotrophs with close relationships to full mycoheterotrophs also exist in other orchid genera, such as *Cephalanthera* (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006) and *Corallorhiza* (Zimmer et al. 2008; Cameron et al. 2009). In addition, initial mycoheterotrophy is ubiquitous among orchid species (Bernard 1899; Rasmussen 1995), and thus, it is likely that partially mycoheterotrophic orchids originated from initially mycoheterotrophic ancestors. A scenario for the evolution toward full mycoheterotrophy in Orchidaceae therefore seems to include shifts from initial mycoheterotrophy to partial mycoheterotrophy and from partial mycoheterotrophy to full mycoheterotrophy (Fig. 5.2). Whether this is a universal model for the evolution of full mycoheterotrophy in plants remains unclear. Initial and partial mycoheterotrophic ancestors of extant full mycoheterotrophic species in other families may have disappeared over the course of evolution. A similar pattern in the evolution of mycoheterotrophy seems to be present in Pyroleae (Ericaceae). Species of Pyroleae produce dust seeds, and some evidence suggests that germination and early development depends on fungal nutrition (Leake

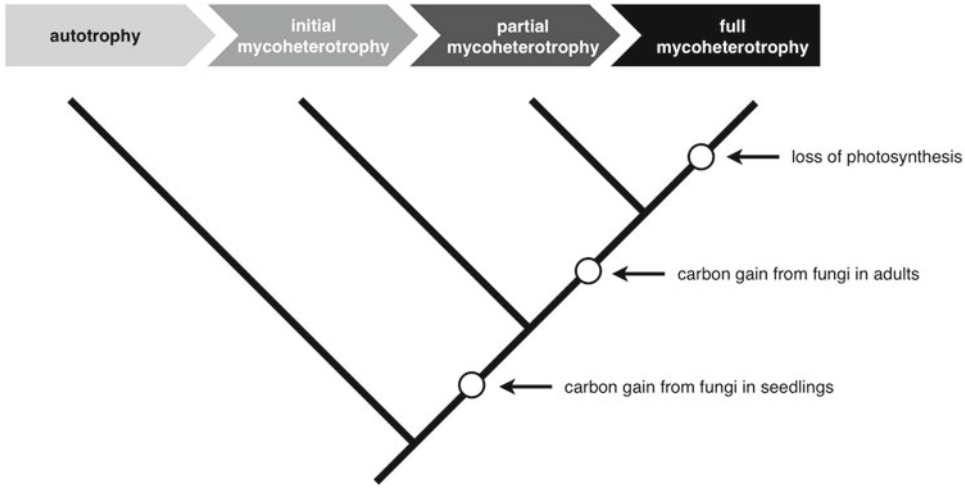

Fig. 5.2 Model for the evolution of mycoheterotrophy in orchids. Key innovations are indicated on the tree

1994; Smith and Read 2008; Eriksson and Kainulainen 2011). Moreover, in *Pyrola,* partial mycoheterotrophy has been detected in the relatives of the full mycoheterotroph *Pyrola aphylla* (Tedersoo et al. 2007; Zimmer et al. 2007; Hynson et al. 2009), which indicates that partial mycoheterotrophy preceded full mycoheterotrophy in the evolution of *Pyrola*. Some evidence suggests that partial mycoheterotrophy occurs in arbuscular mycorrhizal Gentianaceae as well, although not in lineages closely related to full mycoheterotrophs (Cameron and Bolin 2010). In general, autotrophic Gentianaceae are known to be difficult to germinate under laboratory conditions. This has been attributed to specific abiotic requirements (Bouman et al. 2002), but it is also possible that a symbiotic interaction with a mycorrhizal fungus is necessary for seedling development (Ramsbottom 1922). These observations show that Gentianaceae deserve further interest as they may include undiscovered initial and partial mycoheterotrophs.

For some partially mycoheterotrophic orchid species, non-chlorophyllous phenotypes are known. From an evolutionary point of view, these "albino" variants offer a fascinating model for the shift from partial to full mycoheterotrophy. Achlorophyllous specimens have been reported for *Epipactis microphylla* (Selosse et al. 2004), *E. helleborine* (Salmia 1989), *Cephalanthera longifolia* (Abadie et al. 2006), and *C. damasonium* (Julou et al. 2005). Stable isotope analyses indicate that they are fully mycoheterotrophic and thus obtain all of their carbon from associated fungi. They seem to have lost the ability to perform photosynthesis and respire less than chlorophyllous individuals (Julou et al. 2005). In some cases, forms with variegated leaves are known, demonstrating that there is a continuum of leaf chlorophyll concentrations between green and albino individuals. For *Cephalanthera damasonium,* there is a linear relationship between leaf chlorophyll concentrations and the proportional reliance on fungi as a carbon source (Stöckel et al. 2011). While these non-chlorophyllous individuals are able to persist for multiple years, they are extremely rare and produce less seeds than their chlorophyllous phenotypes. Thus, they seem to have a reduced fitness, and consequently, they are sometimes regarded to represent an evolutionary stage of a failed transition from partial mycoheterotrophy to full mycoheterotrophy (Selosse and Roy 2009; see also Chap. 8).

5.3.5 Evolutionary Drivers of Mycoheterotrophy

What drives plants toward mycoheterotrophy? This is one of the most compelling questions about

mycoheterotrophy, and while yet unanswered, some data allow us to at least speculate about the evolutionary drivers behind the shift to a mycoheterotrophic mode of life.

In orchids and probably also in Pyroleae, the occurrence of initial mycoheterotrophy seems to be the first step in the evolutionary trajectory toward full mycoheterotrophy. Dependence on fungal carbon for germination and early development reduces the requirement of maternal resources. This means that smaller seeds can be produced at lower cost and thus in larger numbers. Indeed, initially mycoheterotrophic orchids and Pyroleae, as well as most fully mycoheterotrophic plant species in general, produce large amounts of very small, dust-like seeds (Eriksson and Kainulainen 2011). Having many seeds promotes dispersal and increases the likelihood of reaching microsites with suitable host fungi. From an evolutionary point of view, this involves a paradox: seeds are small because they have to be so numerous to successfully reach suitable hosts, yet they can only be so small because they completely depend on their hosts for successful establishment. It is unlikely that extremely small seeds evolved before a dependency on mycorrhizal fungi because that would make an impossible way of living. But on the other hand, evolution of a high dependency on (a narrow range of) host fungi before reduction of seed size and increase of seed number is equally unlikely. Thus, both trends of increased host dependence and reduction of seed size must have evolved gradually, reciprocally reinforcing each other (Eriksson and Kainulainen 2011). Moreover, at least in some cases, increased dependence on host fungi seems to be coupled with an increased specialization toward narrow ranges of fungi which further increases the need for maximal seed dispersal capabilities.

Thus, reduction of seed size and fungal dependency are tightly coupled, and neither trend seems to be the evolutionary driver of the other. Eriksson and Kainulainen (2011) argue that evolution of dust seeds in mycoheterotrophic flowering plant lineages coincided with the development of closed-canopy forests. Initial mycoheterotrophy would thus allow plants to establish in forest understory habitats where there is strong completion for light. Indeed, several authors have argued that mycoheterotrophy evolved as an adaption to living in deep shade (e.g., Leake 1994; Bidartondo 2005). Although initial mycoheterotrophy may also help plants to establish in nitrogen-limited habitats (Read and Perrez-Moreno 2003) or serve as an escape from water stress. Initial mycoheterotrophic Lycopodiaceae gametophytes, for example, sexually reproduce undercover in moist soil and litter environments that provide protection from desiccation (Leake et al. 2008). Thus, several factors may contribute to the evolution of initial mycoheterotrophy in plants.

As mentioned above, partial and full mycoheterotrophy is often seen as an adaptation to low-light environments. This seems evident since nearly all fully mycoheterotrophic plants grow in the dark understory of primary forests, while partially mycoheterotrophic plants are often found in more open forests, where irradiance is higher (Selosse and Roy 2009). Indeed, studies using stable isotope natural abundances suggest that light availability plays a major role in the degree of mycoheterotrophy at least in a few partially mycoheterotrophic orchids and Pyroleae (Zimmer et al. 2007; Liebel et al. 2010; Preiss et al. 2010; Hynson et al. 2012; see also Chap. 8). However, differences in ^{13}C abundances of partially mycoheterotrophic populations growing under different environmental conditions cannot always be explained by differences in irradiance only (e.g., Zimmer et al. 2007; Hynson et al. 2012). Thus, in these cases, light availability is not the only driver of the heterotrophy level. It has been suggested that C gain in partially mycoheterotrophic plants may in some cases arise as a "side-product" of N and P gain from mycorrhizal fungi (Selosse et al. 2009; Chap. 8). Thus, the evolution toward increasing levels of mycoheterotrophy may be driven by multiple factors. However, since fully mycoheterotrophic plants successfully abandoned photosynthesis *and* mostly occur in habitats where light availability is extremely low, it can be hypothesized that light availability is one of the most important drivers behind the evolution of mycoheterotrophy.

