PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2009) **276**, 761–767
doi:10.1098/rspb.2008.1225
Published online 11 November 2008

Evidence for novel and specialized mycorrhizal parasitism: the orchid *Gastrodia confusa* gains carbon from saprotrophic *Mycena*

Yuki Ogura-Tsujita[1,*], Gerhard Gebauer[2], Toshimasa Hashimoto[3], Hidetaka Umata[4] and Tomohisa Yukawa[1]

[1]*Tsukuba Botanical Garden, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba 305-0005, Japan*
[2]*Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany*
[3]*Nippon Steel Kankyo Engineering Co., Ltd, Kisarazu, Chiba 292-0, Japan*
[4]*Faculty of Agriculture, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan*

We investigated the physiological ecology of the Asian non-photosynthetic orchid *Gastrodia confusa*. We revealed its mycorrhizal partners by using molecular identification and identified its ultimate nutritional source by analysing carbon and nitrogen natural stable isotope abundances. Molecular identification using internal transcribed spacer and large subunit nrDNA sequences showed that *G. confusa* associates with several species of litter- and wood-decomposer *Mycena* fungi. The carbon and nitrogen isotope signatures of *G. confusa* were analysed together with photosynthetic plant reference samples and samples of the ectomycorrhizal epiparasite *Monotropa uniflora*. We found that *G. confusa* was highly enriched in ^{13}C but not greatly in ^{15}N, while *M. uniflora* was highly enriched in both ^{13}C and ^{15}N. The ^{13}C and ^{15}N signatures of *G. confusa* were the closest to those of the fruit bodies of saprotrophic fungi. Our results demonstrate for the first time using molecular and mass-spectrometric approaches that myco-heterotrophic plants gain carbon through parasitism of wood or litter decaying fungi. Furthermore, we demonstrate that, several otherwise free-living non-mycorrhizal, *Mycena* can be mycorrhizal partners of orchids.

Keywords: myco-heterotrophy; parasite; stable isotope; Mycenaceae

1. INTRODUCTION

More than 80 per cent of land-plant families are estimated to be mycorrhizal (Trappe 1987; Wang & Qiu 2006), and although mycorrhizal associations are typically mutualistic and generalistic, hundreds of non-photosynthetic plants are known to nutritionally exploit their target mycorrhizal fungi (Bidartondo 2005). Recent studies have shown that such 'myco-heterotrophic' plants (MHPs) associate with narrow clades of ectomycorrhizal fungi, such as Thelephoraceae (Taylor & Bruns 1997; McKendrick *et al.* 2000), Russulaceae (Taylor & Bruns 1999; Girlanda *et al.* 2006), *Sebacina* (McKendrick *et al.* 2002; Selosse *et al.* 2002) and *Tricholoma* (Bidartondo & Bruns 2001; Yokoyama *et al.* 2005) or arbuscular mycorrhizal fungal clades (Bidartondo *et al.* 2002; Merckx & Bidartondo 2008), that are simultaneously mycorrhizal with neighbouring photosynthetic plants (e.g. mycorrhizal trees). This has been called mycorrhizal 'epiparasitism' or 'cheating' (Björkman 1960; Cullings *et al.* 1996; Bidartondo *et al.* 2002) because MHPs obtain photosynthetic products from neighbouring autotrophic plants via a mycorrhizal network, instead of supporting the carbon needs of mycorrhizal networks as most other plants do (Smith & Read 2008).

