The importance of associations with saprotrophic non-\textit{Rhizoctonia} fungi among fully mycoheterotrophic orchids is currently under-estimated: novel evidence from sub-tropical Asia

Yung-I Lee$^{1,2}$, Chih-Kai Yang$^{3,4}$ and Gerhard Gebauer$^{5,*}$

$^1$Biology Department, National Museum of Natural Science, No 1, Kuan-Chien Rd, Taichung, Taiwan; $^2$Department of Life Sciences, National Chung Hsing University, Taichung 40227, Taiwan; $^3$The Experimental Forest, College of Bio-Resources and Agriculture, National Taiwan University, 12 Chienshan Rd., Sec. 1, Chushan Township, Nantou 55750, Taiwan; $^4$Department of Life Science, National Taiwan Normal University, 88 Tingchow Rd., Sec. 4, Taipei 11677, Taiwan and $^5$Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, D-95440 Bayreuth, Germany

* For correspondence. E-mail gerhard.gebauer@uni-bayreuth.de

Received: 18 December 2014 Returned for revision: 4 February 2015 Accepted: 27 April 2015 Published electronically: 25 June 2015

- **Background and Aims** Most fully mycoheterotrophic (MH) orchids investigated to date are mycorrhizal with fungi that simultaneously form ectomycorrhizas with forest trees. Only a few MH orchids are currently known to be mycorrhizal with saprotrophic, mostly wood-decomposing, fungi instead of ectomycorrhizal fungi. This study provides evidence that the importance of associations between MH orchids and saprotrophic non-\textit{Rhizoctonia} fungi is currently under-estimated.
- **Methods** Using microscopic techniques and molecular approaches, mycorrhizal fungi were localized and identified for seven MH orchid species from four genera and two subfamilies, Vanilloideae and Epidendroideae, growing in four humid and warm sub-tropical forests in Taiwan. Carbon and nitrogen stable isotope natural abundances of MH orchids and autotrophic reference plants were used in order to elucidate the nutritional resources utilized by the orchids.
- **Key Results** Six out of the seven MH orchid species were mycorrhizal with either wood- or litter-decaying saprotrophic fungi. Only one orchid species was associated with ectomycorrhizal fungi. Stable isotope abundance patterns showed significant distinctions between orchids mycorrhizal with the three groups of fungal hosts.
- **Conclusions** Mycoheterotrophic orchids utilizing saprotrophic non-\textit{Rhizoctonia} fungi as a carbon and nutrient source are clearly more frequent than hitherto assumed. On the basis of this kind of nutrition, orchids can thrive in deeply shaded, light-limiting forest understoreys even without support from ectomycorrhizal fungi. Sub-tropical East Asia appears to be a hotspot for orchids mycorrhizal with saprotrophic non-\textit{Rhizoctonia} fungi.

**Key words:** Orchids, Orchidaceae, mycoheterotrophy, mycorrhiza, Vanilloideae, Epidendroideae, \textit{Gastrodia}, stable isotopes, carbon, nitrogen, saprotrophic fungi.

**INTRODUCTION**

In nature, orchids are known to begin their life cycle as mycoheterotrophs (Rasmussen, 1995; Leake, 2004). Because of the rudimentary embryo and the lack of endosperm in seeds, the germination of orchid seeds is dependent on the formation of a mycorrhizal association, which supplies young seedlings, i.e. protocorms, with all carbon (C) and mineral nutrients until the seedlings develop green leaves and become putatively autotrophic (Leake, 1994; Merckx, 2013). Mycorrhizal partners of the majority of these adult green orchids are widely distributed fungi of the polyphyletic \textit{Rhizoctonia} group, including \textit{Tulasnella}, \textit{Ceratobasidium}, \textit{Thanatephorus} and \textit{Sebacina} clade B (Deamaley \textit{et al.}, 2012). In contrast to the putatively autotrophic nutritional mode of chlorophyllous orchids, a few orchids remain achlorophyllous and depend on their mycorrhizal partners for C and mineral nutrient supplies throughout their entire life cycle. These achlorophyllous orchids are known as mycoheterotrophic (MH) plants (Leake, 1994; Merckx, 2013).

