Evolutionary histories and mycorrhizal associations of mycoheterotrophic plants dependent on saprotrophic fungi

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Abstract
Mycoheterotrophic plants (MHPs) are leafless, achlorophyllous, and completely dependent on mycorrhizal fungi for their carbon supply. Mycorrhizal symbiosis is a mutualistic association with fungi that is undertaken by the majority of land plants, but mycoheterotrophy represents a breakdown of this mutualism in that plants parasitize fungi. Most MHPs are associated with fungi that are mycorrhizal with autotrophic plants, such as arbuscular mycorrhizal (AM) or ectomycorrhizal (ECM) fungi. Although these MHPs gain carbon via the common mycorrhizal network that links the surrounding autotrophic plants, some mycoheterotrophic lineages are associated with saprotrophic (SAP) fungi, which are free-living and decompose leaf litter and wood materials. Such MHPs are dependent on the forest carbon cycle, which involves the decomposition of wood debris and leaf litter, and have a unique biology and evolutionary history. MHPs associated with SAP fungi (SAP-MHPs) have to date been found only in the Orchidaceae and likely evolved independently at least nine times within that family. Phylogenetically divergent SAP Basidiomycota, mostly Agaricales but also Hymenochaetales, Polyporales, and others, are involved in mycoheterotrophy. The fungal specificity of SAP-MHPs varies from a highly specific association with a single fungal species to a broad range of interactions with multiple fungal orders. Establishment of symbiotic culture systems is indispensable for understanding the mechanisms underlying plant–fungus interactions and the conservation of MHPs. Symbiotic culture systems have been established for many SAP-MHP species as a pure culture of free-living SAP fungi is easier than that of biotrophic AM or ECM fungi. Culturable SAP-MHPs are useful research materials and will contribute to the advancement of plant science.

Keywords In vitro culture · Litter decay fungi · Orchid · Stable isotopes · Wood decay fungi

Introduction
Mycoheterotrophic plants (MHPs) are non-photosynthetic and thus completely reliant on mycorrhizal fungi for carbon uptake throughout their lifecycle (Leake 1994). Most MHPs have small vegetative organs and have an underground root/rhizome system as the main body, emerging aboveground only for reproduction. Such extreme evolution occurred independently over 40 times in all divisions of land plants, and there are ca. 580 species of MHPs (Jacquemyn and Merckx 2019). The evolution of mycoheterotrophy was accompanied by dramatic changes in a variety of characteristics, such as morphology (Leake 1994), mycorrhizal symbiosis (Ogura-Tsujita et al. 2012), pollination systems (Suetsugu 2015), seed dispersal systems (Suetsugu et al. 2015; Suetsugu 2018), and genome size and content (Barrett and Davis 2012). MHPs are therefore expected to be useful models in plant science.
Mycorrhizal symbiosis between plants and fungi is a ubiquitous type of mutualism, in which autotrophic plants exchange photosynthesized carbon for mineral nutrients obtained by mycorrhizal fungi (Smith and Read 2008). However, mycoheterotrophy represents a breakdown of this mutualism, as plants obtain carbon from fungi without photosynthesis (Merckx and Bidartondo 2008). Molecular studies for mycobiont identification have revealed two main mycorrhizal systems supporting carbon gain by MHPs; namely, via the mycorrhizal fungi of autotrophic plants—arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi—and via free-living saprotrophic (SAP) fungi (Waterman et al. 2013). These two mycorrhizal systems use different carbon sources—MHPs associated with AM (AM-MHPs) and ECM fungi (ECM-MHPs) obtain carbon from surrounding autotrophic plants through shared mycorrhizal fungi, whereas MHPs associated with SAP fungi (SAP-MHPs; Fig. 1) obtain carbon from plant debris through the decomposition of wood and leaf litter. Most of the litter- or wood-decay fungi associated with SAP-MHPs are rarely found as mycorrhizal fungi in autotrophic land plants. The biology of AM- and ECM-MHPs has been reviewed by others (Bidartondo 2005; Leake 1994; Merckx 2013). However, the diversity of SAP-MHPs was elucidated only recently despite its discovery by Kusano in 1911. A critical advantage of SAP-MHPs is the feasibility of symbiotic culture. Because a pure culture of free-living SAP fungi is easier than that of biotrophic AM or ECM fungi, culture systems for several SAP-MHPs have been established (Burgeff 1936; XU and Guo 2000; Yagame et al. 2007). These enable key questions of mycoheterotrophy to be addressed and facilitate the conservation of endangered species. Here, we review SAP-MHPs with emphasis on their evolutionary history and mycorrhizal associations. We also introduce case studies of symbiotic culture of SAP-MHPs and discuss future perspectives.

Mycoheterotrophy

The evolution from autotrophy to mycoheterotrophy is a stepwise process involving the reduction of foliage leaves and chlorophyll content. Leafless and achlorophyllous MHPs are categorized as fully mycoheterotrophic; partial and initial mycoheterotrophy are also recognized in land plants (Merckx 2013). Partial MHPs retain normal chlorophyllous leaves and have the ability to obtain carbon from both photosynthesis and mycorrhizal fungi (Gebauer and Meyer 2003). Initial MHPs are dependent on their mycorrhizal fungi for carbon supply during the early stages of their life history and subsequently develop into autotrophic mature plants (Merckx 2013). Initial mycoheterotrophy has been observed in seed plants producing small dust-like seeds, such as in Orchidaceae and Pyroleae in Ericaceae, and also in the gametophytes of lycophytes and pteridophytes (Merckx 2013). Partial and initial mycoheterotrophy are thought to be an intermediate stage in the transition from autotrophy to mycoheterotrophy and provide insight into the evolution of the latter (Ogura-Tsujita et al. 2012).

The definitions of partial and full mycoheterotrophy are unclear. Foliage leaves are substantially reduced but still develop in some species, such as Cephalanthera subaphylla Miyabe and Kudô (Orchidaceae). Furthermore, some leafless MHPs have chlorophyllous reproductive shoots, indicating that photosynthesis is active during flowering and fruiting (Suetsugu et al. 2018; Zimmer et al. 2008 but also see Cameron et al. 2009). Partial and full mycoheterotrophy can be distinguished by their stable isotope signatures (Suetsugu et al. 2018; Zimmer et al. 2008; see also the section “Isotopic signature of SAP-MHPs”), but the level of mycoheterotrophy has not been evaluated for most SAP-MHPs. Therefore, in this review, species that lack foliage leaves are defined as full MHPs. Leafless species that develop chlorophyllous reproductive shoots are included as full MHPs because photosynthesis is limited to the reproductive phase. Species that have small, chlorophyllous foliage leaves are excluded from full MHP status because the leaves, the principal photosynthetic apparatus, function during the growth period. Further, leafless lianas with chlorophyllous stems such as Pseudovanilla foliata (F.Muell.) Garay and Vanilla aphylla Blume (Orchidaceae), and leafless epiphytes with chlorophyllous roots such as Dendrophyllax and Tueniohypilium (Orchidaceae) are not considered MHPs because the stems or roots are photosynthetic and function throughout the life history of such species.