5.4 Phylogenetic Aspects

5.4.1 Number of Origins

Our increasing knowledge about the phylogenetic relationships of fully mycoheterotrophic plants allows us to estimate the number of origins of a fully mycoheterotrophic mode of life in land plants. Hereby it is essential to assume that reversals to partial mycoheterotrophy or autotrophy are impossible. There is no direct evidence that supports this assumption. However, the relaxed evolutionary constraints on photosynthesis genes of fully mycoheterotrophic plants leads to a profound increase of substitution rates, frameshift mutations, and even the loss of complete genes (Barrett and Freudenstein 2008; Wickett et al. 2008a, 2008b; Delannoy et al. 2011). Therefore, it seems highly unlikely that the reduced plastid genomes of fully mycoheterotrophic plants are able to regain their essential role in functional photosynthesis.

By adding up estimates of the number of independent origins of full mycoheterotrophy in distinct clades of plants, we find evidence of at least 47 origins of full mycoheterotrophy in land plants (or 46 if *Parasitaxus* is not classified as a mycoheterotroph) (Table 5.1). This number is probably an underestimation due to the uncertain phylogenetic position of several mycoheterotrophic species and genera in Burmanniaceae, Orchidoideae, and Epidendroideae. Thus, full mycoheterotrophy has likely more than four times the number of evolutionary origins compared with haustoria-forming holoparasitic plants. The number of evolutionary origins of mycoheterotrophy *sensu lato*—that is, the ability to obtain carbon from mycorrhizal fungi—is probably lower. There may be only a single origin of initial mycoheterotrophy in Orchidaceae and Ericaceae, and this is perhaps also the case for Burmanniaceae and Gentianaceae. On the other hand, initial and partial mycoheterotrophy may remain to be detected in plant groups that do not include fully mycoheterotrophic species. Undiscovered initial mycoheterotrophy may be present in diverse plant families with species that produce dust seeds, such as Rubiaceae, Buddlejaceae, and Gesneriaceae (Eriksson and Kainulainen 2011), and partial mycoheterotrophy may occur in plant families that are adapted to grow in the low-light conditions of forest understories (Selosse and Roy 2009). Also, mycoheterotrophy evolved independently in lycophytes and ferns, of which several lineages have putative fully mycoheterotrophic gametophytes.

5.4.2 Diversity

Mycoheterotrophy occurs in almost every major lineage of land plants (Fig. 5.3). Mycoheterotrophic interactions are unknown in the hornworts and in the mosses. The latter may be explained by the putative nonmycorrhizal status of mosses (Smith and Read 2008). Here we provide a short overview of mycoheterotrophic interactions in major lineages of land plants. See Chap. 2 for a detailed overview.

5.4.2.1 Liverworts

In liverworts, which comprises ca. 5,000 species, at least one species (*Aneura mirabilis*) is fully mycoheterotrophic (Bidartondo et al. 2003). Another closely related species awaits investigation (Crum and Bruce 1996). *A. mirabilis* is specialized on *Tulasnella* species that form ectomycorrhizas with surrounding trees (Bidartondo et al. 2003). Other Aneuraceae species associate nearly exclusively with *Tulasnella* species as well (Bidartondo and Duckett 2010). This interaction is unique because Aneuraceae are the only thalloid group with basidiomycetous mycobionts and the only liverwort group known to have *Tulasnella* symbionts (Preussing et al. 2010).

5.4.2.2 Lycophytes

Lycophytes comprise ca. 1,200 species in three families: Lycopodiaceae, Isoëtaceae, and Selaginellaceae (Christenhusz et al. 2011). Several of the ca. 300 species of Lycopodiaceae have achlorophyllous gametophytes that are presumably mycoheterotrophic on arbuscular mycorrhizal fungi (Winther and Friedman 2008; Chap. 2).

5.4.2.3 Ferns

The gametophytes of several fern species in the families Ophioglossaceae, Psilotaceae,

Table 5.1 Overview of independent occurrence of full mycoheterotrophy in land plants

Clade	Number of origins of full mycoheterotrophy	Associated fungi (see Chap. 6)	References
Aneuraceae	1[a]	Basidiomycota (*Tulasnella*)	Wickett and Goffinet (2008)
Podocarpaceae	1[b]	Unknown	Sinclair et al. (2002); Feild and Bodribb (2005)
Petrosaviaceae	1	Glomeromycota	Cameron et al. (2003)
Triuridaceae	1	Glomeromycota	Rudall and Bateman (2006)
Burmanniaceae	≥8[c]	Glomeromycota	Merckx et al. (2008)
Thismiaceae	1–2[d]	Glomeromycota	Merckx and Bidartondo (2008)
Corsiaceae	1[e]	Glomeromycota	Neyland and Hennigan (2003); Davis et al. (2004)
Iridaceae	1[f]	Glomeromycota	Goldblatt et al. (2008)
Orchidaceae—Vanilloideae	2–3[g]	Ascomycota and Basidiomycota	Cameron (2009)
Orchidaceae—Orchidoideae	≥9[h]	Basidiomycota	See Chap. 2
Orchidaceae—Epidendroideae	≥14[i]	Ascomycota and Basidiomycota	Freudenstein (1994); Chase et al. (2003); Freudenstein et al. (2004); Rothacker (2007), Roy et al. (2009); Pedersen et al. (2009)
Polygalaceae	1[j]	Glomeromycota	Eriksen (1993)
Ericaceae	2–3[k]	Ascomycota and Basidiomycota	Kron (1996); Bidartondo and Bruns (2002)
Gentianaceae	4[l]	Glomeromycota	Albert and Struwe (1997); Struwe and Albert (2002); Yuan et al. (2003); Kissling et al. (2009)

[a]*Aneura mirabilis* is the only fully mycoheterotrophic species in this family

[b]*Parasitaxus usta* is the only fully heterotrophic species in this family, but it is debatable whether it is a true mycoheterotroph

[c]The high number of independent origins of full mycoheterotrophy in this family is due to the polyphyletic status of chlorophyllous *Burmannia* species

[d]It is unclear whether Thismiaceae are monophyletic. A paraphyletic status for the family would mean two separate origins of full mycoheterotrophy

[e]Corsiaceae may not be monophyletic as suggested by Neyland and Hennigan (2003). In that case, more than a single origin of full mycoheterotrophy might be needed to explain the evolution of full mycoheterotrophy

[f]*Geosiris*, which includes two closely related species, is the only fully mycoheterotrophic genus in Iridaceae

[g]Phylogenetic evidence indicates that full mycoheterotrophy evolved independently in *Lecanorchis* and the common ancestor of the *Erythrorchis–Pseudovanilla–Galeola–Cyrtosia* clade. If *Pseudovanilla* species are not considered full mycoheterotrophs, three origins are needed to explain the evolution of full mycoheterotrophy in Vanilloideae

[h]Full mycoheterotrophy probably evolved separately in the genera *Arthrochilus*, *Brachycorythis*, *Corybas*, *Cryptostylis*, *Cystorchis*, *Odontochilus*, *Platanthera*, and *Platythelys* because these genera also contain chlorophyllous species. Due to the uncertain phylogenetic position of *Burnettia*, *Chamaegastrodia*, *Danhatchia*, *Degranvillea*, and *Rhizanthella,* it remains unknown how many independent shifts they represent

[i]Due to taxonomy and phylogenetic placement probably independent origins of full mycoheterotrophy in *Neottia*, *Cephalanthera*, *Cremastra*, *Cymbidium*, *Corallorhiza*, *Stereosandra*, *Tropidia*, *Epipogium*, *Malaxis*, *Hexalectris*, *Eulophia*, *Yoania*, the common ancestor of *Aphyllorchis* and *Limodorum*, and Gastrodieae (including *Didymoplexiella*, *Didymoplexis*, *Gastrodia*, *Uleiorchis*, *Auxopus*, *Neoclementia*). Due to the uncertain phylogenetic position of *Kalimantanorchis*, *Pogoniopsis*, *Risleya*, *Silvorchis*, and *Stereosandra,* it remains unclear how many independent shifts they represent

[j]Probably a single origin of full mycoheterotrophy in the genus *Epirixanthes*

[k]Independent origins in the tribes Pyroleae, Monotropeae, and Pterosporeae, although a single origin in the ancestor of the two latter tribes is also possible

[l]Independent origins for *Voyria*, *Voyriella*, and mycoheterotrophic *Exochaenium* and *Exacum* species