In temperate forests, MH orchids usually associate with narrow clades of ectomycorrhizal (ECM) fungi and obtain photosynthates from neighbouring trees through underground mycorrhizal networks (Taylor and Bruns, 1997; Hynson \textit{et al.}, 2013). In tropical and sub-tropical forests, a few MH orchids have been reported to associate with saprotrophic (SAP) non-\textit{Rhizoctonia} fungi and obtain nutrients through the ability of the fungi to cause wood or litter decay. For example, \textit{Gastrodia} spp. have been shown to associate with the litter- and/or wood-decomposing fungi \textit{Armillaria}, \textit{Mycena}, \textit{Resinicium} and \textit{Campanella} or \textit{Marasmius} (Kusano, 1911; Kikuchi \textit{et al.}, 2008; Martos \textit{et al.}, 2009; Ogura-Tsujita \textit{et al.}, 2009; Deamaley and Bougoure 2010). \textit{Epipogium roseum} associates with a litter-decomposing species of Coprinaceae in culture conditions (Yamato \textit{et al.}, 2005), \textit{Wollschaegelia aphylla} associates with litter-decaying species of \textit{Gymnopus} and \textit{Mycena} (Martos \textit{et al.}, 2009), \textit{Eulophia zollingeri} associates with another litter decomposer, \textit{Psaltryrella cf. candolleana} (Ogura-Tsujita and Yukawa 2008), and \textit{Erythrorchis} spp. associate...
with a wide range of wood-rotting fungi of Hymenochaetae and Polyporaceae (Umata, 1995, 1997a; Dearnaley, 2007). In addition, some MH orchids in tropical and sub-tropical forests also associate with ECM fungi, but lack mycorrhizal specificity as in temperate forests (Roy et al., 2009). A recent molecular approach indicates that Lecanorchis associates with diverse ECM fungi, e.g. Lactarius, Russula, Atheliaeaceae and Sebacina clade A (Okayama et al., 2012).

The analysis of $^{13}$C and $^{15}$N (nitrogen) isotope abundances has been extensively used to elucidate the nutritional resources utilized by organisms in ecosystems. Along food chains most organisms have isotope values similar to their food resources (Fry, 2006). ECM fungi are significantly enriched in $^{13}$C and $^{15}$N as compared with autotrophic plants (Gebauer and Dietrich, 1993; Gleixner et al., 1993), and consequently, MH plants associated with ECM fungi have isotope signatures close to those of ECM fungi (Gebauer and Meyer, 2003; Trudell et al., 2003). As compared with ECM fungi, wood-decaying SAP fungi are even more enriched in $^{13}$C, but less enriched in $^{15}$N, and, therefore, MH plants associated with wood-decaying SAP fungi should have isotope signatures similar to this group of SAP fungi. Unfortunately, little information about the isotopic composition of MH plants associated with non-Rhizoctonia SAP fungi is available to date (Ogura-Tsujita et al., 2009; Martos et al., 2009; Dearnaley and Bougoure, 2010; Sommer et al., 2012; Hynson et al., 2013). Furthermore, the currently available knowledge provides no clue about whether MH plants associated with wood-decaying or litter-decaying fungi are different in terms of their isotopic composition. Such a distinction should be expected from the different isotopic composition of the substrates on which these fungi live (Gebauer and Schulze, 1991; Cernusak et al., 2009).

A great diversity of MH orchids (>120 species) occurs in tropical and sub-tropical Asia. In Taiwan, among the approx. 400 native orchids, >50 fully MH orchids in 15 genera have been recorded (Su, 2000). The Xitou Experimental Forest located in central Taiwan has long humid seasons with warm temperatures that obviously favour the growth of MH orchids. According to the report by Yang et al. (2010), nine fully MH orchids, including representatives of the genera Cyrtosia, Galeola and Lecanorchis (subfamily Vanilloideae) and Gastrodia and Epipogium (subfamily Epipogioideae) occur in this misty forest with abundant litter and dead wood. Among these MH orchids in the Xitou Experimental Forest, three Gastrodia spp., G. appendiculata, G. fontinalis and G. nantoensis, occur sympatrically in a bamboo forest, whereas in another bamboo forest, two vanilloid orchids, C. javanica and L. thalassica, grow sympatrically.

The richness of MH orchids in the sub-tropical forests in central Taiwan allows us to test the following questions. (1) What are their mycorrhizal partners? Current knowledge about the identity of mycorrhizal fungi in the vanilloid genera Cyrtosia and Erythrorchis, close relatives of Galeola, are primarily based on only in vitro isolations (Hamada, 1939; Umata, 1995, 1997a). Here we identify the fungal associates of seven MH orchids using molecular methods. (2) Gastrodia spp. occur mainly in Asia, Africa and Australia. How much diversity is there in mycorrhizal partners over the range of Gastrodia spp.? We compare the fungal composition in mycorrhizas of sympatric and allopatric species. (3) Although the mycorrhizal partners of Cyrtosia, Galeola and Lecanorchis of subfamily Vanilloideae have already been investigated, their nutritional resources are still not clear. Cyrtosia and Galeola appear to associate with SAP fungi, whereas Lecanorchis presumably associates with ECM fungi. However, Lyophyllum shineji, an ECM fungus, could stimulate seed germination in vitro of Erythrorchis, a close relative genus of Galeola (Umata, 1997b), suggesting the possible recruitment of an ECM mycorrhizal partner in the natural environment. In this study we analyse for the first time the C and N stable isotope abundances of three vanilloid orchids and four Gastrodia spp. to reveal their nutritional resources, either ECM fungi or wood-decaying or litter-decaying non-Rhizoctonia SAP fungi.