While mycorrhizal epiparasitic plants are now universally recognized to be ecologically and evolutionarily diverse, direct mycorrhizal 'parasitism' is also suspected among MHPs. Several myco-heterotrophic orchids, mainly distributed in Asia, are shown to associate with free-living litter- or wood-decomposer fungi. By contrast with mycorrhizal epiparasites, direct mycorrhizal parasites would have to bring free-living fungi into the mycorrhizal symbiosis to obtain nutrition. An Asian non-photosynthetic orchid, *Gastrodia elata*, has long been reported to associate with the pathogenic wood-decay fungus *Armillaria* (Kusano 1911; Xu & Mu 1990; Kikuchi *et al.* 2008*a*,*b*) that was also reported from the non-photosynthetic orchid *Cyrtosia septentrionalis* (Hamada 1939; Cha & Igarashi 1996). Species of the genus *Gastrodia* and its related genus *Didymoplexis* are suspected to associate with several decomposer fungi such as *Armillaria*, *Marasmius* and *Fomes* (Burgeff 1932; Campbell 1962, 1964). The world's largest non-photosynthetic plant, *Erythrorchis ochobiensis*, is reported to form mycorrhizas with a wide range of wood-rotting fungi (Hamada & Nakamura 1963; Umata 1995, 1997, 1998*a*,*b*). Mycorrhizal parasitism is also supported by recent work in the non-photosynthetic orchid *Epipogium roseum* with a litter-decomposing Coprinaceae spp. in laboratory conditions (Yamato *et al.* 2005) and *Eulophia zollingeri* associates with another litter decomposer, *Psathyrella* cf. *candolleana* (Ogura-Tsujita & Yukawa 2008). However, it is still unclear whether these plants

* Author for correspondence (oguy@kahaku.go.jp).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2008.1225 or via http://journals.royalsociety.org.

Received 31 August 2008
Accepted 15 October 2008

obtain nutrition from truly saprotrophic fungi (SF) under field conditions because some fungal groups that were not known to include ectomycorrhizal lineages, such as *Tulasnella* (Bidartondo *et al.* 2003), are now known to form ectomycorrhizas with trees and targeted by non-photosynthetic plants. Furthermore, there is no conclusive evidence for plant specificity towards litter- or wood-decomposing fungi; previous studies have examined a small number of individuals from a small number of nearby locations.

The natural abundance of ^{13}C and ^{15}N in organisms is a powerful tool for the elucidation of nutritional sources. Along food chains isotope values of heterotrophic consumers are generally enriched relative to their ultimate sources and they are closest to those of their immediate source. Mycorrhizal epiparasitic plants show highly enriched isotope signatures relative to autotrophic plants (Gebauer & Mayer 2003; Bidartondo *et al.* 2004) and their signatures are most like those of coassociated ectomycorrhizal fungi (Trudell *et al.* 2003). In their biomass, litter- and wood-decomposing fungi are generally enriched in ^{13}C (Kohzu *et al.* 1999; Boström *et al.* 2008) but depleted in ^{15}N relative to ectomycorrhizal fungi (Gebauer & Taylor 1999; Kohzu *et al.* 1999; Trudell *et al.* 2004). Thus, isotope signatures can be used to distinguish between mycorrhizal fungi living in humus and decomposing fungi feeding on litter and wood. We hypothesized that the isotope signatures of direct mycorrhizal parasitic plants targeting litter- and wood-decomposing fungi would be enriched in ^{13}C but depleted in ^{15}N relative to the indirect mycorrhizal epiparasites targeting mycorrhizal fungi, and that the direct mycorrhizal parasites' isotope signature would be the closest to that of litter- and wood-decomposing fungal tissues.

In this study, we tested this hypothesis with the Japanese myco-heterotrophic orchid *Gastrodia confusa*. There are *ca* 50 species of *Gastrodia* distributed through Siberia and Japan to Malesia, Australasia and Madagascar, where several species are endangered. They are all terrestrial non-photosynthetic plants from moist and sheltered forests (including *G. elata* that is cultivated commercially by collecting tubers from the wild that are then grown in special facilities). *Gastrodia confusa* is a rare plant of bamboo forests in Japan and Taiwan. We examined: (i) the mycorrhizal fungi of *G. confusa* at ten distantly located sites to identify their symbionts, and (ii) the abundance of ^{13}C and ^{15}N stable isotopes to reveal its nutritional sources. The mycorrhizal fungus of this orchid has never been identified before, but Tashima *et al.* (1978) isolated fungi from its mycorrhizas that were able to induce seed germination *in vitro*.