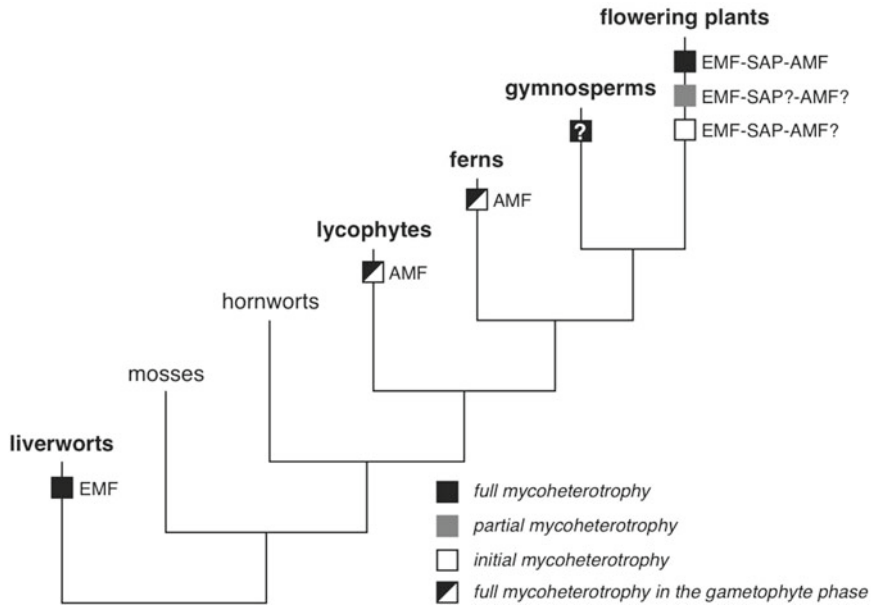

Fig. 5.3 Mycoheterotrophic interactions in land plants. Phylogenetic relationships of land plants based on Palmer et al. (2004) and Forrest et al. (2006). Occurrence of different types of mycoheterotrophy is plotted on the tree with symbols shown in the legend. The identity of fungi involved is indicated. *AMF* arbuscular mycorrhizal fungi; *EMF* ectomycorrhizal fungi; *SAP* saprotrophic fungi. *Question marks* indicate putative interactions (Based on data obtained from Chap. 2)

Gleicheniaceae, and Schizeaceae are achlorophyllous and presumably mycoheterotrophic on arbuscular mycorrhizal fungi (Chap. 2). Further study on mycoheterotrophy of achlorophyllous gametophytes of ferns is needed.

5.4.2.4 Gymnosperms

The species *Parasitaxus usta* (Podocarpaceae) sprouts from roots and trunks of another podocarp, *Falcatifolium taxoides*. Fungi are thought to play an important role in this unique interaction, and therefore, the plant may be a mycoheterotroph rather than a holoparasite. However, the identity and the exact role of the fungi involved in this interaction remain undetermined.

5.4.2.5 Angiosperms

There are 10 angiosperm families with fully mycoheterotrophic species, and full mycoheterotrophy probably evolved at least 45 times independently within flowering plants (Table 5.1). In terms of number of species, most fully mycoheterotrophic angiosperms are monocots (ca. 468 species), while full mycoheterotrophy occurs in only 47 species of eudicots (Fig. 5.4). In contrast, haustorial parasitism has 11 independent origins in flowering plants and is limited to ca. 390 species of eudicots (Barkman et al. 2007; Heide-Jørgensen 2008).

In ca. 236 species, full mycoheterotrophy is supported by exploitation of arbuscular mycorrhizal fungi (Glomeromycota). All full mycoheterotrophic species in Ericaceae, and most in Orchidaceae, exploit ectomycorrhizal fungi. Some tropical fully mycoheterotrophic orchids rely on an association with saprotrophic fungi. However, for many species of full mycoheterotrophs, in particular, tropical species of Orchidaceae, the identity of their fungal associates remains to be investigated. Partial mycoheterotrophy has been detected in Orchidaceae and Ericaceae. In most cases, it seems to occur through ectomycorrhizal fungi, although some orchids may receive limited amounts of carbon through saprotrophic fungi (see Chap. 8). Putative partial mycoheterotrophy through arbuscular mycorrhizal fungi has been reported in Gentianaceae (Cameron and Bolin 2010). All three families also contain fully mycoheterotrophic species. Many putatively partially mycoheterotrophic taxa, also in

Fig. 5.4 Species diversity of full mycoheterotrophy in angiosperms. Phylogenetic relationships are based on APG (2009). Orders with fully mycoheterotrophic species are highlighted in *bold*. The families to which these species belong are written after the dash. The *gray bars* represent the number of fully mycoheterotrophic species in each order. Species numbers were derived from Chap. 2

other families, remain to be investigated. Chlorophyllous species that are closely related to fully mycoheterotrophic species are prime candidates for undiscovered partial mycoheterotrophy. Similarly, initial mycoheterotrophy has only been recorded in Orchidaceae, mostly with saprotrophic fungi and more rarely with ectomycorrhizal fungi. However, initial mycoheterotrophy

may be present in other lineages that produce dust seeds (Eriksson and Kainulainen 2011).

5.4.3 Timing

Fossil evidence to trace the evolutionary history of mycoheterotrophy is lacking. There is only one series of fossils that might be assigned to an extant mycoheterotrophic lineage. These fossils are from the Upper Cretaceous (about 90 Ma) and show affinities with extant Triuridaceae (Gandolfo et al. 2002). However, it remains questionable whether these fossilized plants were in fact mycoheterotrophic (Gandolfo et al. 2002) and members of the Triuridaceae (Furness et al. 2002). Nevertheless, based on the taxonomic and phylogenetic data that is available, it is obvious that the fully mycoheterotrophic mode of life has evolved during various epochs of plant diversification. A relatively recent origin of full mycoheterotrophy can be inferred for species that are imbedded in genera that also contain autotrophic species. Examples include *Exacum* and *Exochaenium* in Gentianaceae (Yuan et al. 2003; Kissling et al. 2009) and *Cymbidium* in Orchidaceae (Motomura et al. 2010). For widespread, species-rich clades that contain only fully mycoheterotrophic species, it is likely that full mycoheterotrophy evolved in the common ancestor of these species and thus represents a much older origin of full mycoheterotrophy (e.g., Triuridaceae, Thismiaceae).

Due to the difficulties associated with molecular dating methods, in particular, the use of fossil calibration points, the absence of fossil data for mycoheterotrophic groups, the paucity of data, and the increased substitution rates of mycoheterotrophic clades, only very few mycoheterotrophic lineages have been dated using these methods. In Dioscoreales, fully mycoheterotrophic lineages are estimated to have diverged from as early as the Cretaceous to the Miocene (Merckx et al. 2010). The fully mycoheterotrophic genus *Wullschlaegelia* (Orchidaceae) has been estimated to have an Eocene (Ramirez et al. 2007) or Oligocene (Gustafsson et al. 2010) origin. Based on the results of Yuan et al. (2003), mycoheterotrophic *Exacum* species (Gentianaceae) diverged not before the Miocene. However, a divergence time estimate of the origin of a fully mycoheterotrophic clade ("stem node" age) is not an accurate estimate for the origin of mycoheterotrophy, since it has to be assumed that mycoheterotrophy could evolve between the origin of the mycoheterotrophic clade and the beginning of the diversification of the clade ("crown node") (Fig. 5.5a). This assumption considerably broadens age estimates for the evolution of mycoheterotrophy. But even when crown node ages are considered as upper boundaries for the evolution of mycoheterotrophy, some shifts from autotrophy to mycoheterotrophy must have occurred several tens of million years ago. The diversification of Thismiaceae, for example, probably started during the Eocene; therefore, a shift to mycoheterotrophy in the ancestor of this clade has occurred at least around that time.

In some clades, full mycoheterotrophs may be preceded by partial mycoheterotrophs or initial mycoheterotrophs. In these cases, the first occurrence of mycoheterotrophy predates the origin of fully mycoheterotrophic species (Fig. 5.5b). For example, under the assumption that most orchids are initial mycoheterotrophs, the origin of mycoheterotrophy in Orchidaceae may be traced back to the common ancestor of all Orchidaceae species. As a result, the evolution of mycoheterotrophy in Orchidaceae may have started during the Cretaceous (Ramirez et al. 2007). Despite the difficulties to accurately estimate the timing of the evolution of mycoheterotrophy, the examples above indicate that for at least some groups of mycoheterotrophs, the origin of mycoheterotrophy is relatively ancient. This demonstrates that mycoheterotrophic lineages were able to emerge, persist, and diverge over a considerable amount of time and thus are remarkably evolutionarily stable.