**MATERIALS AND METHODS**

**Sample collection and locations**

Specimens of seven fully MH orchids (Figs 1 and 2) were sampled from four sites in Central Taiwan from 2011 to 2012 (Supplementary Data Table S1). The four sites are located approx. 500–3000 m from each other in the Xitou Experimental Forest (College of Bio-resources and Agriculture, National Taiwan University), Nantou County, Taiwan at 1000 m above sea level. The climate is sub-tropical moist, with a mean annual temperature of 16.6 °C and a mean annual precipitation of 2600 mm. Site A (23°69′29″N, 120°79′12″E) consists of a broadleaf forest on organic soil (pH 3.7) dominated by Phoebe formosana and Machilus japonica (Lauraceae) with some understory plants (see Table S2). The target plant at this site was the MH orchid Galeola falconeri. Site B (23°40′44″N, 120°45′53″E) consists of a dense bamboo (Phyllostachys edulis) forest mixed with Cryptomeria japonica trees on organic soil (pH 4.4) with only few understorey plants (see Table S2). The MH orchids Cyrtosia javanica and Lecanorchis thalassica grow sympatrically at this site. Site C (23°40′26″N, 120°47′45″E) consists of a coniferous forest on organic soil (pH 4.0) dominated by Cryptomeria japonica with some understorey plants (see Table S2). The target orchid at this site was the MH Gastrodia flabilabella. Site D (23°40′41″N, 120°47′34″E) consists of a dense bamboo (Phyllostachys edulis) forest on organic soil (pH 4.4) with only few understorey plants (see Table S2). The MH orchids Gastrodia appendiculata, G. fontinalis and G. nantoensis co-occur at this site. Voucher specimens of G. falconeri (Yung-I Lee 201225), C. javanica (Yung-I Lee 201223), L. thalassica (Yung-I Lee 2011224), G. appendiculata (Yung-I Lee 2011222), G. fontinalis (Yung-I Lee 2011217), G. nantoensis (Yung-I Lee 2011212) and G. flabilabella (Yung-I Lee 2011117) have been deposited in the herbarium of the National Museum of Nature and Science, Taichung, Taiwan. Light climate data of the four sites were measured with a LM-8000 Lux meter (Lutron Electronic Enterprise Co., Ltd, Taipei, Taiwan) at 20 cm from ground level at three different points in each site. Site A received a mean of 3670 lux (= 6 %), site B a mean of 980 lux (= 2 %), site C a mean of 3050 lux (= 5 %) and site D a mean of 950 lux (= 2 %), whereas outside of the forests at the same time a mean of 57800 lux (= 100 %) was measured.
Microscopy

Mycorrhizal roots were collected and fixed in 2.5 % glutaraldehyde and 1.6 % paraformaldehyde buffered with 0.05 M phosphate buffer, overnight at 4 °C. After fixation, the samples were dehydrated using an ethanol series, and embedded in Technovit 7100 (Kulzer & Co., Wehrheim, Germany). Sections of 3 μm thickness were obtained using Ralph knives on a Reichert-Jung 2040 Autocut rotary microtome. Sections were stained with 0.05 % (w/v) toluidine blue O (TBO) in benzoate buffer for general histology (Yeung, 1984). The sections were examined and the images were captured using a digital camera attached to a microscope (Axioskop 2, Carl Zeiss AG, Jena, Germany).