2. MATERIAL AND METHODS

(a) *Molecular identification of mycorrhizal fungus*

A total of 44 plants of *G. confusa* was sampled from 10 sites (5–1000 km apart) in Japan from 2005 to 2007. For the study site and the number of samples see the electronic supplementary material A and B. All sites were dense bamboo forests dominated by arbuscular mycorrhizal *Phyllostachys heterocycla* and/or *Phyllostachys bambusoides*, which have been widely naturalized in Japan, with shaded and sparse herbaceous understories. Microscopic observation revealed that the mycorrhizal fungus mainly colonizes long-branched roots derived from the underground tuber and tissues that have a dark brownish colour. Accordingly, fresh and brownish mycorrhizal root tissues were used for fungal identification. Collected roots were washed in water and kept at −80°C until use. Voucher specimens of *G. confusa* were deposited in the herbarium of the National Museum of Nature and Science (TNS773656–773660, 8500020–8500022, 8500024, 8500028, 8500041, 8500042, 8500055, 8500060).

DNA was extracted from root samples, at least one per plant, using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Fungal internal transcribed spacer (ITS) sequences of nrDNA were amplified with the primer combination ITS1F/ITS4 or ITS1F/ITS4B (White *et al.* 1990; Gardes & Bruns 1993). For phylogenetic analysis, the large subunit (LSU) nrDNA sequences of each fungal ITS type were amplified using primer combinations LR0R/LR5 (Moncalvo *et al.* 2000) or LR0R/LR3 (Vilgalys & Hester 1990). PCR amplification and sequencing were carried out as described by Ogura-Tsujita & Yukawa (2008). An additional sequencing primer, LR3R (Hopple & Vilgalys 1999), was used for LSU sequences. PCR products that were difficult to sequence directly were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). GenBank accession numbers (http://www.ncbi.nlm.nih.gov) of ITS sequences were AB454381–AB454413. DNA sequences were analysed using a BLAST search (Altschul *et al.* 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank) to find the closest sequence matches in the database.

Large-subunit sequences from mycorrhizal roots (908 bp) were used for phylogenetic analysis. LSU sequences of fungal types I, II and III (electronic supplementary material C) were obtained from individuals from site S2 and of type IV from S10. LSU sequences of Mycenaceae from GenBank were also added to the analysis by referring to Moncalvo *et al.* (2000, 2002), Walther *et al.* (2005) and Matheny *et al.* (2006), and sequences of *Tricholoma matsutake*, *Catathelasma ventricosum*, *Entoloma prunuloides* and *Lyophyllum decastes* were used as outgroup taxa. DNA sequences were aligned using CLUSTALX (Thompson *et al.* 1997), followed by manual adjustment. Phylogenetic analyses were conducted with PAUP* v. 4.0.b6 (Swofford 2001). Distance trees were obtained using the neighbour-joining (NJ) method (Saitou & Nei 1987) with a Kimura two-parameter correction (Kimura 1980). For assessing the relative robustness for branches, the bootstrap method (Felsenstein 1985) was used with 1000 replicates.

(b) *Stable isotope abundance analysis*

Samples for isotope analysis were collected from *Gastrodia* site S6 (electronic supplementary material A and C) and a neighbouring reference site in September 2007. At this location, *G. confusa* grows in the floor of dense bamboo (*P. heterocycla*) forest with *Piper kadsura*, *Gynostemma pentaphyllum* and *Thelypteris acuminata* patchily covering the understorey. An evergreen broadleaf forest dominated by the ectomycorrhizal tree *Lithocarpus edulis* neighbours the bamboo forest, where an ectomycorrhizal epiparasite plant, *Monotropa uniflora*, grows on the forest floor with *Damnacanthus indicus*, *P. kadsura* and *Trachelospermum asiaticum*. Since *M. uniflora* is known to be an epiparasite associated with the ectomycorrhizal fungal family Russulaceae (Bidartondo & Bruns 2001; Young *et al.* 2002; Yang & Pfister 2006), this species was collected as a reference for comparison. Five 1 m^2