5.4.4 Diversification

Clades of fully mycoheterotrophic plants generally contain only few species. In some cases, this may reflect their relatively recent origin. For example, the mycoheterotrophic species in the

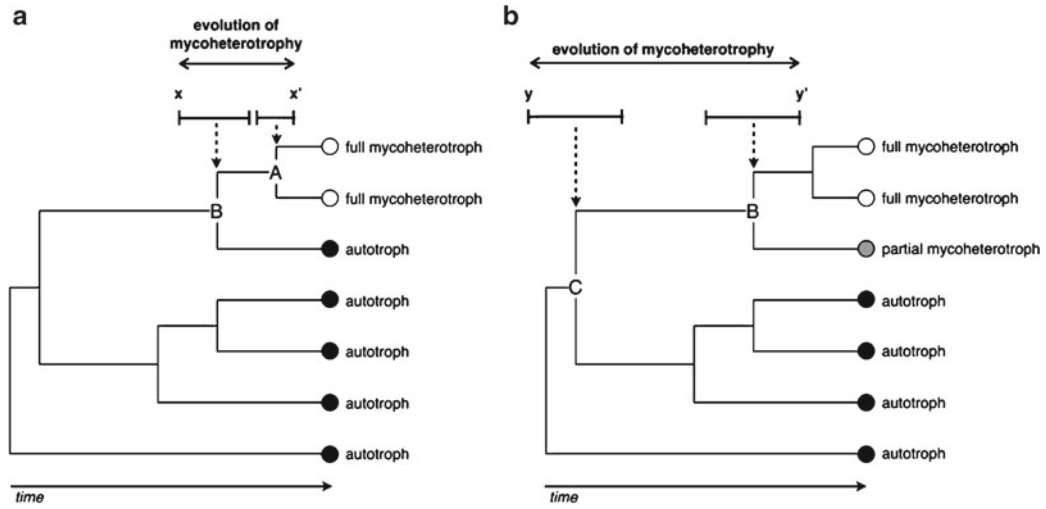

Fig. 5.5 Estimating the age of the origin of mycoheterotrophy using molecular clock methods. (**a**) When a monophyletic clade of fully mycoheterotrophic species is assessed, it can be assumed that full mycoheterotrophy has evolved somewhere between the node where this mycoheterotrophic clade branched from its autotrophic ancestors ("B") and the node where diversification started ("A"). Age estimates of these nodes will encompass 95% confidence intervals. When these are taken into account, evolution of mycoheterotrophy occurred between time x and x'. (**b**) Similarly in this example, with a clade of partial and full mycoheterotrophs, it can be derived that mycoheterotrophy evolved between time y and y'. Accurate taxon sampling is essential in this context. Failure to include extant lineages (particularly earliest diverging mycoheterotrophic species and most closely related autotrophs) will influence the upper and lower boundaries of the age estimates

genera *Exacum* and *Exochaenium* (Gentianaceae) and several Orchidaceae genera may have diverged in relatively recent times (see above). However, some early diverging lineages of full mycoheterotrophic plants (e.g., *Geosiris*, *Petrosavia*) are species poor as well. Relatively speciose lineages include Thismiaceae (ca. 65 species) and Gastrodieae (ca. 47 species), but even in these clades, species numbers are not particularly high. This suggests that diversification rates in lineages of fully mycoheterotrophic plants are low, extinction rates are high, or a combination of both. Diversification through time has only been studied in detail for Burmanniaceae, for which it has been suggested that the diversification and radiation of the pantropical genera *Burmannia* and *Gymnosiphon* significantly increased during the Eocene (Merckx et al. 2008; Fig. 5.6). The beginning of the Eocene was characterized by high global temperatures, which allowed tropical rain forests to expand and reach high levels of diversity (Jaramillo 2002; Jaramillo et al. 2006). Dense closed-canopy rainforest is the prime habitat for mycoheterotrophic species of Burmanniaceae. The increase in rainforest area may have resulted in an increase in mycoheterotrophic plant diversity in tropical regions during the Eocene. Larger regions can support more species, which enhance both regional and local diversity by reducing the risk of extinction and increasing niche opportunities (Leigh et al. 2004; Linder 2008). Subsequent cooling during the end of the Eocene caused a global retraction of rainforest and a decrease in plant diversity (Jaramillo et al. 2006), perhaps leading to a decrease in mycoheterotrophic plant diversity as well. It remains to be investigated whether this also influenced the diversity of other tropical mycoheterotrophic groups such as Triuridaceae and Thismiaceae, which were likely to be present in rainforest during that period as well (Gandolfo et al. 2002; Merckx et al. 2010). In Orchidaceae, major diversification is also estimated to have occurred during the early Eocene (Ramirez et al. 2007), although dates suggested by Gustafsson et al. (2010) place orchid diversification during the cooler period at the end of the Eocene and into the Oligocene.

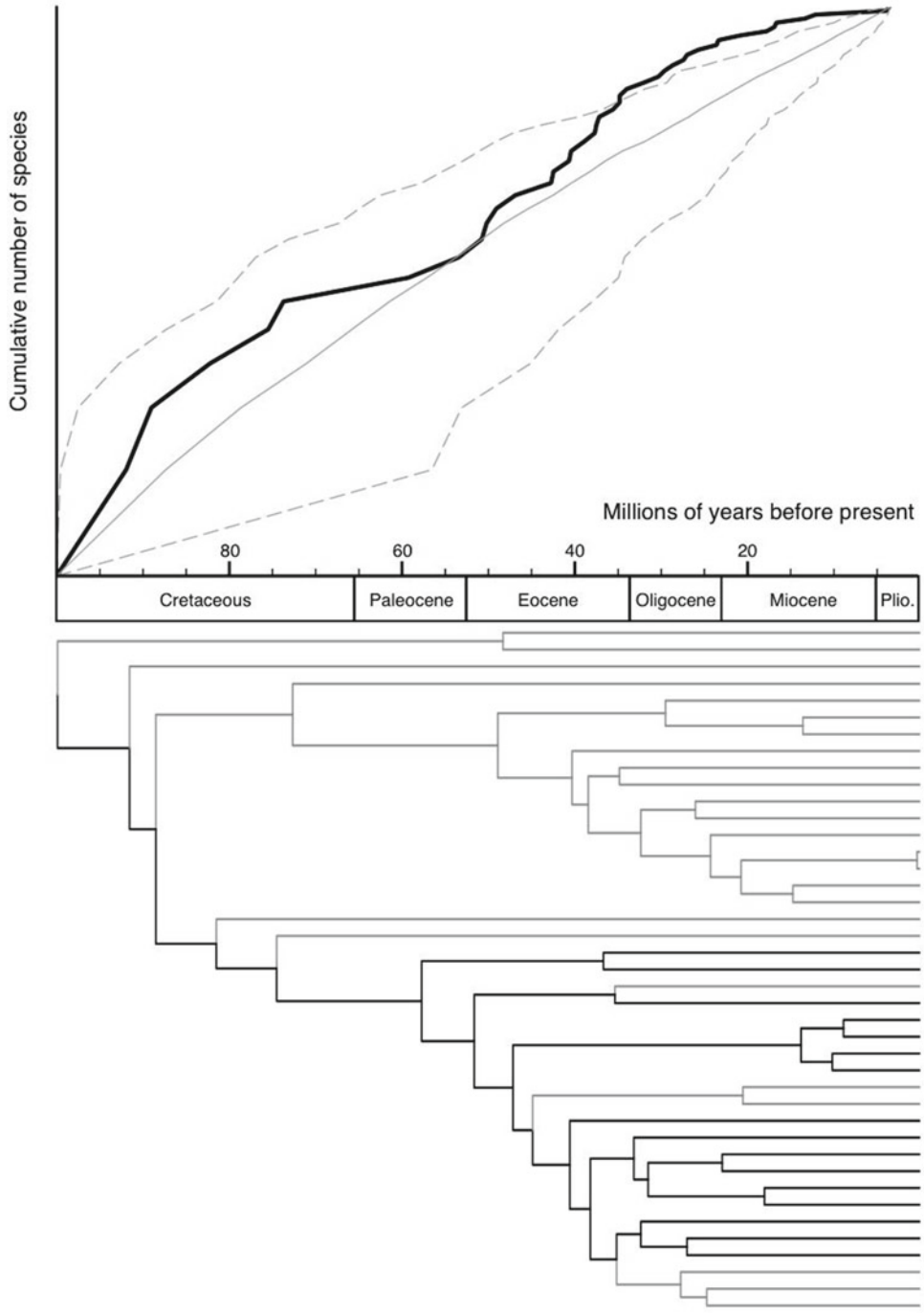

Fig. 5.6 An example of species diversification through time estimated from a molecular phylogeny. Semilogarithmic lineage-through-time (LTT) plot of Burmanniaceae (*black*) calculated from the chronogram shown below. A simulated LTT plot with 95% confidence intervals under a constant death–birth rate of 0.5 is shown in gray for comparison. *Gray* branches in the chronogram represent fully mycoheterotrophic species (Adapted from Merckx et al. (2008))

The species diversity of mycoheterotrophic lineages is not solely to be explained by ancient fluctuations in diversification rates. In some lineages, the occurrence of closely related species over a more or less continuous and restricted geographic range suggests that the diversity in these lineages is the result of a recent rapid diversification. The species in the *Hexalectris spicata* species complex, for example, are likely to have evolved only recently (Kennedy and Watson 2010; Kennedy et al. 2011). Other candidates for recent rapid diversification include the *Corsia* species in New Guinea, *Didymoplexiella* species on Borneo, and *Lecanorchis* species in Japan.