Molecular identification of mycorrhizal fungi

After checking for fungal colonization by free-hand sections under the microscope, mycorrhizal roots were washed in water and kept at −80 °C until use. DNA was extracted from each sample by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of the fungal nuclear rRNA gene was amplified with the primer combinations ITS1F/ITS4 or ITS1F/ITS4B (White et al., 1990; Gardes and Bruns, 1993). The large subunit (LSU) nuclear ribosomal DNA (nrDNA) sequences were amplified using primer combinations LR0R/LR5 (Moncalvo et al., 2000) or LR0R/LR3 (Vilgalys and Hester, 1990). PCR amplification and sequencing were carried out as described by Ogura-Tsujita et al. (2009). PCR products that were difficult to sequence directly were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). Sequences were identified (Supplementary Data Table S3) using a BLAST search against the NCBI sequence database (National Center for Biotechnology Information, GenBank) to find the closest sequence matches in the database. For phylogenetic analysis, LSU marasmioid sequences from GenBank were added to the analysis by referring to Moncalvo et al. (2000, 2002), Wilson and Desjardins (2005), Matheny et al. (2006), Martos et al. (2009) and Ogura-Tsujita et al. (2009), and sequences of Cyphella digitalis, Nia vibrissa and Henningsomyces candidus were used as outgroup taxa. LSU sequences of Polyporales from GenBank were added to the analysis by referring to Justo and Hibbett (2011) and Binder et al. (2013), and sequences of Coltriciella oblectabilis was used as outgroup taxa. ITS sequences of Russula from GenBank were added to the analysis by referring to Okayama et al. (2012), and sequences of Arcangeliana campphorata and Lactarius quietus were used as outgroup taxa. DNA sequences were aligned using CLUSTALX (Thompson et al., 1997), followed by manual adjustment. Phylogenetic relationships were analysed by a model-based Bayesian approach using MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003). The ‘best-fit’ model of evolution was selected under the Akaike information criterion test (Akaike, 1974) as implemented in MrModeltest 2.2 (Nylander, 2004). The general time reversal plus invariant rates and a gamma distribution (GTR+I+C) was selected for the analyses. Two separate runs of four Monte Carlo Markov chains (MCMCs; Yang and Rannala, 1997) were performed for 10 000 000 generations until the mean deviation of split frequency dropped below 0.01, and a tree was sampled every 1000th generation. The first 25 % of generations were discarded using the ‘burn-in’ command, and the remaining trees were used to calculate a 50 % majority-rule consensus topology and to determine the posterior probability (PP) for individual branches. The alignment data sets were further analysed by maximum
parsimony (MP) using PAUP* version 4.0b10 (Swofford, 2002). Support for groups was evaluated using the bootstrap method (Felsenstein, 1985) with 1000 replicates. The trees obtained in these analyses were drawn with the TreeGraph 2 software (Stover and Muller, 2010).

Stable isotope abundance analysis

Five 1 m² plots were selected at each site; each plot included fully MH orchids and four to five autotrophic reference plant species. Flower stalks of seven fully MH orchids, leaves of four to five autotrophic reference plants and soil samples from the organic layer were taken from each of the five plots at each site. The reference plants collected at the respective sites are listed in Supplementary Data Table S2. In addition, on site B, fruit bodies of a litter-decaying SAP fungus (Marasmius sp.) were found and collected in five replicates.

Samples were dried at 105 °C, ground to a fine powder and stored in a desiccator with silica gel until analysed. Relative N and C isotope abundances of the samples were measured using a dual-element analysis mode with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo et al. (2004). Measured abundances are denoted as δ values that were calculated according to the given equation

$$\delta^{15}N = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad \text{[‰]}$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases (N₂ and CO₂) were calibrated with respect to international standards by using the reference substances N₁ and N₂ for N isotopes and ANU sucrose, NBS 18 and NBS 19 for C isotopes, provided by the International Atomic Energy Agency (Vienna, Austria). Reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measuring the test substance acetanilide (Gebauer and Schulze, 1991). At least six test substances with varying sample weight were routinely analysed in each batch of 50 samples. The maximum variation of δ15C and δ15N within and between batches was always <0.2 ‰. To compare isotope abundances of orchids and reference plants from different sites, the data were normalized. Enrichment factors (ε) were calculated per plot: $\epsilon = \delta_S - \delta_{\text{REF}}$, with S as a single δ13C or δ15N value of an adult

Fig. 2. Flower morphology of Gastrodia species. (A) Gastrodia appendiculata, scale bar = 1 cm; (B) Gastrodia fontinalis, scale bar = 1 cm; (C) Gastrodia flabellata, scale bar = 1 cm; (D) Gastrodia nantoensis, scale bar = 1 cm.
orchid and REF as the mean value of non-orchid reference plants from the respective plot (Preiss and Gebauer, 2008). The original $\delta^{13}$C and $\delta^{15}$N values of orchids, the respective reference plants, fungi and soil samples are available in Supplementary Data Table S2. Total N concentrations in leaf, stem, fungus and soil samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on acetanilide measurements (Gebauer and Schulze, 1991). Acetanilide has a constant N concentration of 10.36%. Enrichment factors and total N concentrations of orchids and reference plants were tested for normal distribution. Enrichment factors $\epsilon^{13}$C and $\epsilon^{15}$N were not normally distributed and therefore these data were tested for statistical differences using the Kruskal–Wallis non-parametric test followed by a post-hoc Mann–Whitney U-test with an adjusted significance level according to Holm (1979). The autotrophic reference plants were treated as one group after confirming insignificant differences among the data of each species. Total N concentrations were normally distributed and thus the Student’s t-test was used to test total N concentrations in orchids and reference plants for statistical differences.