Mycorrhizal symbiosis in MHPs

Three phylogenetically and physiologically distinct fungal groups are involved in mycorrhizal symbiosis in MHPs—AM, ECM, and SAP fungi (Waterman et al. 2013). AM or ECM fungi obtain carbon from their autotrophic host plants through mutualistic relationships. The AM association is the most dominant mycorrhizal symbiosis type in land plants, with more than 71% of mycorrhizal plant species associated with AM fungi (Brundrett and Tedersoo 2018). ECM fungi are mostly associated with particular tree families, such as Pinaceae and Fagaceae, and are the dominant mycorrhizal type in boreal and temperate forests (Smith and Read 2008).
Laeliinae  
Pleurothallidinae  
Ponerinae  
Bletiinae  

Calyposinae  
Agrostophyllinae  
Collabieae

Podochieae  
Angraecinae  
Aeridinae  
Adrorhizinae  
Polystachyinae  
Cymbidinae

Cyrtopodinae  
Stanhopeinae  
Coelopsisdinae  
Maxillarinae  
Eripidinae  
Zygopetalinae  
Oncidinae  
Catasetinae  
Eulophininae

Dendrobiinae  
Malaxidinae  
Coelogyginae  
Arethusinae

Thaieae  
Nervilinae  
Epiopininae  
Gastrodieae

Xerorchideae  
Triphorinae  
Dicerotostelinae  
Triphoreae

Wullschlaegelieae  
Tropidieae  
Sobralieae  
Neottieae

Coryciinae  
Disinae  
Orchidinae  
Brownleeinae  
Codonorchideae

Thelymitrinae  
Megastylidinae  
Drakeaeinae  
Cryptostylidinae  
Diuridinae  
Prasophyllinae  
Caladeniinae  
Acanthinae  
Rhzantelinae  
Chloraenae  
Pterostylidinae  
Goodvertinae  
Galosottelinae  
Mannielinae  
Spiranthis  
Disciphinae  
Cranichidinae  
Cyripedioidae  
Pogonieae  
Vanileae

Apostasioidae

Epidendreae  
Hexalectris

Corallorhiza, Cremas*  
Danxiaorchis, Yoania  
Risleya

$\text{SAP-MHP}$  
$\text{ECM-MHP}$

* Mixed with leafy and leafless plants

Cymbidium*  
Dipodium*, Eulophilia*

Liparis*, Crepidium*

Tropidia*

Epipogium, Stereosandra  
Auxopus, Didymoplexiella, Didymoplexis, Gastrodia, Uleiionchis

Aphyllorchis, Cephalanthera*, Limodorum, Neottia*

Brachycorythis*, Platanthera*, Silvorchis

Burnettia  
Arthrochilus*

Cryptostyla*, Stigmatodactylus*

Corybas*  
Rhizanthes*

Aspidogyne*, Chamaegastrodia, Cystorchis*, 
Danhatchia, Odontochilus*

Degranvillea
AM or ECM fungi are simultaneously associated with MHPs, and thus, AM- or ECM-MHPs obtain photosynthesized carbon from the surrounding autotrophic plants via shared mycorrhizal mycelia. This tripartite symbiosis allows MHPs access to the common mycorrhizal networks of AM or ECM fungi that link the surrounding autotrophic plants.

By contrast, SAP-MHPs depend on nonliving biomass. Mycoheterotrophic associations with free-living litter- or wood-decay fungi are dependent on the forest carbon cycle (Ogura-Tsujita et al. 2018; Suetsugu et al. 2020b). The decomposition of woody debris and leaf litter by SAP fungi plays a key role in regulating carbon and nutrient cycles in forest ecosystems (Berg and McClaugherty 2008). Woody debris is a major component of forest biomass, and this large carbon store represents up to 20% of the total aboveground biomass (Bradford et al. 2009; Laiho and Prescott 1999). MHPs dependent on SAP fungi can access the carbon pool via associations with litter- or wood-decay fungi, a pathway of carbon gain unique among land plants. Although carbon flow in tripartite symbiosis has been studied using stable isotopic signatures (Gebauer and Meyer 2003; Hynson et al. 2016) and labeled isotopes (Bougoure et al. 2010; Mc Kendrick et al. 2000), carbon acquisition from plant debris in SAP-MHPs is less well understood than that in AM- and ECM-MHPs.

**Phylogeny and evolution of SAP-MHPs**

Among the MHPs for which the mycorrhizal fungi have been surveyed, SAP-MHPs include 28 species from 10 genera, all belonging to Orchidaceae (Fig. 2; Table 1). The evolutionary tracks of mycoheterotrophy within Orchidaceae were traced in a phylogenetic tree covering all of the major clades of the family (Chase et al. 2015; Fig. 2). It is likely that full mycoheterotrophy evolved independently at least 41 times, and that SAP-associated mycoheterotrophy (SAP-MH) evolved at least nine times within Orchidaceae (Fig. 2). When a group includes only SAP-MHPs, SAP-MH evolved in their common ancestor, whereas when a group comprises a mixture of SAP-MHPs and species associated with other mycorrhizal types, SAP-MH evolved in that clade. SAP-MHPs are found in the second-basalmost subfamily Vanilloideae and five tribes of the latest diverged subfamily Epidendroideae, showing that SAP-MH evolved in various lineages in the family.

Mycoheterotrophy dependent on SAP fungi probably evolved twice or more in Vanilloideae, which encompasses three SAP-MH genera—Galeola, Cyrtosia, and Erythrochis. Cameron et al. (2009) and Cameron (2011) showed that the paired genera Galeola-Cyrtosia and Erythrochis-Pseudovannila form a clade (Fig. S1). Pseudovanilla is the only chlorophyllous genus in this clade. There are two alternative hypotheses on the evolution of mycoheterotrophy in this clade. One is that mycoheterotrophy evolved twice from the common ancestor of Galeola-Cyrtosia and the ancestor of Erythrochis. The other is that mycoheterotrophy evolved once from the common ancestor of the four genera and was subsequently reversed in Pseudovanilla, a photosynthetic plant. The latter is implausible because evolutionary processes to achieve full mycoheterotrophy cause the loss of multiple genes regulating photosynthesis (Delannoy et al. 2011; Li et al. 2020), and reversal from full mycoheterotrophy to autotrophy requires the reorganization of these functional genes. Thus, SAP-MHPs likely evolved at least twice in Vanilloideae.