The fragmented but often widespread distribution patterns of many mycoheterotrophic lineages (Chap. 3) suggest that extinction may have played a significant role in the current distribution and diversity of mycoheterotrophic plants. The comparative low diversity of mycoheterotrophic plants in African rain forests, for example, is likely the result of the degrading effect of significant climatic changes during the Cenozoic, and more recently to Pleistocene climatic fluctuations, resulting in a drier climate throughout most of equatorial Africa (Chap. 3). However, the fossil record of mycoheterotrophic plant is virtually nonexistent and thus prevents the detection of extinction events in these lineages. Also, episodes of rapid extinction do not leave a clear signal in phylogenies based on extant species which makes it hard to estimate extinction rates based on a phylogenetic approach (Harvey et al. 1994; Purvis 2008; Tarver and Donoghue 2011).

5.5 Common Evolutionary Trends

5.5.1 Morphology

The shift from autotrophy to full mycoheterotrophy is accompanied by profound morphological changes, and despite the very diverse range of families and genera of mycoheterotrophic plants, these show some quite remarkable parallel evolutionary forms.

5.5.1.1 Subterranean Morphology

The subterranean parts of mycoheterotrophic plants show considerable trends of convergent evolution, consistent with a change in function from organs of absorption to organs of storage: (1) root hairs are mostly absent, (2) there is trend toward stout, clumped roots and rhizomes mostly with a specialized fungal colonization pattern, and (3) an increased width of the root cortex often accommodates mycorrhizal infection and stores of carbohydrates and other materials obtained from the fungal host (Leake 1994). See Chap. 4 for a detailed overview of root morphology of mycoheterotrophic plants.

5.5.1.2 Shoots

Many fully mycoheterotrophic species have exceptionally slender and threadlike stems, resulting in a hyaline appearance. Vascular tissues are often reduced to a single narrow cylinder of bicollateral bundles or, minimally, to four or six narrow bundles in the cortex. Most species lack secondary thickening, and their stems are succulent and brittle (e.g., *Rhizanthella*) or hyaline and slender (e.g., *Gymnosiphon*). Lignification is generally confined to a narrow ring of xylem vessels, or rarely only a few scalariform xylem vessels are present (e.g., *Voyria tenella*). Phloem is present in very small amounts and then mainly as parenchyma with sieve tubes frequently recorded as narrow and possibly with adherent sieve plates (Leake 1994).

5.5.1.3 Leaves

In fully mycoheterotrophic plants, leaves no longer serve a useful function. As a result, leaves are typically reduced to widely spaced achlorophyllous scales on the inflorescence axis. Occasionally, they are present only on underground rhizomes or tubers or even totally absent. The vascular supply to the leaf scales is often reduced to a single trace or may be absent. Stomata are generally absent as well, although some species retain vestigial stomata on their leaves and shoots (Leake 1994).

5.5.1.4 Seeds

An extreme reduction of seed size and seed complexity is one of the most common and significant

modifications seen in fully mycoheterotrophic plants. Most species of mycoheterotrophic plants have extremely small seeds, which are commonly described with the term "dust seeds." For example, some of the smallest seeds known are found in the genus *Voyria* in the Gentianaceae (Maas and Ruyters 1986) although the seeds of some orchids are equally reduced in size (Arditti and Ghani 2000). This reduction in seed size is coupled with a reduction of endosperm and a lack of differentiation of the embryo at maturity. Some parasitic plant families are also characterized by the occurrence of very small seeds (e.g., Orobanchaceae, Rafflesiaceae, Balanophoraceae, Hydnoraceae). And dust seeds are also present in a few angiosperm lineages that have not been associated with heterotrophic interactions, such as Rubiaceae, Buddlejaceae, and Gesneriaceae (Eriksson and Kainulainen 2011). The small dust seeds of mycoheterotrophic plants are often produced in very large numbers. A single capsule of the mycoheterotrophic orchid *Galeola altissima* contains about 18,000 seeds (Arditti and Ghani 2000). And *Pterospora andromedea* (Ericaceae) produces 2,000–4,800 seeds per capsule (Bakshi 1959). However, not all full mycoheterotrophic plants produce large numbers of seeds; individuals of *Rhizanthella gardneri* (Orchidaceae), for example, only produce 20–50 seeds (George and Cooke 1981).

5.5.2 Biomass Reduction

In general, full mycoheterotrophic species are considerably smaller than their most closely related autotrophic/partially mycoheterotrophic species. In many cases, this reduction in biomass cannot solely be explained by the absence of leaves. Perhaps biomass production of fully mycoheterotrophic plants is limited by the amount of carbon they can obtain from their associated fungal networks. Or it may be the result of positive selection toward smaller plants because these can minimize the amount of carbon needed from the fungus, which makes it easier to subvert the mycorrhizal mutualism while remaining undetected.

5.5.3 Habitats

Fully mycoheterotrophic plants generally occur in shaded habitats, like the ground layer of closed-canopy forest (Chap. 3). These habitats are often characterized by a lack of understory plants, which is attributed to the absence of sufficient light for photosynthetic plants to survive. Partially mycoheterotrophic orchids, Ericaceae, and Gentianaceae often grow in forest habitats as well, although partially mycoheterotrophic orchids and gentians (*Bartonia*) also can be found in open vegetations, such as bogs and meadows (Matthews et al. 2009; Girlanda et al. 2011). Interestingly, Preiss et al. (2010) demonstrated that light availability is a major determinant of the degree of mycoheterotrophy in two partially mycoheterotrophic species of *Cephalanthera* (Orchidaceae). These observations provide support for a strong correlation between irradiance levels and dependence on fungal carbon. Therefore, an evolutionary switch from autotrophy to full mycoheterotrophy seems to be accompanied by a shift toward more shaded habitats.

5.5.4 Reduction of the Chloroplast Genome

In holoparasitic plants, the loss of photosynthesis has led to a loss of various chloroplast genes (Bungard 2004; Barbrook et al. 2006). Similar trends are observed in fully mycoheterotrophic plants, although only few taxa have been studied in detail. The RuBisCO large-subunit gene (*rbcL*) was amplified in a few mycoheterotrophic Dioscoreales and showed significantly increased substitution rates (Caddick et al. 2002). Davis et al. (2004) noticed that *rbcL* could not be amplified from species of *Arachnitis* (Corsiaceae), *Thismia* (Thismiaceae), *Lacandonia*, *Sciaphila*, and *Triuris* (Triuridaceae). Cameron (2004) reported putative pseudogenes of *rbcL* in *Cyrtosia* but not for *Erythrorchis* and *Pseudovanilla* (Orchidaceae). The evolution of *rbcL* was studied in detail in *Corallorhiza* orchids by Barrett and Freudenstein (2008). Pseudogenes were detected in some lineages of *Corallorhiza* but not in others.

This is probably indicative of the early stages of pseudogene formation and the result of a recent switch from autotrophy to mycoheterotrophy. The maturaseK gene (*matK*) is probably a pseudogene in species of *Corallorhiza* as well. But because large 5′ deletions and frameshift indels occur in closely related leaf-bearing species of *Aplectrum* and *Oreorchis,* major changes in the functionality of *matK* apparently precede the transition to full mycoheterotrophy (Freudenstein and Senyo 2008).