RESULTS

Histological studies

In the three vanilloid study species, the below-ground structure of Galeola falconeri had a long rhizome with a few thick roots. Cyrtosia javanica had a short rhizome with a few thick roots. Lecanorchis thalassica had a slender rhizome with a number of thick roots (Supplementary Data Fig. S1). The exodermal cells of roots in the three vanilloid orchids were characterized by the thickened outer and lateral walls. Colonization by fungal hyphae in the middle cortex cells could be observed (Fig. 3), and their exodermal and outer cortex layers were occasionally colonized (Fig. 3B).

The below-ground structures of Gastrodia appendiculata, G. fontinalis and G. nantoensis were similar, having thick rhizomes with a few slim roots (Supplementary Data Fig. S1E). The epidermal cells remained intact or became collapsed without fungal colonization. The outer and inner cortex cells were usually uncolonized, whereas the middle cortex cells were filled with fungal hyphae (Fig. 4A, B, D). Gastrodia flabilabella had a tuberous rhizome with several coralloid roots (Supplementary Data Fig. S1D). As observed in the other three Gastrodia spp., the epidermal, outer and inner cortical layers were rarely colonized. The middle cortex cells were heavily colonized by fungal hyphae. It is worth noting that several papilae-like cell wall thickenings could be observed at the adjoining walls between the outer and middle cortex cells (Fig. 4A, C, D).

Molecular identification of mycorrhizal fungi

Two of the three MH vanilloid study species were associated with SAP non-Rhizoctonia fungi known to be wood-decaying. The ITS sequences obtained from seven G. falconeri individuals (22 samples) and those obtained from five C. javanica individuals (20 samples) had a high DNA sequence homology with species of Meripilaceae (order Polyporales) by BLAST analysis. Thus, wood-decaying Meripilaceae have to be considered as the exclusive fungal associates of G. falconeri and C. javanica (Fig. 5). In contrast, for the third MH vanilloid orchid L. thalassica, the ITS sequences obtained from seven individuals (24 samples) demonstrate a high DNA sequence homology with species of the ECM fungus Russula (Fig. 6).

For all Gastrodia spp. studied, associations with SAP non-Rhizoctonia fungi were found. For the three Gastrodia spp. growing sympatrically in a bamboo forest (G. appendiculata, 15 samples of five individuals; G. fontinalis, 22 samples of six individuals; G. nantoensis, 20 samples of six individuals), the ITS sequences demonstrate a high DNA sequence homology with Mycena spp. The ITS sequences of Mycena obtained from the roots of G. appendiculata, G. fontinalis and G. nantoensis (57 root samples) are grouped into three types (Supplementary
Fig. 4. Histology of mycorrhizas in four Gastrodia species. Their epidermal, outer and inner cortical layers are rarely colonized. (A) Light micrograph showing a transverse section of a root of G. appendiculata. The middle cortex cells are heavily colonized by fungal hyphae. The thickened papillae-like cell walls could be observed at the adjoining walls between the outer and middle cortex cells (arrows). (B) Light micrograph showing a transverse section of a root of G. fontinalis. (C) Light micrograph showing a transverse section of a root of G. flabilabella. (D) Light micrograph showing a transverse section of a root of G. nantoensis. A few papillae-like cell walls occur at the adjoining walls between the outer and middle cortex cells (arrows). E, epidermal cells; H, fungal hyphae; IC, inner cortex; MC, middle cortex; OC, outer cortex. Scale bar = 50 μm.

Data Table S4). Type I was only detected in G. fontinalis, and type II was only detected in G. appendiculata. Type III was detected in both G. appendiculata and G. nantoensis. In addition, the mycorrhizal roots of G. fontinalis (eight samples) were also colonized by Gymnopus spp. For G. flabilabella (21 samples of seven individuals), collected in a coniferous forest, the generated ITS sequences indicate homology with species of the fungal genus Hydropus (Fig. 7).

Stable isotope natural abundance and total N concentrations

Comparisons of enrichment factors \( \epsilon^{13}C \) (H = 77; d.f. = 7; \( P < 0.001 \)) and \( \epsilon^{15}N \) (H = 77; d.f. = 7; \( P < 0.001 \)) among the seven orchid species and the set of reference plants revealed highly significant differences in our data set. All study orchids were enriched by 7.9 ± 0.2 \%o (G. appendiculata) to 12.1 ± 0.5 \%o (G. flabilabella) in \( ^{13}C \) and by 4.7 ± 0.6 \%o (G. flabilabella) to 8.8 ± 2.7 \%o (L. thalassica) in \( ^{15}N \) in comparison with autotrophic reference plants growing at the same sites (Fig. 8). Based on post-hoc tests, this enrichment in \( ^{13}C \) and \( ^{15}N \) was highly significant for all orchid species (in all cases \( U = 0; P < 0.001 \)).