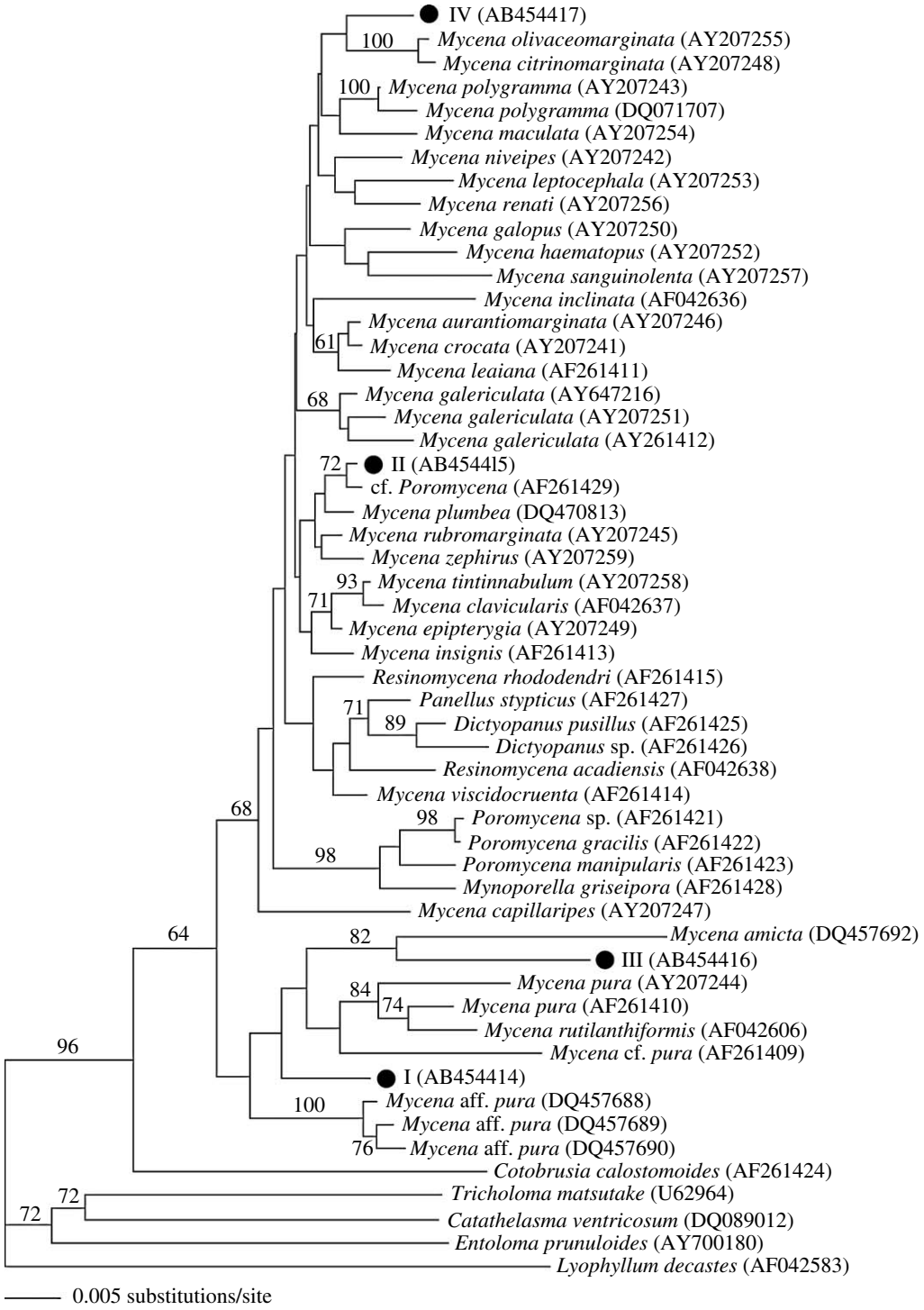

Figure 1. Phylogenetic relationships of the mycorrhizal fungi of *G. confusa* (black circles) based on LSU ribosomal DNA sequences of Mycenaceae available in GenBank. GenBank accessions are shown in parentheses. The numbers shown to the right of black circles indicate the fungal ITS sequence type shown in electronic supplementary material C. Phylogenetic analysis was conducted using NJ with 1000 bootstrap replicates (values of more than 60% are near branches).