In Epidendroideae, SAP-MH likely evolved at least seven times, viz., Wullschlaegelia, Gastrodia, Didymoplexis, Epipogium roseum (D.Don) Lindl., Eulophia zollingeri (Rchb.f.) J.J.Sm., Cremastra aphylla T.Yukawa, and Yoania (Fig. 2). Wullschlaegelia comprises two mycoheterotrophic species and W. calcarea Benth. is recognized as an SAP-MHP (Hatté et al. 2020; Martos et al. 2009). Gastrodia is the largest genus of SAP-MHPs and includes ca. 100 species (WCSP 2020), among which 13 have been reported to be SAP-MHPs (Table 1). Didymoplexis comprises 20 species, two of which—D. micradenia Hemsl. (synonym, D. minor J.J.Sm.) and D. pallens Griff.—exhibit SAP-MH (Burgeff 1932, 1936). The mycoheterotrophic genus Epipogium comprises both SAP- and ECM-associated species. Epipogium roseum was reported to be an SAP-MHP (Yamato et al. 2005). Eulophia and Cremastra contain both leafy and leafless species, and SAP-MH was reported for both Eulophia zollingeri (Ogura-Tsujita and Yukawa 2008; Suetsugu et al. 2020b) and C. aphylla (Yagame et al. 2018). The mycoheterotrophic genus Yoania includes four species, three of which are SAP-MHPs (Suetsugu et al. 2020b; Yamashita et al. 2020). In Cremastra and Epipogium, speciation stopped occurring subsequent to SAP-MH evolution. By contrast, SAP-MH did not lead to an evolutionary dead end in Gastordia, and this genus was likely diversified by the establishment of novel symbioses with various SAP fungi (Kinoshita et al. 2016).
| Plant taxa | Taxonomic affiliation of mycobionts | Analyses\(^a\) | References | Notes |
|------------|-----------------------------------|----------------|------------|-------|
| Cremastra aphylla | Agaricales | *Coprinellus domesticus*, *Coprinellus* sp. | Molecular identification, sporocarp formation | Yagame et al. (2018) | One of the isolates was identified as *C. domesticus*. |
| Cyrtosia javanica | Polyporales | Meripilaceae | Molecular identification, stable isotopes | Lee et al. (2015) | The Meripilaceae fungi were identified as *Physisporinus* by Yamashita et al. (2020). |
| *C. septentrionalis* (Galeola septentrionalis)\(^b\) | Agaricales | *Armillaria mellea* | Rhizomorph morphology, isolate characteristics | Hamada (1939, 1940) | |
| | | *Armillaria mellea* | Symbiotic culture | Terashita (1985) | Aseptic seedlings formed mycorrhizae with *A. mellea*. |
| | | *Armillaria tabescens* | Sporocarp formation | Terashita and Chuman (1987) | Possibly *A. borealis*, but further identification is required. |
| | | *Armillaria borealis*, *A. cepistipes*, *A. gallica* (*A. bulbosa*), *A. mellea*, *A. tabescens* | SI test | Terashita and Chuman (1989), Terashita (1996) | |
| | | *Armillaria* | Isozyme | Matsushita et al. (1996) | The fungal isolates were assigned to four biological species. |
| | | *Armillaria jezoensis* | SI test | Cha and Igarashi (1996) | |
| | | *Armillaria jezoensis* | PCR-RFLP | Terashima et al. (1998) | |
| | | *Armillaria mellea* | SI test, RAPD | Ota et al. (2000) | |
| | | *Armillaria gallica*, *A. mellea*, *A. tabescens* | Symbiotic culture | Umata et al. (2013) | Seed germination was stimulated, but no further growth was observed. |
| Polyporales | Meripilaceae | | Symbiotic culture, molecular identification | Umata et al. (2013) | Seed germination and following seedling growth were promoted. The Meripilaceae fungus was identified as *Physisporinus* by Yamashita et al. (2020). |
| Russulales | Xylobolus annosus | | Symbiotic culture | Umata et al. (2013) | Seed germination was stimulated, but no further growth was observed. |
| Cantharellales | Rhizoctonia repens | | Symbiotic culture | Masuhara and Katsuya (1991) | Aseptic seedlings formed mycorrhizae with *R. repens*. |
| | | | | Nakamura et al. (1975), Nakamura (1982) | The aseptic seed germination was observed. |
| | | | | Umata et al. (2006) | No fungal peloton was observed in the protocorms obtained from *in situ* seed germination. |
| | | | | Motomura et al. (2010) | |
| | | | | Suetsugu et al. (2020b) | |
Table 1 (continued)

| Plant taxa | Taxonomic affiliation of mycobionts | Analysesa | References | Notes |
|------------|------------------------------------|-----------|------------|-------|
| **Didymoplexis micradenia (D. minor)**<sup>b</sup> | Agaricales | *Marasmius coniatus var. didymoplexis* | Symbiotic culture, sporocarp formation | Burgeff (1932, 1936, 1959) |
| *D. pallens* | Agaricales | *Marasmius coniatus var. didymoplexis* | Symbiotic culture, sporocarp formation | Burgeff (1932, 1936, 1959) |
| Epipogium roseum | Agaricales | *Coprinellus (Coprinus)*<sup>b</sup>, *Psathyrella* | Molecular identification | Yamato et al. (2005) |
| | Agaricales | *Coprinellus* | Molecular identification, symbiotic culture | Yagame et al. (2007) |
| Erythrorchis altissima (<i>Galeola altissima, E. ochobiensis</i>)<sup>b</sup> | Hymenochaetales | *Coprinellas disseminatus* | Sporocarp formation | Yagame et al. (2008) |
| | Hymenochaetales | *Erythromyces crocicreas (<i>Hymenochaete crociceras</i>)*<sup>b</sup> | Isolate characteristics | Hamada and Nakamura (1963) |
| | Agaricales | *Lentinula edodes* | Symbiotic culture | Umata (1998a) |
| | | *Lyophyllum shineji* | Symbiotic culture | Umata (1997a) |
| | | *Pleurotus ostreatus* | Symbiotic culture | Umata et al. (2000a) |
| | | *Gymnopus, Hypholoma, Mycena, Neonothopanus* | Molecular identification | Ogura-Tsujita et al. (2018) |
| Atheliales | Athelia | | Molecular identification | Ogura-Tsujita et al. (2018) |
| Auriculariales | *Auricularia polytricha* | Symbiotic culture | Umata (1997b) |
| Cantharellales | Ceratobasidium, *Tulasiella* | Molecular identification | Ogura-Tsujita et al. (2018) |
| Corticales | *Vuilleminia* | Molecular identification | Ogura-Tsujita et al. (2018) |
| Hymenochaetales | *Erythromyces crocicreas* | Symbiotic culture | Umata (1995, 1998b) |
| | *Phellinus sp.* | Symbiotic culture | Umata (1995, 1998b) |
| | *Phellinus gilvus, Phellinus wahlbergii* | Symbiotic culture | Umata et al. (2000a) |
| | *Fuscoporia, Hymenochaetaceae* | | Molecular identification | Ogura-Tsujita et al. (2018) |
| | *Fomitopsis vinosa, Lentinus sajor-caju, Panus tigrinus* | Symbiotic culture | Umata et al. (2000a) |