The orchids themselves fall into three distinct groups. The ECM-associated L. thalassica was significantly more enriched in \( ^{15}N \) than all other orchids associated with SAP fungi (\( U = 0 \) to \( U = 3; P < 0.001 \) to \( P = 0.003 \); mean difference 3.7 \%o) and significantly less enriched in \( ^{13}C \) than the group composed of G. falconeri, C. javanica and G. flabilabella (\( U = 0; P < 0.001 \); mean difference 3.0 \%o). However, L. thalassica was not significantly distinguishable in its \( ^{13}C \) enrichment from the orchid group composed of G. nantoensis, G. appendiculata and G. fontinalis (\( U = 25; P = 0.295 \)). The group composed of G. falconeri, C. javanica and G. flabilabella was more enriched in \( ^{13}C \) than the group composed of G. nantoensis, G. appendiculata and G. fontinalis (\( U = 0; P < 0.001 \); mean difference 3.5 \%o). However, both of these groups were not significantly different in their \( ^{15}N \) enrichments (\( U = 107; P = 0.836 \)).

The investigated litter-decaying SAP fungus Marasmius sp. was enriched by 9.4 ± 0.5 \%o in \( ^{15}C \) and by 4.3 ± 0.2 \%o in \( ^{15}N \) compared with autotrophic reference plants (Fig. 8) and thus had similar enrichments in \( ^{13}C \) and \( ^{15}N \) to the orchid group composed of G. nantoensis, G. appendiculata and G. fontinalis.

Orchid flower stems had a slightly higher mean total N concentration (2.88 ± 0.50 mmol g d. wt\(^{-1}\); \( n = 35 \)) than reference plant leaves (2.67 ± 0.67 mmol g d. wt\(^{-1}\); \( n = 90 \); Supplementary Data Table S2). However, this slight difference was not significant (\( t = 1.671; P = 0.097 \); d.f. = 123).

DISCUSSION

Fungal colonization in the roots

In three MH vanilloid orchids, mycorrhizal colonization of wood-decaying and ECM fungi was mainly observed in the cortical layers of the old roots, a finding similar to reports for...
mycorrhizal colonizations in chlorophyllous Vanilla spp. by Porras-Alfaro and Bayman (2007). The outermost layers of old roots in MH vanilloid orchids are characterized by thickened exodermal layers, whereas the epidermal cells are no longer alive. Colonization of fungal hyphae was also found in the thickened exodermal layers (Fig. 3B), suggesting their roles for maintenance of nutrient uptake by older roots (Esnault et al., 1994).

In the Gastrodia spp. investigated, fungal colonization was restricted to a few cortical layers of root systems, but was not commonly observed in rhizomes. In the colonized cortical layers, the papillae-like cell wall thickenings were abundant at the adjoining walls between the outer and middle cortex cells, corresponding to pathways for fungal hyphae (Fig. 4A, C, D). The presence of papillae-like cell wall thickenings could be potentially underdeveloped structures of wall ingrowths in specialized transfer cells as described in symbiotic associations by Pate and Gunning (1972), suggesting a specific nutrient transport network in the mycorrhiza (Martos et al., 2009).

**Mycorrhizal partners**

Subfamily Vanilloideae contain a number of non-photosynthetic genera (40% of the 15 genera of vanilloid orchids), e.g. Cyrtosia, Erythrorchis, Galeola, Pseudovanilla and Lecanorchis (Cameron, 2009). Chlorophyllous Vanilla spp. have been shown to associate with a wide range of Rhizoctonia fungi, including Ceratobasidium, Thanatephorus and Tulasnella (Porras-Alfaro and Bayman, 2007), whereas the MH vanilloid taxa Cyrtosia and Erythrorchis mainly associate with wood-decaying fungi, such as Armillaria and species of Hymenochaetaceae and Polyporaceae (Umata, 1995, 1997a; Cha and Igarashi, 1996; Dearnaley, 2007). In this study, G. falconeri and C. javanica were identified to associate exclusively with wood-decaying fungi of Meripilaceae (order Polyporales).