To date, complete chloroplast genomes have been sequenced of only three species of fully mycoheterotrophic plants: *Aneura mirabilis* (Aneuraceae), *Rhizanthella gardneri,* and *Neottia nidus-avis* (Orchidaceae) (Wickett et al. 2008a; Delannoy et al. 2011; Logacheva et al. 2011). In *A. mirabilis,* all *ndh* genes are either absent or pseudogenes. Five of 15 *psb* genes are pseudogenes, as are 2 of 6 *psa* genes and 2 of 6 *pet* genes. In addition, pseudogenes of *cysA*, *cysT*, *ccsA*, and *ycf 3* were also detected. The remaining genes retained intact open reading frames. Chlororespiratory genes are the most affected of any functional category in *A. mirabilis*, with the partial or complete loss of all genes (Wickett et al. 2008a). All gene losses and pseudogenes are limited to *Aneura mirabilis* and were not found in closely related photosynthetic species of *Aneura* suggesting that they are likely correlated with the loss of photosynthesis in this liverwort (Wickett et al. 2008b). With 108,007 base pairs and a structure that is remarkably collinear with its distant relative, *Marchantia polymorpha*, the plastid genome of *A. mirabilis,* probably represents a genome in the early stages of decay following the relaxation of selection pressures. In contrast, the plastid genome of *Rhizanthella gardneri* consists of only 59,190 base pairs and contains only 37 genes. It is the least gene-rich plastid genome known apart from the fragmented plastid genome of some dinoflagellates. In comparison with the plastid genome of *Phalaenopsis aphrodite* (Orchidaceae), an estimated 70% of the original genes are lost or transferred to the nucleus. These missing genes include those coding for the plastid-encoded RNA polymerase (PEP), the maturase-like protein MatK, all of the genes required for photosynthesis (encoding subunits of photosystem I, photosystem II, cytochrome b_6f complex, and ATP synthase), as well as 6 genes encoding ribosomal proteins and 27 genes encoding tRNAs. Despite rampant gene loss, the plastid genome of *R. gardneri* retains a minimal set of protein-encoding and tRNA genes and appears to be the basis of a functioning gene expression system, with transcription, splicing, and RNA editing all detected and translation likely (Delannoy et al. 2011). The plastid genome of *Neottia nidus-avis* was also found to be reduced in both genome size and gene content. However, these reductions are not as drastic as in *Rhizanthella*: the plastome of *Neottia nidus-avis* lacks all genes encoding photosynthetic proteins and RNA polymerase subunits, but retains most genes of the translational apparatus (Logacheva et al. 2011).

5.5.5 High Substitution Rates in Nuclear and Mitochondrial Genomes

High rates of molecular evolution have also been observed in nuclear and mitochondrial genomes of mycoheterotrophic plants. Merckx et al. (2006, 2009) reported that branch lengths in 18S rDNA gene trees of mycoheterotrophic Dioscoreales (particularly in Thismiaceae) are up to 6.5-fold longer than those of related autotrophic species (see also Yokoyama et al. 2008). Lemaire et al. (2011) measured 18S rDNA substitution rates of heterotrophic species across the phylogeny of the angiosperms and observed that branch lengths of many included mycoheterotrophic species are significantly longer than those of related autotrophic lineages. Substitution rates of 18S rDNA are particularly high in mycoheterotrophic species of Thismiaceae, Corsiaceae, Orchidaceae (*Rhizanthella*), and Triuridaceae (*Kupea*). However, in many other lineages of mycoheterotrophic plants, no significantly faster substitution rates of 18S rDNA were detected. In lineages where substitution rates of 18S rDNA are high, only few mutations occur in major functional and structural regions of the small ribosomal

molecule, suggesting that the efficiency of the translational apparatus in nonphotosynthetic plants has not been affected (Lemaire et al. 2011). Although data is more limited, high substitution rates have been demonstrated to occur in some mitochondrial genes of fully mycoheterotrophic species as well (Merckx et al. 2006, 2009).

Interestingly high substitution rates in nuclear and mitochondrial genomes have also been observed in holoparasitic plants and have, for example, frustrated attempts to infer the phylogenetic position of the world's largest flower, *Rafflesia* (Barkman et al. 2004). The underlying causes of accelerated substitution rates in heterotrophic plants are unknown, but numerous hypotheses have been proposed. The long-term effects of a small effective population size resulting in a genetic bottleneck effect (Wu and Li 1985), the influence of a short generation time and the correlated higher number of mutation-generating reproductive events (Wu and Li 1985), an increased tolerance of mutations due to a relaxation of selective constraints, variations in mutation rate (Sniegowski et al. 2000), DNA repair efficiency (Modrich and Lahue 1996), and speciation rates (Barraclough and Savolainen 2001) are possible factors that trigger high substitution rates in mycoheterotrophic and parasitic plants. However, none of these hypotheses can unequivocally explain higher substitution rates in parasitic plants (Nickrent and Starr 1994; dePamphilis et al. 1997; Young and dePamphilis 2005) and mycoheterotrophic plants (Lemaire et al. 2011).

5.5.6 Mycorrhizal Specificity

Both the arbuscular and the ectomycorrhizal symbiosis are generally characterized by low specificity between plants and fungi. In contrast to autotrophic mycorrhizal plants, however, mycoheterotrophic plants often show high specificity toward fungi even though the fungi remain generalists (e.g., Taylor and Bruns 1997; Bidartondo et al. 2002, 2003; Chap. 6). In the Monotropoideae (Ericaceae), five related mycoheterotrophic plant lineages are each specialized on one of five distantly related basidiomycete fungal lineages (Bidartondo 2005). Species of mycoheterotrophic orchids often show specificity toward various lineages of either ectomycorrhizal or saprotrophic fungi (e.g., Taylor and Bruns 1997; Ogura-Tsujita and Yukawa 2008). Similarly, specificity toward narrow lineages of fungi is also observed in mycoheterotrophs that are living on arbuscular mycorrhizal fungi, such as *Arachnitis uniflora* (Corsiaceae) (Bidartondo et al. 2002), *Kupea martinetugei* (Triuridaceae) (Franke et al. 2006), *Afrothismia* (Thismiaceae) (Merckx and Bidartondo 2008), and *Petrosavia sakuraii* (Petrosaviaceae) (Yamato et al. 2011). High specificity toward host fungi is also observed in non-angiosperm mycoheterotrophs (Bidartondo et al. 2003; Winther and Friedman 2007, 2008, 2009). See Chap. 6 for a detailed overview on the interactions between mycoheterotrophs and their host fungi.

Association with compatible fungi is essential for the establishment of a fully mycoheterotrophic plant. In absence of their specific fungus, many mycoheterotrophic plants will not germinate or develop (Bruns and Read 2000). Even if germination is triggered by a close relative of the host fungus, the seedling may not survive past the early stages of development (Bidartondo and Read 2008). In an evolutionary context, highly specialized full mycoheterotrophs have evolved from ancestors with more generalist fungal associations. There are two mechanisms that may drive this evolutionary trend toward increased fungal specificity: (1) a mycoheterotrophic plant selects from the potential fungal community the best target to meet its nutrient demands, and (2) a mycoheterotrophic plant, because of its increasingly parasitic interaction with fungi, is "denied" access to most members of the fungal community except for a few fungal lineages that fail to detect or exclude the plant (Bruns et al. 2002; Egger and Hibbett 2004; Bidartondo 2005; Merckx et al. 2009).

Mycorrhizal specificity of mycoheterotrophic plants is not always targeted toward a single fungal lineage. Some mycoheterotrophic species associate with distinct sets of fungal lineages. For example, *Corallorhiza maculata* (Orchidaceae) associates with exclusive sets of fungi, spanning ca. 22 described species across the Russulaceae (Taylor et al. 2004). It was demonstrated that *C. maculata* consists of several distinct fungal

host-associated races, and even when different orchid genotypes were found growing in close proximity, they maintained their distinct fungal associations (Taylor et al. 2004). Similarly, the closely related species *Corallorhiza striata* targets a narrow breadth of fungi in the genus *Tomentella* (Thelephoraceae)—specifically *T. fuscocinerea*. A population-level analysis of *C. striata* and its mycorrhizal associates across the entire distribution range of the orchid shows that distinct orchid clades associate with divergent sets of fungi. The associations between *C. striata* and its mycorrhizal fungi are strongly determined by geography and phylogenetic affinity of the orchid populations (Barrett et al. 2010). Levels of mycorrhizal specificity are also high in species of *Hexalectris* (Orchidaceae). The phylogenetic breadth of mycorrhizal associations between the species ranges from specificity toward a single narrow clade of ectomycorrhizal fungi (e.g., *H. brevicaulis*, *H. grandiflora*) to specificity toward multiple clades of different fungal groups in *H. spicata*. However, fungal associations of *H. spicata* showed geographic variation (Kennedy et al. 2011). In Ericaceae, the fully mycoheterotrophic species *Monotropa uniflora* has a widespread distribution in North America. The species specializes on various Russulaceae species within *Russula*, *Lactarius*, and *Martellia*, but fungal associations on population level appear to be geographically structured as well (Bidartondo and Bruns 2001; Bidartondo 2005). Specifically, *M. uniflora* populations in Oregon associated exclusively with *Russula brevipes*, while a single population in Vermont, USA, associated with three *Russula* species groups and *Lactarius theiogalus*. These examples demonstrate that the interactions between mycoheterotrophs and their mycorrhizal fungi may resemble a geographic mosaic (Thompson 2005). The geographic mosaic theory of coevolution hypothesizes that three processes are the primary drivers of coevolutionary dynamics: geographic selection mosaics, coevolutionary hot spots, and trait remixing. Selection mosaics occur when natural selection on interactions varies among different communities. While this has yet to be tested for mycoheterotrophic plants, fitness was found to vary in vitro across different fungus–orchid combinations in the orchid *Tolumnia variegata*, suggesting potential for natural selection to act on these relationships (Otero et al. 2005). Hot spots are communities in which interacting species have reciprocal effects on fitness and are often embedded within surrounding communities in which interspecific selection affects only one or neither species (cold spots). Finally, a combination of gene flow, random genetic drift, and extinction/colonization dynamics continually reshapes the genetic landscape over which future selection takes place (trait remixing) (Gomulkiewicz et al. 2000; Thompson 2005). This tripartite coevolutionary process should produce three general ecological patterns: different combinations of coevolved traits in different regions, local maladaptation within some interactions, and few geographically uniform coevolved traits (Thompson 2005).