<sup>a</sup> Analyses include: Symbiotic culture, sporocarp formation, molecular identification, symbiotic culture, isolate characteristics, and additional information as noted.
| Plant taxa | Taxonomic affiliation of mycobionts | Analyses | References | Notes |
|------------|-----------------------------------|----------|------------|-------|
|            | **Order** **Taxa**                |          |            |       |
| **Russula**| **Hericiaceae, Xylolus amnus**    | Molecular identification | Umata et al. (2000a) | |
|            | **Asterostroma, Coniophora, Russula, Scytinostroma** | Symbiotic culture | Umata et al. (2000a) | |
|            | **Sebacinales Russula**           | Molecular identification | Ogura-Tsujita et al. (2018) | |
|            | **Trechisporales**                | Molecular identification, stable isotopes | Ogura-Tsujita et al. (2018) | |
|            | **Erythrorchis cassythoides**     | Molecular identification | Dearnaley (2006) | The Gymnopus sequence was nested within the Marasmiellus clade (Fig. S2). |
|            | **Eulophia zollingeri**           | Molecular identification | Ogura-Tsujita and Yukawa (2008) | |
|            | **Galeola falconeri**             | Radiocarbon | Suetsugu et al. (2006) | The Meripilaceae fungus was identified as Physisporinus by Yamashita et al. (2020). |
|            | **G. nudifolia (G. hydra)**       | Molecular identification, stable isotopes | Ogura-Tsujita et al. (2018) | |
|            | **Gastrodia appendiculata**       | Molecular identification, stable isotopes | Lee et al. (2015) | |
|            | **G. callosa**                    | Mycelia without clamp connection | Burgeff (1932, 1959) | |
|            | **G. confusa**                    | Molecular identification, stable isotopes | Ogura-Tsujita et al. (2009) | Mycena was the most dominant. The sequences of Clitocybula and Gymnopus were nested within the hydropoid and Marasmiellus clades, respectively (Fig. S2). |
| Plant taxa         | Taxonomic affiliation of mycobionts | Analysesa                                      | References                      | Notes                                                                 |
|-------------------|-------------------------------------|-----------------------------------------------|---------------------------------|----------------------------------------------------------------------|
| Order             | Taxa                                |                                               |                                 |                                                                      |
| Cantharellales    | *Mycena*                            | Molecular identification, symbiotic culture   | Shimaoka et al. (2017)          |                                                                      |
| G. cunninghamii   | *Ceratobasidium*                    | Molecular identification                      | Ogura-Tsujita et al. (2009)     | Rhizomorphs were attached to the tuber surfaces.                     |
| Agaricales        | *Armillaria mellea*                 | Morphology of rhizomorph                      | Campbell (1962)                 |                                                                      |
|                   |                                     |                                               |                                 |                                                                      |
| G. elata          | *Armillaria mellea*                 | Rhizomorph morphology                         | Kusano (1911)                   | See also the review by Xu and Guo (2000) and Liu et al. (2010) for G. elata study. |
| A. gallica        | SI test                             |                                               | Mohammed et al. (1994)          |                                                                      |
| A. gallica, A. jezoensis, A. ostoyae, A. sinapina, A. singula | SI test, isozyme                     |                                               | Cha and Igarashi (1995)        |                                                                      |
| A. cepistipes, A. gallica, A. nabsnoma | SI test                           |                                               | Kikuchi et al. (2008a)          |                                                                      |
| A. nabsnoma       | SI test, molecular identification    |                                               | Sekizaki et al. (2008)          |                                                                      |
| Armirallia (seven lineages) | Molecular identification     |                                               | Guo et al. (2016)               |                                                                      |
| Armirallia        | Molecular identification, symbiotic culture |                                               | Yeh et al. (2017)              |                                                                      |
| *Mycena anoectochila* | Symbiotic culture                  |                                               | Guo et al. (1997)               |                                                                      |
| *Mycena dendrobl* | Symbiotic culture                   |                                               | Guo et al. (1999), Pan et al. (2015) |                                                                      |
| *Mycena orchidicola* | Symbiotic culture                 |                                               | Fan et al. (1996)               |                                                                      |
| *Mycena osmundicola, Mycena osmundicola* | Symbiotic culture             |                                               | Hong et al. (2002)              |                                                                      |
| *Mycena osmundicola* | Sporocarp formation, symbiotic culture |                                               | Xu and Guo (1989)               |                                                                      |
| *Armillaria mellea, Mycena osmundicola* | Symbiotic culture               |                                               | Kim et al. (2006)               |                                                                      |
| *Mycena*          | Molecular identification, symbiotic culture |                                               | Park and Lee (2013a)            |                                                                      |
| *Armillaria mellea, Mycena* | Symbiotic culture              |                                               | Park and Lee (2013b)            |                                                                      |
| *Mycena*          | Symbiotic culture, TEM              |                                               | Li et al. (2020)                |                                                                      |
| Agaricales and others | Unidentified Agaricales and others | Illumina sequencing                           | Chen et al. (2019)              | Identified from seedlings.                                           |
| Hymenochaetales   | *Resinicium*                        | Illumina sequencing                           | Chen et al. (2019)              | Identified from seedlings.                                           |
|                   |                                     | Radiocarbon                                   | Suetsugu et al. (2020b)         |                                                                      |
| Plant taxa | Taxonomic affiliation of mycobionts | Analyses<sup>a</sup> | References | Notes |
|-----------|------------------------------------|----------------------|------------|-------|
| G. flabilabella | Agaricales | *Hydropus* | Molecular identification, stable isotopes | Lee et al. (2015) |
| G. fontinalis | Agaricales and others | *Mycena* and others | Illumina sequencing | Liu et al. (2015) |
| G. javanica | Agaricales | *Xerotus javanicus* | Sporocarp formation | Burgeff (1936, 1959) |
| G. lacista | – | – | Stable isotopes | Sommer et al. (2012) |
| G. minor | – | Clamp bearing fungus | Isolate characteristics | Campbell (1963) |
| G. nanoensis | Agaricales | *Mycena* | Molecular identification, stable isotopes | Lee et al. (2015) |
| G. nipponica | Agaricales | *Crinipellis, Clitocybula, Gymnopus, Marasmiellus, Marasmius, Mycena* | Molecular identification | Kinoshita et al. (2016) |

<sup>a</sup> The sequences of *Crinipellis, Clitocybula, Gymnopus, Marasmiellus* and *Marasmius* were spread into the Omphalotaceae, Marasmiaceae and hydropoid clades (Fig. S2).
### Table 1 (continued)

| Plant taxa | Taxonomic affiliation of mycobionts | Analyses$^a$ | References | Notes |
|------------|-------------------------------------|-------------|------------|-------|
| **Polyporales** |                                     |             |            |       |
| Diplomitoporus rimosus | Molecular identification | Kinoshita et al. (2016) | Fungal ITS sequences had 100% similarity with *D. rimosus*. |
| Diplomitoporus rimosus | Molecular identification, symbiotic culture | Shimaoka et al. (2017) | Fungal ITS sequence from protocorm shared 558/559 bp identity with that from *D. rimosus*. |
| – | Symbiotic culture | Umata et al. (2000b) |         |
| **G. sesamoides** | Polyporales | Probably *Fomes mastoporus* | Field observation | Campbell (1964) | Mycelium, that was similar to the mycobiont of *G. sesamoides*, was traced to the sporocarp of *F. mastoporus*. |
| Agaricales | Campanella, Marasmius | Molecular identification, stable isotopes | Dearnaley and Bougoure (2010) | The sequences of *Campanella* and *Marasmius* were nested within the campanelloids and Omphalotaceae clades, respectively (Fig. S2). |
| – | Clamp bearing fungus | McLenan (1959) |             |
| – | Stable isotopes | Gomes et al. (2020) |         |
| **Resinicium, Mycena, Gymnopus** | Hymenochaetales | Molecular identification, stable isotopes | Martos et al. (2009) | *Resinicium* is the most dominant. The *Gymnopus* sequence was nested within the *Marasmiellus* clade (Fig. S2). |
| **G. similis** | Clamp bearing isolates from *G. nipponica* and *G. verrucosa* | Symbiotic culture | Tashima et al. (1978) | Plant identification is erroneous and may represent either *Gasrotina confusa* or *G. pubilabia* (H. Umata, personal communication). |
| **Wallischlaegelia calcarata** | Agaricales | *Gymnopus, Mycena* | Molecular identification | Martos et al. (2009) | A species was wrongly identified as *W. aphylla* in Martos et al. (2009) (see Hatté et al. 2020). The *Gymnopus* sequences were nested within the two clades of *Omphalotaceae* (Fig. S2). |
| **Yoania amagiensis** | Polyporales | *Physisporinus* (four OTUs) | Molecular identification | Yamashita et al. (2020) | Asymbiotic culture was also achieved. |
| **Y. flavia** | – | Unidentified isolate from *Y. flavia* rhizome | Symbiotic culture | Tsuda et al. (2004) | A single *Physisporinus* OTU is dominantly detected. |
| Polyporales | *Physisporinus* (a single OTU), Thelephoraceae$^c$ | Molecular identification | Yamashita et al. (2020) | |
Diversity of mycobionts in SAP-MHPs

Mycobionts of SAP-MHPs include leaf-litter or wood basidiomycete fungi (Table 1). Leaf-litter-decaying fungi colonize the topsoil and decompose plant leaf litter and other soil organic matter (Osono 2007), whereas wood-decaying fungi inhabit living trees, the trunks of standing dead trees, stumps, or fallen logs and degrade wood lignocellulose (Stokland et al. 2012). The activities of enzymes that catalyze the degradation of natural polymers, including lignin and plant cell-wall polysaccharides (mainly cellulose and hemicellulose), in both fungal groups are higher than those in AM and ECM fungi (Kohler et al. 2015).

Because of their saprotrophic nature, mycobionts of MHPs isolated from plant roots are amenable to pure culture, facilitating the identification of fungal species. Fungal isolates often develop basidiocarps on culture medium and can be identified morphologically (Burgeff 1932; Kikuchi et al. 2008a; Terashita and Chuman 1987; Xu and Guo 2000; Yagame et al. 2008, 2018).