plots were selected in both *Gastrodia* and *Monotropa* sites; each plot included orchids or monotropoids and at least two autotrophic reference plant species. Flower stalks of a non-photosynthetic plant, leaves of three to two reference autotrophic plants and soil samples from the organic layer were taken from each of the five plots at each site. The reference plants for the *Gastrodia* site were *P. kadsura*, *G. pentaphyllum* and *T. acuminata* and those for the *Monotropa* site were *D. indicus*, *P. kadsura* and *T. asiaticum*. Five samples were collected for each plant with the exception of *P. kadsura* ($n=3$) at the *Monotropa* site and *G. pentaphyllum* ($n=4$). A total of 14 sporocarps of saprotrophic fungal species (four *Marasmiellus*, three *Hydropus*, two *Gymnopus*, one *Conocybe*, one *Gymnopilus*, one *Lepiota*, one *Paneolus*, one unknown), which mostly occurred on decayed bamboo stems (DS), leaves or roots, were also collected within 30 m of the *Gastrodia* population. The sporocarps of representative species were deposited as dried herbarium specimens (TNS-F-18424–18428). DNA was extracted from all sporocarps, and ITS was sequenced with the same methods described earlier. DS and leaves, which are expected to be the main substrate for the SF, were also collected from the five *Gastrodia* plots.

Samples were dried at 105°C, ground to a fine powder and stored in a desiccator with silica gel until analysed. Relative nitrogen and carbon 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 $\delta^{15}\mathrm{N}$ or $\delta^{13}\mathrm{C} = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000‰$, where R_{sample} and R_{standard} are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases (nitrogen and carbon dioxide) were calibrated with respect to international standards by using the reference substances N1 and N2 for N isotopes and ANU sucrose and NBS 19 for C isotopes, provided by the International Atomic Energy Agency (Vienna, Austria).

The 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.

3. RESULTS AND DISCUSSION

(a) *Fungal identification*

Our results from the molecular identification of mycorrhizal fungi revealed that *G. confusa* consistently associates with Mycenaceae fungi. A total of 106 roots from 44 individuals from 10 sites were analysed and fungal ITS sequences were successfully amplified from 105 roots. The ITS sequences obtained from 94 roots of 41 individuals had high-DNA sequence homology with those species of the basidiomycete *Mycena* by BLAST analysis, and grouped into four types (electronic supplementary material C). The most frequent types were I and II, which were found at eight and six sites, respectively. Types III and IV were rare and found at only two and one site(s), respectively. The fungal DNA sequences from single individuals were identical, with the exception of four individuals simultaneously associated with two types. Three individuals lacked *Mycena* ITS sequence but alternatively generated sequences that closely matched those of *Marasmiellus ramealis* (DQ450030; 90–93% identical) or *Clitocybura* (DQ192179; 84% identical). The *Marasmiellus*-like ITS sequence from orchid roots was completely identical to the sequences from five sporocarps used in isotope analysis. Thus, there is the possibility of a geographical mosaic of mycorrhizal specificity in *G. confusa* (*sensu* Thompson 2005).

To clarify the phylogenetic relationships among the four *Mycena* types, the LSU sequences from each type were analysed together with those from GenBank (figure 1). All LSU sequences were monophyletic with those of fungi belonging to Mycenaceae with bootstrap support 96 per cent. The LSU sequences from *G. confusa* roots were paraphyletic among the sequences of Mycenaceae fungi that are known as free-living litter and wood decomposers (Maas Geesteranus 1992; Cannon & Kirk 2007). Although, several *Mycena* species, such as *Mycena osmundicola* and *Mycena orchidicola*, were reported to be mycorrhizal in orchids (Fan *et al.* 1996; Xu & Fan 2001), this is the first cultivation independent molecular report to reveal that Mycenaceae species are mycorrhizal partners of any plant.