According to the findings of Okayama et al. (2012), Lecanorchis spp. in Japanese mountain abundantly with a broad range of ECM fungi, including Lactarius, Russula, Atheliaceae and Sebacina, with Lactarius and Russula dominating. In this study, Russula was identified as the preferred mycorrhizal partner of L. thalas-sica. In phylogenetic analyses, MH vanilloid orchids, i.e. Cyrtosia, Erythrorchis, Galeola, Pseudovanilla and Lecanorchis, are closely related to chlorophyllous Vanilla spp. in tribe Vanilleanae (Cameron, 2009). All these results together suggest that the nutritional shift from autotrophy to mycoheterotrophy in vanilloid orchids correlates with shifts in fungal partners from Rhizoctonia fungi to wood-decaying non-Rhizoctonia fungi or to ECM fungi. A shift towards either a wood-decaying fungus or an ECM fungus can even happen for sympatrically growing closely related species, as in our case for C. javanica and L. thalas-sica. Liebel et al. (2015) recently argued that this shift in fungal partners is essential for the MH mode of nutrition. The annual C and N flux from Rhizoctonia fungi to their orchid partners is rather low (Stöckel et al., 2014). This low matter flux is obviously sufficient to support growth of the tiny initially MH orchid protocorms, but appears to be insufficient to support growth of adult MH orchids.
Fig. 6. Phylogenetic relationships of the mycorrhizal fungi of *Lecanorchis thalassica* based on the Bayesian analysis of partial ITS ribosomal DNA sequences of Russulaceae available in GenBank (with the position of mycorrhizal fungi found in *Lecanorchis* by Okayama et al., 2012). GenBank accession numbers are shown in parentheses. The values above branches are bootstrap percentages and Bayesian posterior probabilities (> 50 %), respectively. ‘–’ indicates that the node was not supported in MP analysis.
FIG. 7. Phylogenetic relationships of the mycorrhizal fungi of Gastrodia appendiculata, G. fontinalis, G. flabilabella and G. nantoensis based on the Bayesian analysis of LSU ribosomal DNA sequences of marasmioid available in GenBank (Moncalvo et al., 2000, 2002; Wilson and Desjardins, 2005; Matheny et al., 2006; Martos et al., 2009; Ogura-Tsujita et al., 2009). The values above branches are bootstrap percentages and Bayesian posterior probabilities (> 50 %), respectively. ‘–’ indicates that the node was not supported in MP analysis.
Despite the fairly high number of fully MH species in subfamily Vanilloideae, no partially MH species have been reported so far to occur among green species of Vanilloideae.

All seven of the MH study orchids turned out to be significantly enriched in the heavy isotopes $^{13}\text{C}$ and $^{15}\text{N}$ in comparison with autotrophic plants growing in identical habitats. This finding confirms that fungi also known to be enriched in $^{13}\text{C}$ and $^{15}\text{N}$ (Gebauer and Dietrich, 1993; Gleixner et al., 1993) serve as their C and N source. Though leafless, some putative MH orchids have green flowering stems and thus a low photosynthetic activity. One leafless orchid with frequently green flowering stems and green seed capsules is *Corallorhiza trifida*. This species is associated with an ECM fungus and is less enriched in $^{13}\text{C}$ and $^{15}\text{N}$ than fully MH orchids also associated with ECM fungi (Zimmer et al., 2008). Thus, *C. trifida* has been classified as being partially MH. All study orchids of this investigation provided no indications for chlorophyllous flowering stems and had isotope signatures typical of various types of fully MH plants. According to their pattern in $^{13}\text{C}$ and $^{15}\text{N}$ enrichment, the MH orchids fall into three groups. (1) The ECM-associated *L. thalassica* is the species most enriched in $^{15}\text{N}$. This pattern is in agreement with current knowledge (Hynson et al., 2013) and can be traced back to the fact that ECM fungi are more enriched in $^{15}\text{N}$ than sympatrically growing wood- or litter-decaying SAP fungi (Gebauer and Taylor, 1999). (2) The two orchid species associated with wood-decaying Meripilaceae, *Galeolea falconeri* and *Cyrtosia javanica*, belong to the group most enriched in $^{13}\text{C}$. The specifically high $^{13}\text{C}$ enrichment of this group of MH orchids is expected due to the fact that wood is more enriched in $^{13}\text{C}$ than leaf tissue (Gebauer and Schulze, 1991; Cernusak et al., 2009) and confirms previous observations of similar patterns in MH orchids associated with wood-decaying fungi (Ogura-Tsujita et al., 2009; Martos et al., 2009; Hynson et al., 2013). The third MH orchid belonging to this group is the *Hydropus*-associated *Gastrodia flabilabella*, suggesting that this *Hydropus* sp. should be a wood-decaying fungus. (3) The third MH orchid cluster characterized by a significantly lower $^{15}\text{N}$ enrichment than (1) and a significantly lower $^{13}\text{C}$ enrichment than (2) is composed of the three *Gastrodia* spp. growing sympatrically in a bamboo forest, *G. appendiculata*, *G. fontinalis* and *G. nantoensis*. The isotopic pattern of this cluster of MH orchids provides strong evidence that their mycorrhizal associates, *Mycena* of three different types and *Gynopous*, are all litter-decaying fungi. This
evidence is further strengthened by the rather similar isotopic pattern found for the fruit bodies of the litter-decaying fungus *Marasmius* sp. This is the first report we are aware of that indicates a significantly different isotopic pattern for MH orchids associated with either wood-decaying or litter-decaying fungi. The only hint towards an isotopic distinction between MH orchids associated with wood- or litter-decaying fungi was previously given by Martos et al. (2009), who investigated the isotopic compositions of the MH orchids *Gastrodia similis* associated with wood-decaying *Resinicium* from *La Réunion* and *Wullschlaegelia aphylla* mycorrhizal orchids with litter-decaying *Gymnopus* and *Mykoca* from Guadeloupe. Unfortunately, comparisons of the data from the study of Martos et al. with each other and with our investigation based on normalized enrichment factors is not possible due to a lack of stable isotope data for forest ground plants growing in micro-environments identical to the MH orchids.