Most leaf-litter-decaying fungi that associate with MHPs belong to families in Agaricales, such as Mycenaceae, Marasmiaceae, and Omphalotaceae, which are the main fungal partners of Gastrodia and Didymoplexis (Table 1). These fungi are found worldwide as common saprobes in decaying plant materials (Kirk et al. 2008), but their taxonomic status is controversial (Wilson and Desjardin 2005). We conducted a phylogenetic analysis using published internal transcribed spacer (ITS) sequences of mycobionts of SAP-MHPs, including fungi molecularly identified as Marasmius, Marasmiellus, Gymnopus, Clitocybula, Crinipellis, Campanella, and Hydropus (Fig. S2). Recent studies that updated the phylogenetic placement of these fungal lineages (Antonín et al. 2019; Oliveira et al. 2019; Sandoval-Leiva et al. 2016) were employed as references. The sequences mostly clustered in Marasmiaceae and Omphalotaceae, with some in the hydropoid clade, which mainly includes wood-decaying fungi (Antonín et al. 2019). Most of the sequences clustered in the Marasmiellus clade, and the mycobionts of Gastrodia similis Bosser, Gastrodia pubilabiata Sawa, and Gastrodia confusa Honda and Tuyama were closely related with 100% bootstrap support (BS). Furthermore, mycobionts of G. pubilabiata and Gastrodia nipponica (Honda) Tuyama were closely related within the Marasmiellus, campanelloid, and Porotheleum clades (99–100% BS). These results indicate that particular fungal lineages are associated with SAP-MHPs, although a variety of fungal species participate in mycoheterotrophy. Members of Mycena are the most common mycobionts of Gastrodia species (Table 1). Although Mycena species are pure saprophytes, recent in vitro investigations revealed that several Mycena species
have saprotrophic and biotrophic capacities (Thoen et al. 2020). These species can penetrate tree roots, and one Mycena species facilitated nutrient transfer to the plant. Interestingly, mycobionts of G. pubilabiata and G. nipponica detected by Kinoshita et al. (2016) exhibit high sequence similarity (>98%) with three of these Mycena species—Mycena galopus (Pers.) P.Kumm., Mycena albidoilacea Kühner and Maire, and Mycena olivaceo-marginata (Massée) Massée. Therefore, Mycena species that associate with SAP-MHPs could have biotrophic potential, and further evaluation of their trophic mode is warranted.

The wood-decaying fungi that associate with SAP-MHPs are predominantly members of Agaricales (Basidiomycota), such as Armillaria (Physalacriaceae), Psathyrella (Psathyrellaceae), and Coprinellus (Psathyrellaceae) (Table 1). Armillaria species are the main symbionts of Gastrodia elata Blume (Kusano 1911) and Cyrtosia septentrionalis (Rehb.f.) Garay (Fig. 1c; Hamada 1939). Mycobionts from G. elata and C. septentrionalis formed sporocarps and were identified as Armillaria gallica Maxm. & Romagn. (Kikuchi et al. 2008a) and Armillaria tabescens (Scop.) J.E. Lange (Yagame et al. 2008) and Coprinellus domesticus (Bolton) Vilgalys, Hopple & Jacq. Johnson (Yagame et al. 2018), respectively, by sporocarp morphology and molecular identification. Besides Agaricales fungi, SAP symbionts include wood-decay fungi from other Basidiomycota orders. Gastrodia similis, a tropical MHP, associates with Resinicium of the Hymenochaetales (Martos et al. 2009). Wood-decay basidiomycetes of the Trechisporales, Polyporales, Corticiaceae, Russulaceae, and Atheriales were found in Epipogium roseum and Eupholia zollingeri (Ogura-Tsujita and Yukawa 2008; Yamato et al. 2005). Mycobionts of E. roseum and Cremastra aphylia (Fig. 1a) were identified as Coprinellus disseminatus (Pers.) J.E. Lange (Yagame et al. 2008) and Coprinellus domesticus (Bolton) Vilgalys, Hopple & Jacq. Johnson (Yagame et al. 2018), respectively, by sporocarp morphology and molecular identification. Besides Agaricales fungi, SAP symbionts include wood-decay fungi from other Basidiomycota orders. Gastrodia similis, a tropical MHP, associates with Resinicium of the Hymenochaetales (Martos et al. 2009). Wood-decay basidiomycetes of the Trechisporales, Polyporales, Corticiaceae, Russulaceae, and Atheriales were found in roots of a climbing MH orchid, Erythrorchis altissima (Blume) Blume (Fig. 1d; Ogura-Tsujita et al. 2018). Symbioses between multiple species of wood-decay fungi and E. altissima were confirmed by in vitro symbiotic culture by Umata (1995, 1997ab, 1998ab, 1999; Table 1). Further, Yamashita et al. (2020) found Physisporinus (Meripilaceae, Polyporales) as a fungal partner of SAP-MHPs. This genus is predominantly associated with Yoania species (Fig. 1e) as well as two other SAP-MH genera, Cyrtosia and Galeola. Although the litter-decay fungi found in SAP-MHPs comprise three Agaricales families; i.e., Mycenaceae, Marasmiaceae, and Omphalotaceae, highly divergent wood-decay fungal families are involved in SAP-MH associations.

A wood-decaying fungus, Armillaria mellea (Vahl) P. Kumm. sl, was reported to be a symbiont of SAP-MHPs by Kusano (1911) and Hamada (1939). This fungus is one of the largest and longest-lived terrestrial organisms and has been reported to cover an area of up to 965 ha with an age of up to ca. 8650 years (Ferguson et al. 2003). Therefore, associating with A. mellea sl allows MHPs access to the huge carbon pool in forests. This fungus has been recognized as a species complex (Korhonen 1978), and seven Armillaria species associated with Japanese Gastrodia elata have been recognized for A. mellea sl (Cha and Igarashi 1995; Kikuchi et al. 2008b). At least seven Armillaria lineages are associated with Chinese G. elata (Guo et al. 2016). Although A. mellea sl includes pathogens that cause root-rot disease in woody plants, Armillaria gallica, a major symbiont of G. elata, is a weak pathogen that inhabits decayed wood and litter (Mohammed et al. 1994). Rhizomorphs, i.e., linear mycelial organs, are well-developed in A. mellea sl (Roll-Hansen 1985) and are often attached to the tuber surface of G. elata (Fig. 1g; Kusano 1911). Such rhizomorphs can be traced from plant tubers or roots to decayed wood (Cha and Igarashi 1996; Kikuchi et al. 2008b). A. mellea rhizomorphs transport water and phosphate efficiently (Cairney et al. 1988), indicating that fungal rhizomorphs play an important role in nutrient transport between MHPs and rhizomorph-forming mycobionts.

ECM fungi occasionally associate with SAP-MHPs although dominant fungal partners are SAP fungi (more than half of total abundance). In such cases, SAP-MHPs are simultaneously associated with both ECM and SAP fungi. ECM Russula fungi were found to associate with Erythrorchis cassythoides (A.Cunn. ex Lindl.) Garay (Dearnaley 2006) and Erythrorchis altissima (Ogura-Tsujita et al. 2018). Mycobionts of Gastrodia nipponica (Fig. 1f) included ECM fungi in Russulaceae (8.6% in frequency) and Sebacinaeae (6.2%) as well as SAP fungi (Kinoshita et al. 2016). Because those ECM fungi are the main symbionts in ECM-MHPs (Ogura-Tsujita et al. 2012; Selosse et al. 2002; Taylor and Bruns 1999), they could also be symbiotic with SAP-MHPs although they are not main symbionts.