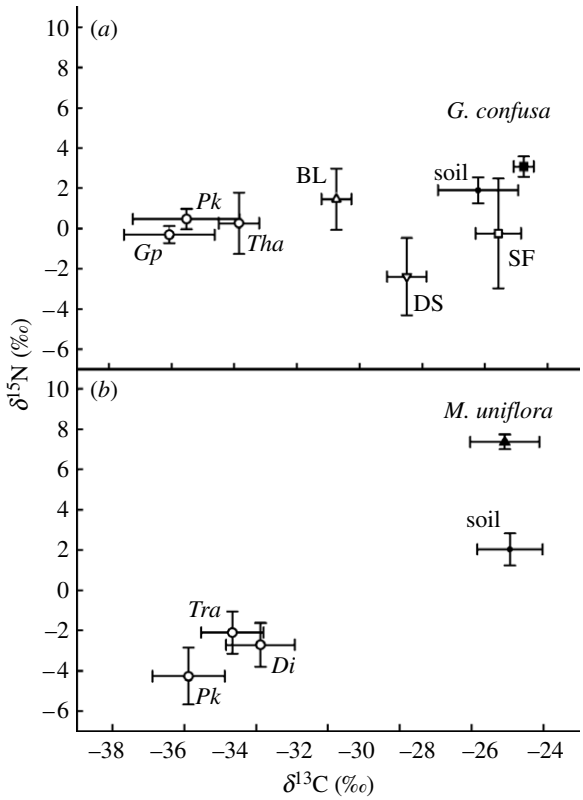

Figure 2. Mean ($\pm$1 s.d.) $\delta^{13}\mathrm{C}$ and $\delta^{15}\mathrm{N}$ values in flower stalks of two myco-heterotrophic species (*G. confusa* and *M. uniflora*), leaves of five autotrophic species (open circles), sporocarps of saprotrophic fungi (SF, open squares), bamboo litter (BL, open triangles), decayed bamboo stems (DS, upward-pointing open triangle) and in soil samples collected from (*a*) a dense bamboo forest (*G. confusa*) and (*b*) its neighbouring evergreen broadleaf forest, (*M. uniflora*, filled triangle). Plant species: *Di*, *Damnacanthus indicus*; *Gp*, *Gynostemma pentaphyllum*; *Pk*, *Piper kadsura*; *Tha*, *Thelypteris acuminata*; *Tra*, *Trachelospermum asiaticum*.

(b) *Isotope signatures*

The results of stable isotope abundance analysis strongly support the hypothesis that *G. confusa* gains carbon from litter and/or wood SF. Isotope signatures were quite different between directly parasitic and epiparasitic MHPs, especially in $^{15}\mathrm{N}$. The $\delta^{13}\mathrm{C}$ and $\delta^{15}\mathrm{N}$ values of *G. confusa* were significantly higher than those of autotrophic reference plants (figure 2*a*), but the differences were greater in $^{13}\mathrm{C}$ (enrichment factor $\varepsilon_{Gastrodia-\text{reference plants}} = 10.2 \pm 1.3‰$) than $^{15}\mathrm{N}$ ($\varepsilon_{Gastrodia-\text{reference plants}} = 2.9 \pm 0.5‰$). The mycorrhizal epiparasite *M. uniflora* was highly and significantly enriched in both $^{13}\mathrm{C}$ ($\varepsilon_{Monotropa-\text{reference plants}} = 7.5 \pm 0.8‰$) and $^{15}\mathrm{N}$ ($\varepsilon_{Monotropa-\text{reference plants}} = 10.2 \pm 1.0‰$) relative to photosynthetic plants (figure 2*b*). The enrichment factors found in *M. uniflora* are identical to those found in all previously examined epiparasitic MHPs, including a variety of orchids and *Monotropa* (Zimmer *et al.* 2007, 2008; Preiß & Gebauer 2008). However, these enrichment factors are significantly different from those found in *G. confusa*. Since litter- and wood-decaying fungi are generally enriched in $^{13}\mathrm{C}$ but depleted in $^{15}\mathrm{N}$ relative to ectomycorrhizal fungi living in humus, the higher $\delta^{13}\mathrm{C}$ than $\delta^{15}\mathrm{N}$ in SF might be reflected in the isotope signature of *G. confusa*. In fact, the $\delta^{13}\mathrm{C}$ and $\delta^{15}\mathrm{N}$ values of *G. confusa* were the closest to those of the litter- and wood-decaying saprotrophic

fungal species in our habitat (figure 2*a*). The sporocarps of the SF were significantly enriched in ^{13}C but not in ^{15}N compared with their substrates, i.e. decayed bamboo litter (BL) and stems. Because total nitrogen contents of BL (1.10 ± 0.21 mmol gdw^{-1}) and decayed stems (0.21 ± 0.07 mmol gdw^{-1}) are markedly low, no N fractionation may be attributed to these severely N limited substrates.