While high mycorrhizal specificity is often regarded as a hallmark of mycoheterotrophic plants, not all full mycoheterotrophs show high specificity toward fungi. Examples are *Pyrola aphylla* (Ericaceae) (Hynson and Bruns 2009), *Wullschlaegelia aphylla* (Martos et al. 2009), and species of *Aphyllorchis* (Orchidaceae) (Roy et al. 2009). This indicates that identifying a specific fungus that meets the plant's demands need not be the initiating process in the subversion of the mycorrhizal mutualism. Also, high mycorrhizal specificity is not restricted to fully mycoheterotrophic plants. Many green species of orchids (although are all initial mycoheterotrophs and some perhaps partially mycoheterotrophic) show specificity toward mycorrhizal fungi. Specialization trends within some of these lineages appear to be phylogenetically conserved as well (Shefferson et al. 2007, 2010; Jacquemyn et al. 2011).

5.5.7 Host Shifts

Broad shifts of fungal associations in the evolutionary trajectory toward full mycoheterotrophy have probably occurred multiple times in Orchidaceae: while an association with saprotrophic fungi is likely the ancestral state for all orchids (Yukawa et al. 2009), many fully and partially mycoheterotrophic orchids associate with ectomycorrhizal fungi (see Chaps. 6 and 8).

This "host" switch has been described in detail for the genus *Cymbidium* (Orchidaceae) by Motomura et al. (2010). Early diverging *Cymbidium* species are presumably autotrophic as adults and generally have associations with saprotrophic fungi only (mainly Tulasnellaceae). The partially mycoheterotrophic species diverging from this group harbor both Tulasnellaceae and ectomycorrhizal fungi (Russulaceae and others), while the latest diverging branch in this clade comprises the fully mycoheterotrophic species *C. macrorhizon* and *C. aberrans* that associate exclusively with the ectomycorrhizal fungi. These results indicate that the nutritional shift from autotrophy to mycoheterotrophy through partial mycoheterotrophy in *Cymbidium* may correlate with shifts in associated fungi from saprotrophic to ectomycorrhizal fungi. Hashimoto et al. (2012) recently reported a remarkable congruent fungal shift in a Japanese *Pyrola* species (Ericaceae), of which adult plants are supposedly capable of partial mycoheterotrophy. Adult *Pyrola asarifolia* plants, like other Pyroleae, associate with diverse ectomycorrhizal fungal species that are a subset of those that are forming mycorrhiza with surrounding trees. Conversely, seedlings of *Pyrola asarifolia* specifically associate with a lineage of Sebacinales B, which probably derive carbon through saprotrophy (see Chap. 8).

Similarly, in the liverwort family Aneuraceae, an association with tulasnelloid symbionts is a secondary acquisition of recent origin following the loss of the ancestral arbuscular mycorrhizal symbiosis (Bidartondo and Duckett 2010). The evolution of mycoheterotrophy in *Aneura mirabilis* then led to a subsequent specialization toward narrow *Tulasnella* lineages (Bidartondo et al. 2003; Bidartondo and Duckett 2010). In these examples, evolution of mycoheterotrophy is thus preceded by a secondary gain of associations with fungal lineages, which are in a later evolutionary step exploited to obtain carbon.

Shifts of fungal associations within lineages of full mycoheterotrophic plants have not yet been documented, and the mycoheterotrophs' common preference toward narrow fungal lineages suggests that such shifts may be rare. However, the tendency of many species of monotropes and orchids to specialize toward multiple distant lineages of mycorrhizal fungi may be interpreted as the result of radical host jumps. Another possible explanation for this pattern is high lineage attrition creating vast phylogenetic gaps that then appear as host jumps (Bidartondo 2005). Only by studying mycorrhizal specialization in a phylogenetic context it is possible to distinguish host jumps from lineage attrition (Box 5.1). Although the lack of extant species that represent in-between evolutionary steps of this mycorrhizal specialization process may prevent the uncovering of the underlying evolutionary pattern.

Box 5.1 Analysis of Mycorrhizal Interactions in an Evolutionary Framework: A Primer

Fully mycoheterotrophic plants often show extreme specificity in their associations with fungi. Since all fully mycoheterotrophic plants evolved from autotrophic mycorrhizal ancestors, and autotrophic mycorrhizal plants generally show low specificity in their interaction with mycorrhizal fungi, this extreme specificity is the result of an evolutionary specialization process. Estimating effects of evolutionary relatedness on mycorrhizal associations requires information on phylogenetic relationships and plant–fungal interactions. Depending on the questions asked and the available data, phylogenetic patterns can be used to infer different evolutionary processes shaping mycorrhizal interactions (Fig. 5.7).

Mycorrhizal Associations and Plant Phylogenetic Relatedness

Do plant species that are phylogenetically related show similar patterns of mycorrhizal specificity? This question can be answered when a well-supported plant phylogeny is available as well as information on the mycorrhizal fungi that are associated with each plant species in the phylogeny. Plant species can be

(continued)

Box 5.1 (continued)

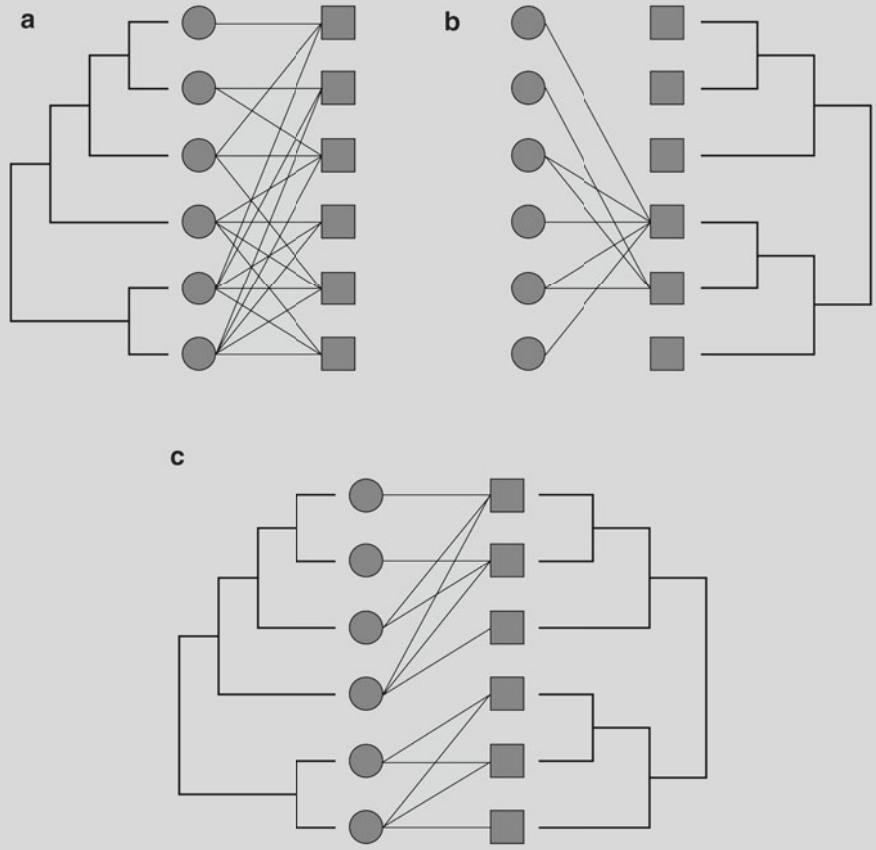

Fig. 5.7 Hypothetical models showing examples of mycorrhizal interactions between plants (*circles*) and fungi (*squares*) influenced by phylogenetic relationships of plants, fungi, or both. (**a**) Example in which the degree of mycorrhizal specificity of host plants is clearly influenced by the phylogenetic relationships between the plants, shown as a progressive loss of associated fungi moving from the *bottom* to the *top* of the phylogeny. (**b**) Example in which plants in a clade associate with a phylogenetically restricted set of fungi. (**c**) Example in which plant–fungal interactions are influenced by the phylogenetic relationships of both plants and fungi

scored according to the number of associated fungi (genotypes or fungal OTU's) or according to the mean pairwise genetic or phylogenetic distance among the associated fungal taxa (e.g., Nei and Tajima 1981). The former method assesses mycorrhizal specificity purely as a function of number of associated fungi per species, without taking the phylogenetic distance between the associated fungi into account. This method results in discrete character states. Calculating genetic distances defines mycorrhizal specificity as a function of phylogenetic breadth of associated fungi, but may be misleading when species tend to specialize on multiple, distant fungal lineages. This method results in continuous character states. Both the number of associated fungal OTU's and distances among the associated fungi can be mapped on the plant phylogeny and used reconstruct evolutionary patterns of mycorrhizal specificity. For this purpose, a wide variety of methods for ancestral state reconstruction are available, for example, in the software packages Mesquite (Maddison and Maddison 2011) and APE (Paradis et al. 2004). In addition, there are different methods

(continued)

Box 5.1 (continued)

to test whether the variation in mycorrhizal specificity between the plant species is influenced by their phylogenetic relationships, such as phylogenetic autocorrelation tests (Gittleman and Kot 1990), and measurements of phylogenetic signal (e.g., *K* statistic of Blomberg et al. 2003). Several of these tests are available in the R packages APE and Picante (Kembel et al. 2010). These methods have been used to study the evolution of mycorrhizal specificity, for example, in the orchid genera *Cypripedium* (Shefferson et al. 2007), *Goodyera* (Shefferson et al. 2010), and *Orchis* (Jacquemyn et al. 2011).