Most orchids are associated with so-called rhizoctonia fungi, including those in the basidiomycete families Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae (Rasmussen 2002; Yukawa et al. 2009). Associations with rhizoctonia fungi are occasionally observed in SAP-MHPs, such as Erythrorchis altissima (Ogura-Tsujita et al. 2018) and Gastrodia species (Kinoshita et al. 2016). Rhizoctonia fungi exhibit divergent trophic strategies; they can be plant pathogens, endophytes, saprophytes, orchid myccorrhizal or ectomycorrhizal fungi (Roberts 1999; Veldre et al. 2013). However, rhizoctonia fungi isolated from leafy orchid roots are saprophytes and are thus able to obtain nutrients from plant debris (Rasmussen 1995). These fungi are involved in initial or partial mycoheterotrophy in leafy Orchidaceae species (Schiebold et al. 2018; Stöckel et al. 2014). Although rhizoctonia fungi are involved in mycoheterotrophy with
the albino forms of usually chlorophyllous orchid species (Suetsugu et al. 2019), saprophytic rhizoctonia fungi are only occasionally associated with fully mycoheterotrophic species. This suggests that rhizoctonia fungi possess insufficient physiological functionality to support the growth of full MHPs (Martos et al. 2009).

Specificity of mycobionts

Most land plants have generalized associations with AM or ECM fungi (Smith and Read 2008), but AM-MHPs (Gomes et al. 2017; Merckx and Bidartondo 2008; Yamato et al. 2011) and ECM-MHPs (Ogura-Tsujita et al. 2012; Selosse et al. 2002; Taylor and Bruns 1999) typically have highly specific associations with a narrow phylogenetic range of fungi. In SAP-MHPs, the specificity varies among plant species, from a highly specific association with a single fungal species to broad interactions with multiple fungal orders. Individuals of the SAP-MHP Eulophia zollingeri from seven populations in Japan, Taiwan, and Myanmar associate exclusively with Psathyrella candolleana (Fr.) Maire sl (Ogura-Tsujita and Yukawa 2008). Most mycobionts of Gastrodia confusa from 10 populations separated by 5–1000 km belong to three fungal groups in the genus Mycena (Fig. 1b; Ogura-Tsujita et al. 2009). By contrast, Gastrodia pubilabiata, a species closely related to G. confusa, associates with multiple groups of litter-decaying fungi in the families Mycenaceae, Marasmiaceae, and Omphalotaceae (Fig. S2; Kinoshita et al. 2016). Further, a close relative of these Gastrodia species, Gastrodia nipponica, associates with wood-decaying and ECM fungi in addition to litter-decaying fungi, and its mycobionts exhibited significantly higher sequence divergence than those of G. confusa and G. pubilabiata (Kinoshita et al. 2016). The giant mycoheterotroph, Erythrorchis altissima, is an extreme example of low fungal specificity in full MHPs. In total, 37 fungal species belonging to nine orders of Basidiomycota, which mainly include wood-decaying fungi but also ECM and rhizoctonia fungi, have been identified in the roots of this MHP (Ogura-Tsujita et al. 2018). Mycobiont specificity in SAP-MHPs often varies within a single host plant genus, as in Gastrodia (Kinoshita et al. 2016) and Yoania (Yamashita et al. 2020). Therefore, specificity can fluctuate greatly during host-plant speciation.

Fungal partner shift during the plant life cycle

The fungal partner often changes during the life cycle of an SAP-MHP. A mycoheterotrophic orchid, Gastrodia elata, switches its fungal partner upon transitioning from the juvenile to adult stage. The litter-decaying fungus, Mycena, induces seed germination, whereas the wood-decaying Armillaria supports further development of the mature plant (Xu and Guo 2000). A recent high-throughput sequencing study suggests that more diverse fungal groups than previously assumed are involved at the juvenile stage of G. elata (Chen et al. 2019). Partner switching also seems to occur in Cyrtosia septentrionalis, the adult stage of which is associated with Armillaria (e.g., Hamada 1939). However, C. septentrionalis seeds failed to germinate with Armillaria isolates in vitro (Terashita 1985), and Physisporinus, a wood-decaying fungus, promoted germination in situ (Umata et al. 2013). Changing the fungal partner at some stage of the life cycle seems riskier than living with the same partner. Switching to a fungus with a large biomass, such as Armillaria, allows access to a large carbon pool, thus possibly outweighing the risk of partner shifting. Armillaria produces abundant rhizomorphs in soil (Smith et al. 1992), which increases the likelihood of successful colonization.

Evolution of mycorrhizal interactions

During the evolution from autotrophy to mycoheterotrophy, the associated mycorrhizal fungi have switched to different fungal communities in some instances (Jacquemyn and Merckx 2019; Ogura-Tsujita et al. 2012; Yagame et al. 2016). Most leafy relatives of SAP-MHPs are associated with rhizoctonia fungi, suggesting that the mycorrhizal community shifted from rhizoctonia to litter- or wood-decaying fungi during the evolution of SAP-MHP lineages. The chlorophyllous genus Vanilla is most closely related to three genera containing SAP-MHPs in the tribe Vanilleae (Cameron 2011; Cameron et al. 2009; Fig. S1) and mainly associates with rhizoctonia fungi, including Tulasnellaecae and Ceratobasidioecae (Porras-Alfaro and Bayman 2007). Mycobionts may have shifted from rhizoctonia to diverse wood-decaying fungi in accordance with the evolution of Galeola-Cyrtosia and Erythorchis. The leafy genus Calypso is likely sister to SAP-MHP-containing Yoania (Freudenstein et al. 2017) and forms associations with Tulasnellaceae and Ceratobasidioecae (Currah et al. 1988; Taylor and McCormick 2008). This suggests that fungal partners have been switched from rhizoctonia fungi to the wood-decaying Physisporinus with the shift to SAP-MH in Yoania. Rhizoctonia fungi are often found as free-living saprotrophs (Roberts 1999), and mycorrhizal associations with these fungi are unique to Orchidaceae (Yukawa et al. 2009). This ability to associate with free-living fungi in orchids might have triggered the evolution of full mycoheterotrophy dependent on saprotrophic non-rhizoctonia fungi.

Mycorrhizal communities are typically switched via a phase of dual association, which involves mycobionts of
both leafy and leafless plants, during the evolution of mycoheterotrophy. In the orchid genus *Cymbidium*, mycobionts were compared within a clade in which mycoheterotrophy evolved (Ogura-Tsujita et al. 2012). A leafy outgroup species, *Cymbidium dayanum* Rchb.f., associates mainly with rhizoctonia fungi from Tulasnellaceae, whereas two MHPs, *Cymbidium macrorhizorum* Lindl. and *Cymbidium aberrans* (Finet) Schltr., mostly associate with ECM fungi in Sebacinae. By contrast, the leafy sister taxa of the two MHPs, *Cymbidium lancifolium* Hook. and *Cymbidium goeringii* (Rchb.f.) Rchb.f., associate with both rhizoctonia fungi and several ECM fungal families, suggesting that fungal partners have switched via a dual association with both rhizoctonia and ECM fungi. Such dual associations could trigger mycorrhizal switching and play an important role in the evolution of MHPs. Another case of evolution via a dual association with wood-decaying fungi was found in the genus *Cremastr*a (Yagame et al. 2018). A SAP-MHP, *Cremastr*a *aphylla*, which mainly associates with *Coprinellus*, a wood-decaying fungus, and its leafy sister species, *Cremastr*a *appendiculata* (D.Don) Makino, can associate with both rhizoctonia fungi and *Coprinellus* (Freudenstein et al. 2017; Nishikawa and Ui 1976; Yagame et al. 2013). This suggests that the type of mycorrhizal fungi in the symbiosis had been switched to wood-decaying fungi via a dual association. A novel association with *Coprinellus* probably triggered the evolution of the SAP-MHP.