Total N concentrations in MH orchids have been reported to be significantly higher than in the majority of co-occurring autotrophic plants (Gebauer and Meyer, 2003; Liebel et al., 2010; Liebel and Gebauer, 2011; Sommer et al., 2012). The most likely reason for these unusually high total N concentrations in MH orchid tissues is their N gain through the fungal source. Fungi are known to have considerably higher total N concentrations in their tissue than autotrophic plants growing in the same environment (Gebauer and Dietrich, 1993; Gebauer and Taylor, 1999). Total N concentrations found in the flower stems of the MH orchid species investigated here (2.88 ± 0.50 mmol g d. wt⁻¹) are rather similar to the total N concentrations reported for other fully or partially MH orchids (Gebauer and Meyer, 2003; Liebel et al., 2010; Liebel and Gebauer, 2011; Sommer et al., 2012). The reference plant leaf total N concentrations investigated here (2.67 ± 0.67 mmol g d. wt⁻¹) were only slightly lower than N concentrations in the flower stems of the MH orchids and statistically not distinguishable. Total N concentrations in the leaves of the understorey plants investigated here from four different forests in Taiwan are about twice as high as leaf total N concentrations in autotrophic forest ground plants from temperate Central Europe (Gebauer et al., 1988; Gebauer and Meyer, 2003) and Mediterranean Italy (Liebel et al., 2010), and three times higher than leaf total N concentrations in autotrophic forest ground plants from severely N-limited regions, such as SW Australia (Sommer et al., 2012) or boreal Norway (Liebel and Gebauer, 2011). The most likely reason for the high total N concentrations in the leaves of autotrophic forest ground plants in Taiwan is a high mineral N availability due to high soil N mineralization rates. Thus, N limitation as a driver for the switch from autotrophy towards mycoheterotrophy is unlikely for the MH orchids investigated here. More likely as a driving factor for the development of mycoheterotrophy is light limitation for photosynthesis on the forest ground. As reported for other habitats of MH plants (Bidartondo et al., 2004; Zimmer et al., 2007; Preiss et al., 2010) only 2–6 % of incoming light reached the ground of our study forests and thus severely limited the photosynthetic capacity of forest ground vegetation.

In conclusion, our data provide further evidence for the importance of associations with non-*Rhizoctonia* SAP fungi among fully MH orchids from sub-tropical Asia. Abundant fallen litter and wood in the warm and humid forests of Taiwan obviously supplies ideal substrates for the continuous growth of SAP fungi. For the MH orchids studied here, as seeds germinate, they can construct efficient mycorrhizal interactions preferentially with wood- or litter-decaying SAP fungal partners, but sympatrically also with ECM fungi, in order to thrive in deeply shaded forest understoreys with low light conditions.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: voucher and sampling sites of seven mycoheterotrophic orchids in Xitou Experimental Forest used in molecular identification of mycorrhizal fungi. Table S2: mean δ¹⁵N and δ¹³C values, mean enrichment factors ε²⁰¹⁵N and ε²⁰¹³C, and mean total N concentrations of all plant and fungal samples in this study. Table S3: putative taxonomic identity of the fungi detected in this study. Table S4: ITS sequence types in *Mykoca* from mycorrhizal roots of three sympatric *Gastrodia* spp. in a bamboo forest. Figure S1: morphology of rhizomes and roots of mycoheterotrophic orchids in this study.

**ACKNOWLEDGEMENTS**

The authors thank Christine Tiroch (BayCEER–Laboratory of Isotope Biogeochemistry, University of Bayreuth) for skilful technical assistance with stable isotope abundance measurements. The authors also thank Yu-Hsiu Cho (National Museum of Natural Science) for molecular identifications of fungal partners, and Sheng-Kun Yu for providing the photo of *Lecanorchis thalassica*. This work is a contribution to the German Research Foundation Project GE 565/7–2. This work was also supported by the funding from National Museum of Natural Science, Taiwan to Y.-I.L.

**LITERATURE CITED**


Kusano S. 1911. *Gastrodia elata*.


Su HJ. 2000. *Orchidaceae*. In TC Huang, ed. *Flora of Taiwan*, 2nd edn, Vol. 5. Editorial Committee of the Flora of Taiwan, Department of Botany, National Taiwan University, Taipei, Taiwan, 720–1086.


Lee et al. — Associations with saprotrophic fungi among fully mycoheterotrophic orchids