(c) *Conclusion*

Our results demonstrate for the first time by using molecular and mass-spectrometric approaches that MHPs gain carbon through parasitism of wood- or litter-decaying fungi. Plant exploitation of fungi turns out to fall into two types, i.e. epiparasitic (tripartite, with a living photosynthetic plant as ultimate host and an intermediary mycorrhizal fungus) and parasitic (bipartite, without the need for a living plant). These two types of lifestyles have evolved independently in non-photosynthetic plants that cannot provide photosynthates to their mycorrhizal fungi and instead extract carbon (and nitrogen) from fungi. The epiparasites break into ancient arbuscular mycorrhizal and ectomycorrhizal symbioses, but the direct parasites recruit free-living fungi into novel orchid mycorrhizal symbioses. In this way, both the two ecologically dominant ways in which fungi obtain carbon—mycorrhization and decomposition—are susceptible to non-photosynthetic plants. Remarkably, these plants are able to evolve high specificity towards their fungi in the context of highly diverse mycorrhizal and saprotrophic fungal communities.

The authors thank Teruo Katsuyama, Shiro Kobayashi, Katsutoshi Maruno, Hiroko Nakayama, Munetaka Nishi, Kazuhiro Suzuki, Schuichiro Tagane, Tadayuki Tsujita and Tetsukazu Yahara for collecting; Christine Tiroch and Hiroko Abe for technical assistance; Kentaro Hosaka for valuable advices. Financial support was received from grants-in-Aid from the ministry of Education, Sports, Culture, Science and Technology of Japan (No. 17370033) and the Kyushu University Foundation.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402. (doi:10.1093/nar/25.17.3389)

Bidartondo, M. I. 2005 The evolutionary ecology of myco-heterotrophy. *New Phytol.* **167**, 335–352. (doi:10.1111/j.1469-8137.2005.01429.x)

Bidartondo, M. I. & Bruns, T. D. 2001 Extreme specificity in epiparasitic Monotropoideae (Ericaceae): widespread phylogenetic and geographical structure. *Mol. Ecol.* **10**, 2285–2295. (doi:10.1046/j.1365-294X.2001.01358.x)

Bidartondo, M. I., Redecker, D., Hijri, I., Wiemken, A., Bruns, T. D., Dominguez, L., Sersic, A., Leake, J. R. & Read, D. J. 2002 Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature* **419**, 389–392. (doi:10.1038/nature01054)

Bidartondo, M. I., Bruns, T. D., Weiss, M., Sergio, C. & Read, D. J. 2003 Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. *Proc. R. Soc. B* **270**, 835–842. (doi:10.1098/rspb.2002.2299)

Bidartondo, M. I., Burghardt, B., Gebauer, G., Bruns, T. D. & Read, D. J. 2004 Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proc. R. Soc. B* **271**, 1799–1806. (doi:10.1098/rspb.2004.2807)

Björkman, E. 1960 *Monotropa hypopitys* L.—an epiparasite on tree roots. *Physiol. Plant.* **13**, 308–327. (doi:10.1111/j.1399-3054.1960.tb08034.x)

Boström, B., Comstedt, D. & Ekblad, A. 2008 Can isotopic fractionation during respiration explain the ^{13}C-enriched sporocarps of ectomycorrhizal and saprotrophic fungi? *New Phytol.* **177**, 1012–1019. (doi:10.1111/j.1469-8137.2007.02332.x)

Burgeff, H. 1932 *Saprophytismus und Symbiose. Studien an tropischen Orchideen*. Jena, Germany: Gustav Fischer.

Campbell, E. O. 1962 The mycorrhiza of *Gastrodia cunninghamii* Hook. f. *Trans. R. Soc. New Zeal.* **1**, 289–296.

Campbell, E. O. 1964 The fungal association in a colony of *Gastrodia sesamoides* R. *Br. Trans. R. Soc. New Zeal.* **2**, 237–246.

Cannon, P. F. & Kirk, P. M. 2007 *Fungal families of the world*. Wallingford, UK: CABI Publishing.

Cha, J. Y. & Igarashi, T. 1996 *Armillaria jezoensis*, a new symbiont of *Galeola septentrionalis* (Orchidaceae) in Hokkaido. *Mycoscience* **37**, 21–24. (doi:10.1007/BF02461451)

Cullings, K. W., Szaro, T. M. & Bruns, T. D. 1996 Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites. *Nature* **379**, 63–66. (doi:10.1038/379063a0)

Fan, L., Guo, S. X., Cao, W. Q., Xiao, P. G. & Xu, J. T. 1996 Isolation, culture, identification and biological activity of *Mycena orchidicola* sp. nov. in *Cymbidium sinense* (Orchidaceae). *Acta Mycol. Sin.* **15**, 251–255.