Symbiotic associations with SAP fungi are occasionally found in leafy orchid species. The wood-decaying fungus *Psathyrella* is often found associating with leafy orchids, such as *Oeceoclades maculata* (Lindl.) Lindl. (Bayman et al. 2016) and *Satyrium nepalense* D.Don (Jyothsna and Purushothama 2014). Seeds of the former orchid species germinated *in vitro* only in association with *Psathyrella*, whereas adult plants associate with rhizoctonia fungi in addition to *Psathyrella* (Bayman et al. 2016). Such high specificity for SAP fungi during seed germination could represent an initial stage of the evolution of an SAP-MHP. *Mycena* fungi are one of the main symbionts of *Gastrodia* SAP-MHPs and also promote seed germination and seedling growth in *Dendrobium* epiphytic green orchids (Guo et al. 1997; Zhang et al. 2012). Interestingly, *Mycena anoectochila* L. Fan & S.X. Guo, which was isolated from the leafy orchid *Anoeectochilus roxburghii* (Wall.) Lindl., induced seed germination in the mycoheterotrophic *Gastrodia elata* (Guo et al. 1997). These results suggest that the same fungus can associate with both leafy and leafless orchids. *Mycena* fungi were also found to associate with the terrestrial orchids *Cymbidium sinense* (Andrews) Willd. (Fan et al. 1996), *Goodyera repens* (L.) R.Br. (Voronina et al. 2018), and *Bletilla striata* (Thunb.) Rchb.f. (Guo and Xu 1992). Members of Trechisporales, Polyporales, Corticiales, and Hymenochaetales, which largely comprise wood-decaying fungi, have been occasionally found in tropical epiphytic orchids (Kartzinel et al. 2013; Martos et al. 2012). Although these cases could reflect opportunistic associations in leafy orchids, a symbiotic relationship with SAP fungi may be more adaptive than that with rhizoctonia fungi in some environments and trigger the evolution of SAP-MHPs (Selosse et al. 2010).

Convergent evolution of mycorrhizal specificity toward particular fungal lineages has occurred in several SAP-MH lineages. Associations with *Armillaria* have been observed for *Cyrtosia septentrionalis* (Subfamily Vanilloideae) and *Gastrodia elata* (Subfamily Epidendroideae) (Fig. 2). For instance, *Armillaria gallica* associates with both these phylogenetically distant orchids exhibiting SAP-MH (Kikuchi et al. 2008a; Terashita 1996). Two SAP-MHPs among several tribes of Epidendroideae, *Epipogium roseum* and *Eulophia zollingeri*, associate with *Psathyrellaceae* fungi (Ogura-Tsujita and Yukawa 2008; Yamato et al. 2005). Sequences of the nuclear ribosomal ITS region of *Psathyrella* fungi isolated from these two orchids had >97% similarity (Ogura-Tsujita and Yukawa 2008). Such convergent evolution has also been found in AM- and ECM-MHPs. AM fungi from AM-MHPs belonging to different plant families, such as Burmanniaceae and Corsiaceae, were grouped into the same taxa (>97% small subunit rRNA sequence similarity; Gomes et al. 2019; Merckx et al. 2012). The mycobionts in Sebacinae from the ECM-MHP *Hexalectris* (Epidendreae) are closely related to those from *Neottia nidus-avis* (L.) Rich. (Neottieae) (>98% ITS sequence similarity; Kennedy et al. 2011). Mycobionts of MHPs may converge on particular fungal taxa that support high nutrient acquisition.

Mycorrhizal interactions have changed during the speciation of MHPs. The species of mycobionts in the associations have changed or fungal specificity has changed from broad to narrow, such that the same mycobiont species is shared among several plant species (Kinoshita et al. 2016). In SAP-MHPs, fungal specificity differed among the three closely related *Gastrodia* species, *G. confusa*, *G. pubilabiata*, and *G. nipponica*, all of which are associated with litter-decaying fungi within Mycenaceae, Marasmiaceae, and Omphalotaceae (Kinoshita et al. 2016). The fungal specificity of *G. confusa* was significantly greater than that of *G. pubilabiata* and *G. nipponica*, indicating that specificity fluctuates during speciation. Interestingly, *G. confusa* exclusively inhabits bamboo thickets, the mycorrhizal communities of which differ significantly from those of other vegetation. This suggests that adaptation to particular fungi inhabiting bamboo thickets triggered the speciation of *G. confusa*. The mycobionts of 15 *Gastrodia* species have been surveyed; Mycenaceae, Marasmiaceae, or Omphalotaceae fungi were reported to associate with ten species (Table 1). This suggests that these litter-decaying fungi are main fungal partners for *Gastrodia* species. Associations with the wood-decaying fungi *Armillaria* and *Resinicium* could have appeared during the
evolution of *Gastrodia elata* and *Gastrodia simillis*. Furthermore, a symbiotic relationship with ECM fungi has been observed in *G. nipponica*, which associates with both litter-decomposing and ECM fungi. Both SAP- and ECM-MHPs have appeared in the genus *Epipogium* (Roy et al. 2009; Yamato et al. 2005). Although because of the poor phylogenetic resolution of this genus it is unclear which MHP type evolved earlier, the fungal partner could be switched to SAP or ECM fungi even in closely related taxa. Fungal partner switching during speciation has been reported in AM- and ECM-MHPs. Two sister ECM-MHPs, *Corallorhiza maculata* (Raf.) Raf. and *Corallorrhiza mertensiana* Bong., specifically associate with different Russulaceae fungal taxa (Taylor and Bruns 1999). Fungal specificity differs among the six ECM-MHPs within *Neottia* (Yagame et al. 2016). Five AM-MHPs in the genus *Afrothisia* exhibit high fungal specificity for different Glomeraceae fungal taxa (Merckx and Bidartondo 2008; Merckx et al. 2012). These results suggest that switching between mycorrhizal partners accelerated the speciation of MHPs.

**Isotopic signature of SAP-MHPs**

Nutrient fluxes between MHPs and mycorrhizal fungi have been studied using stable isotope natural abundance analysis (Gebauer and Meyer 2003; Hynson et al. 2013). Because fungal-derived carbon and nitrogen are highly enriched in $^{13}$C and $^{15}$N, the tissues of MHPs are expected to also be enriched in carbon and nitrogen isotopes compared to the surrounding autotrophic plants (Gebauer and Meyer 2003). This approach has been applied to examine a variety of ECM-MHPs (Liebel and Gebauer 2011; Motomura et al. 2010; Roy et al. 2009). AM-MHPs (County et al. 2011; Gomes et al. 2020; Mercck et al. 2010), and several SAP-MHPs (Lee et al. 2015; Martos et al. 2009; Ogura-Tsujita et al. 2009). The relative enrichment levels of isotopes confirmed the mycoheterotrophy of those species. All three types of MHPs are highly enriched in $^{13}$C, but the $^{15}$N enrichment level is lower in SAP- and AM-MHPs than in ECM-MHPs. The difference is attributable to the greater enrichment of nitrogen isotopes in ECM fungi than in AM and SAP fungi, as a result of their different nitrogen acquisition strategies. Interestingly, litter-decomposing fungi are generally depleted in carbon isotopes relative to wood-decaying fungi (Kohzu et al. 1999), likely because wood is more enriched in $^{13}$C than leaf tissue (Gebauer and Schulze 1991). Lee et al. (2015) showed that $^{13}$C was significantly less enriched in SAP-MHPs associated with the litter-decomposing fungi—i.e., *Gastrodia appendiculata* C.S.Lou & N.J.Chung, *Gastrodia fontinalis* T.P.Lin, and *Gastrodia nantoensis* T.C.Hsu & C.M.Kuo ex T.P.Lin—than in those associated with wood-decaying fungi. However, there are insufficient studies of the isotopic signature of SAP-MHPs, and further work is required to clarify the isotopic signatures of litter- and wood-decomposing fungi and elucidate the physiological ecology of SAP-MHPs.