A drawback of the methodology described above is that it ignores the actual identity of the fungal partners. Related plant species may be associated with the same number of fungal taxa or with a similar phylogenetic range of fungi, yet they may actually grow with completely different sets of mycorrhizal fungi. To test whether phylogenetically related plant species interact with similar fungi, plant phylogenetic distances can be regressed against metrics of fungal community dissimilarity using a Mantel test. The dissimilarity (or distance) in fungal community structure between plant species pairs can be calculated as $1-S$, in which S is the fungal community similarity between species pairs. There are a number of different community similarity statistics available in the ecological literature (reviewed in Koleff et al. 2003; Anderson et al. 2010), but they are generally based on a ratio of shared vs. total fungal taxa hosted between plant species. Differences in the number of associated fungal taxa per plant species may affect estimates of community similarity, and therefore, a partial Mantel test controlling for a number of associated fungi or choice of a metric that controls for differences in richness (e.g., β_{sim}, Koleff et al. 2003) may be more appropriate in most cases. The ZT software package (Bonnet and Van de Peer 2002) and the R package APE can be used to run these Mantel tests, and the R package Vegan (Oksanen et al. 2011) can be used to calculate most metrics of community similarity.

Mycorrhizal Associations and Fungal Phylogenetic Relatedness

Are mycorrhizal interactions evolutionarily conserved across the fungal phylogeny? In theory, the same methods as described above for mycorrhizal interactions in the context of plant relationships can be applied to fungal phylogenies. However, for most fungal taxa, we lack data about the range of associated plant species. Nevertheless, we can test the degree to which fungi associating with particular plant species tend to be close phylogenetic relatives. These tests can be performed at different ecological and evolutionary scales. For example, a fungal phylogeny may represent all lineages of a particular clade of fungi, regardless of their occurrence or their ecology, and may be used to infer whether a particular group of plants shows a preference for fungi based on the phylogenetic relatedness of the latter. Or a fungus phylogeny may be restricted to all mycorrhizal fungi detected at a particular research site and can be used to test whether mycorrhizal associations of particular plants at this site are influenced by fungal phylogenetic relatedness. Choosing the appropriate pool of species is critical to matching the scale of analysis to the scale of the question being asked. A popular method to examine the phylogenetic structure of a "community" (in this case, a set of mycorrhizal fungi detected in a particular sample of plant species) is the calculation of the net relatedness index (NRI) and nearest taxon index (NTI) (Webb et al. 2002). NRI is a standardized measure of the mean pairwise phylogenetic distance of taxa in a sample and quantifies overall clustering of taxa on a tree, while NTI is a measure of the phylogenetic distance to the nearest co-occurring taxon for each taxon in the sample and quantifies the extent of terminal clustering, independent

(continued)

Box 5.1 (continued)

of deep-level clustering (Webb et al. 2002). The NTI and NRI indices are measured as standardized effect sizes based on a null model generated by random permutation of a phylogeny that contains an appropriate pool of species for the given question. Positive NTI and NRI values indicate that species within a sample are more closely related than expected by chance (phylogenetic clustering), and negative values indicate that species within a sample are less related than might be expected by chance (phylogenetic evenness or overdispersion). Phylogenetic clustering and overdispersion are generally interpreted as the result of evolutionary trait conservation or trait convergence as the result of ecological processes such as niche partitioning and competition. However, care must be used when interpreting results as multiple processes can result in patterns of clustering or overdispersion (Kraft et al. 2007). These methods are implemented in the software program Phylocom (Webb et al. 2011) and the R package Picante and have been used by Merckx et al. (2012) to investigate the phylogenetic clustering of arbuscular mycorrhizal fungi detected in mycoheterotrophic interactions. Alternatively, multivariate statistics can be used to analyze phylogenetic distance between fungi and their ecological role (e.g., presence/absence in mycoheterotrophic interactions).

Plant–Fungal Networks

The methods described above evaluate mycorrhizal interactions either from a plant or fungus phylogenetic perspective. A more integrative approach estimates the influence of both phylogenies on a plant–fungal interaction network. This can be achieved by calculating the phylogenetic relationships of both plants and their interacting mycorrhizal fungi and assessing the mycorrhizal network from a classic host–parasite coevolutionary perspective. A simple approach is to calculate congruency between plant and fungal phylogenies (e.g., Penny and Hendy 1985; Farris et al. 1994) and has been used by Bidartondo and Bruns (2002) for Monotropoideae and their associated fungi and Barrett et al. (2010) for *Corallorhiza–Tomentella* interactions. The latter study also applied a more advanced congruence/incongruence statistical test implemented in ParaFit (Legendre et al. 2002). Several other methodologies to analyze host–parasite associations are available (reviewed by Stevens 2004). However, many of these approaches are based on a host–parasite co-speciation model (e.g., Charleston and Page 2002; Ronquist 2002), which may be not an appropriate model to explain the observed branching patterns of mycorrhizal specialization processes. When congruence between both phylogenies is high, co-speciation of congruent plant–fungus nodes can be tested by comparing their absolute ages obtained with molecular clock techniques. When age estimates for congruent nodes overlap, they may be the result of synchronous co-speciation events. When age estimates do not overlap, the pattern may be explained by delayed co-speciation of phylogenetic tracking events, as was observed in fully mycoheterotrophic *Afrothismia* species and their associated *Glomus* fungi (Merckx and Bidartondo 2008).

Food-web approaches are probably more appropriate to explore complex plant–fungus mycorrhizal interactions than host–parasite co-speciation models. A relevant method for evaluating mycorrhizal interactions is that of Ives and Godfray (2006), which calculates the strength of the phylogenetic signal of the two phylogenies on the interaction matrix. It is implemented in the R package Picante and has been used to study phylogenetic effect on *Orchis*–Tulasnellaceae interactions by Jacquemyn et al. (2011).

5.5.8 Phylogenetic Tracking

It has been argued that once an appropriate fungal partner has been found, a mycoheterotroph fine-tunes its physiology to adapt to that particular fungus, and it is therefore largely incapable of host jumps to distantly related fungi (Bidartondo and Bruns 2002). Subsequent speciation, mycorrhizal specialization, and a lack of host switching may force the plants to track the fungus phylogeny. Such phylogenetic tracking has been observed between species of *Afrothismia* (Thismiaceae) and *Glomus* group A fungi (Merckx and Bidartondo 2008). Phylogenetic tracking and host loyalty has been extensively studied in Monotropoideae, where it resulted in extreme mycorrhizal specialization, most extensively toward *Tricholoma* and Russulaceae (Bidartondo 2005).

5.6 Conclusions

Many millions of years of evolution have produced an astonishing array of land plants. Fully mycoheterotrophic plants are perhaps among the most remarkable of these. They clearly demonstrate that the mycorrhizal mutualism, which in case of the AM mutualism has been stable for over 400 million years, can be successfully cheated. A large majority of land plants associate with mycorrhizal fungi, and therefore, it is perhaps not surprising that the ability to cheat this interaction arose many times independently during plant evolution. However, it remains unknown why particular families of mycorrhizal plants contain many fully mycoheterotrophic species, while mycoheterotrophy is absent in most other mycorrhizal plant families. Lineages of fully mycoheterotrophic plants always comprise relatively few species, and those species are often rare and have scattered geographic distributions (see also Chap. 3). This may reflect limited ecological success, at least under recent climatic and biotic conditions. However, detailed evolutionary histories of ancient mycoheterotrophic lineages may reveal pronounced diversification and geographic dispersal—and thus increased ecological success—during past epochs of plant evolution. The drivers behind the evolution toward mycoheterotrophy remain unknown as well, although several lines of evidence suggests that mycoheterotrophy may provide an evolutionary escape route from intense competition for light in forest understory habitats.

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