Stable isotope analysis can be used to distinguish between partial and full mycoheterotrophy (Gebauer and Meyer 2003). The linear two-source mixing model estimates the proportion of carbon and nitrogen gain from photosynthesis and mycorrhizal fungi. The endpoint of this model is a value that falls between those of co-occurring autotrophic plants (0% nutrient gain from fungi) and full MHPs (100% nutrient gain from fungi) (Preiss and Gebauer 2008). The level of mycoheterotrophy has been quantitatively assessed for various leafy plant species associated with ECM (Abadie et al. 2006; Bidartondo et al. 2004; Matsuda et al. 2012) and AM (Suetsugu et al. 2020a) fungi using this model, but little is known of those associated with SAP fungi. A preliminary isotope analysis by Yagame et al. (2015) showed that the leafy orchid *Cremastria appendiculata*, which associates with wood-decaying fungi, is partially mycoheterotrophic. Stable isotope analysis may reveal more diverse partial SAP-MHPs if applied to leafy orchids associated with non-rhizoctonia SAP fungi.

Suetsugu et al. (2020b) estimated the age of carbon in SAP-MHP tissue by measuring the natural abundance of radiocarbon (nuclear weapon-derived $^{14}$C). This approach traces the time elapsed since carbon isotopes derived from the nuclear-weapon tests of the 1950s and 1960s were fixed from atmospheric CO$_2$ by photosynthesis. The carbon utilized by wood decomposing fungus-dependent MHPs was fixed 10–40 years before that fixed by ECM-MHPs (Suetsugu et al. 2020b). The carbon in SAP-MHPs associated with litter-decomposing fungi was estimated to be 6.7–9.9 years old (Hatté et al. 2020), suggesting that SAP-MHPs associated with wood-decaying fungi use older carbon than those associated with litter-decaying fungi. This technique will enable investigations of nutrient flows via mycoheterotrophy from decomposing plant debris.

**Culturing SAP-MHPs**

AM and ECM fungi are almost obligately biotrophic, i.e., dependent on autotrophic plants for their carbon supply. Thus, AM- and ECM-MHPs require a chlorophyllous host plant for co-culture with appropriate symbiotic fungi in vitro (Mckendrick et al. 2000). By contrast, mycobionts of SAP-MHPs can grow in pure culture and stimulate seed germination and further seedling growth in vitro (Burgeff 1932; Yagame et al. 2007). Field or container culture has been established for several SAP-MHPs (Shimaoka et al. 2017;
et al. 1975). Interestingly, the concentrations were similar to those in soil, implying that in its natural habitat, the seed does not require direct mycobiont contact for germination (Umata et al. 2013). Aseptically germinated seedlings developed inflorescences in *Didymoplexus pallens* (Irawati 2002), and rhizome formation was observed in an asymbiotic culture of *Yoania flava* K.Inoue & T.Yukawa (Tsuda et al. 2004). Aseptic propagation via an embryogenic callus was demonstrated in *Gastrodia elata*, and the regenerated tubers continued to grow after inoculation of *Armillaria* isolates (Yeh et al. 2017). Seeds of *Gastrodia pubilabiata* successfully germinated without symbionts, and their subsequent development was controlled by illumination (Godo et al. 2020).

**Future perspectives**

Fully mycoheterotrophic plants associated with SAP fungi have to date been found only in Orchidaceae and have evolved independently at least nine times within two subfamilies, Vanillioideae and Epidendroideae. A variety of litter- and wood-decaying fungi are involved in mycoheterotrophy in association with SAP-MHPs, and several SAP-MHPs can be cultured with or without mycorrhizal fungi. Culturable SAP-MHPs may be key to addressing many unsolved questions regarding mycoheterotrophy and will contribute to a range of scientific fields. A critical event in the evolution from autotrophy to mycoheterotrophy is fungal partner switching, the replacement of the associated fungal community by another. The mycobionts of most SAP-MHPs have been switched from rhizoctonia fungi to leaf-litter- or wood-decaying basidiomycetes. The benefits gained by plants from mycobionts differ between rhizoctonia and SAP fungi. Plants may select the best fungal partners for nutrient acquisition (Jacquemyn and Merckx 2019; Ogura-Tsujita et al. 2012), thus triggering the evolution of mycoheterotrophy. Symbiotic culture will allow direct comparisons of the relative fitness between plants with rhizoctonia and those with SAP mycobionts. Leafy sister species of fully mycoheterotrophic species, such as *Cremastrra appendiculata*, often associate with rhizoctonia and wood-decaying fungi and could be suitable model plants for such assays. Comparing gene expression between SAP-MHPs and their leafy relatives will clarify the mechanism underlying the evolution from autotrophy to mycoheterotrophy. Field samples are subject to environmental effects, but culture systems facilitate comparisons between autotrophs and mycoheterotrophs.

The physiological mechanisms underlying plant–fungus interactions in MHPs are unclear, but recent studies of SAP-MHPs have provided information on the interactions between MHPs and their mycorrhizal fungi. Transcriptomic and proteomic analyses of *Gastrodia elata* co-cultured with *Mycena* fungi revealed differentially accumulated mRNAs...
and proteins involved in energy metabolism, plant defense, molecular signaling, and secondary metabolism (Zeng et al. 2017, 2018). Fungal digestion was demonstrated during seed germination in *G. elata* co-cultured with *Mycena* (Li et al. 2020). The factors transported from the fungus to the plant are unknown in MHPs, but two sucrose transporter-like genes, *GeSUT4* and *GeSUT3*, were highly expressed in *Armillaria*-colonized *G. elata* tubers, suggesting that sucrose is the major sugar transported between the fungus and *G. elata* (Ho et al. 2020). Symbiotic culture of SAP-MHPs enables broader approaches for physiological studies of mycoheterotrophy, such as those investigating the mechanism of recognition between plant and fungus, and of nutrient transfer from fungus to plant. Furthermore, symbiotic culture allows the comparison of gene expression profiles of plants with and without mycorrhizal fungi, thus providing insights into the physiology of mycoheterotrophy.

The ecology of MHPs is poorly understood because they spend most of their life cycle underground and shoot systems appear only during reproductive phases. For example, the processes of plant development and seasonal growth in many MHP species are considered “black boxes”. A culture system would expand the understanding of the life cycle and phenological properties of MHPs. Container culture of *Epipogium roseum* revealed the developmental process of subterranean seedlings, with stolons and rhizomes produced (Yagame et al. 2007). Rapid clonal propagation of this orchid was also achieved, with a single protocorm producing 80 tubers. Development from seed to flower in several SAP-MHPs was successfully monitored in symbiotic or asymbiotic culture and required 6 months in *E. roseum* (Yagame et al. 2007), 4 months in *Gastrodia pubilabiata* (Shimaoka et al. 2017), and 4–6 months in *Didymoplexis pallens* (Irawati 2002). Many MHPs are endangered worldwide because of habitat loss and climate change (Merckx 2013). A culture system would contribute to the recovery of the SAP-MHP populations. The rhizomes of *Yoania flava* that developed under symbiotic culture were transplanted to the natural habitat and survived for 490 days thereafter (Tsuda et al. 2004). Because MHPs require mycobionts for survival, *ex situ* conservation of plants with their mycobionts is a good strategy for preventing extinction. The cryopreservation of seeds and culture of SAP-MHP symbiont isolates will contribute greatly toward the long-term conservation of SAP-MHPs.

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