Felsenstein, J. 1985 Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791. (doi:10.2307/2408678)

Gardes, M. & Bruns, T. D. 1993 ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118. (doi:10.1111/j.1365-294X.1993.tb00005.x)

Gebauer, G. & Meyer, M. 2003 ^{15}N and ^{13}C natural abundance of autotrophic and mycoheterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytol.* **160**, 209–223. (doi:10.1046/j.1469-8137.2003.00872.x)

Gebauer, G. & Taylor, A. F. S. 1999 ^{15}N natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. *New Phytol.* **142**, 93–101. (doi:10.1046/j.1469-8137.1999.00373.x)

Girlanda, M. *et al.* 2006 Inefficient photosynthesis in the mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Mol. Ecol.* **15**, 491–504. (doi:10.1111/j.1365-294X.2005.02770.x)

Hamada, M. 1939 Studien über die Mykorrhiza von *Galeola septentrionalis* Reichb. f.—Ein neuer fall der mykorrhiza-bildung durch intraradicale Rhizomorpha. *Jpn J. Bot.* **10**, 151–211.

Hamada, M. & Nakamura, S. 1963 Wurzelsymbiose von *Galeola altissima* Reichb. F., einer chlorophyllfreien Orchidee, mit dem holzzerstorenden *Pilz Hymenochaete crocicreas* berk et Br. *Sci. Rep. Tohuku Univ. Ser. IV* **29**, 227–238.

Holm, S. 1979 A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**, 65–70.

Hopple, J. S. & Vilgalys, R. 1999 Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. *Mol. Phylogenet. Evol.* **13**, 1–19. (doi:10.1006/mpev.1999.0634)

Kikuchi, G., Higuchi, M., Yoshimura, H., Morota, T. & Suzuki, A. 2008*a* *In vitro* symbiosis between *Gastrodia elata* Blume (Orchidaceae) and *Armillaria* Kummer (Tricholomataceae) species isolated from the orchid tuber. *J. Jpn Bot.* **83**, 77–87.

Kikuchi, G., Higuchi, M., Morota, T., Nagasawa, E. & Suzuki, A. 2008*b* Fungal symbiont and cultivation test of *Gastrodia elata* Blume (Orchidaceae). *J. Jpn Bot.* **83**, 88–95.

Kimura, M. 1980 A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120. (doi:10.1007/BF01731581)

Kohzu, A., Yoshioka, T., Ando, T., Takahashi, M., Koba, K. & Wada, E. 1999 Natural ^{13}C and ^{15}N abundance of field-collected fungi and their ecological implications. *New Phytol.* **144**, 323–330. (doi:10.1046/j.1469-8137.1999.00508.x)

Kusano, S. 1911 *Gastrodia elata* and its symbiotic association with *Armillaria mellea*. *J. Coll. Agric. Imp. Univ. Tokyo* **4**, 1–65.

Maas Geesteranus, R. A. 1992 *Mycenas* of the Northern Hemisphere II. Conspectus of the *Mycenas* of the Northern Hemisphere. *Koninklijke Nederlandse Academie van Wetenschappen, Verhandelingen Afd. Natuurkunde, Tweede Reeks* **90**, 1–493.

Matheny, P. B. *et al.* 2006 Major clades of Agaricales: a multilocus phylogenetic overview. *Mycologia* **98**, 982–995. (doi:10.3852/mycologia.98.6.982)

McKendrick, S. L., Leake, J. R., Taylor, D. L. & Read, D. J. 2000 Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corallorhiza trifida* and characterization of its mycorrhizal fungi. *New Phytol.* **145**, 523–537. (doi:10.1046/j.1469-8137.2000.00603.x)

McKendrick, S. L., Leake, J. R., Taylor, D. L. & Read, D. J. 2002 Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytol.* **154**, 233–247. (doi:10.1046/j.1469-8137.2002.00372.x)

Merckx, V. & Bidartondo, M. I. 2008 Breakdown and delayed cospeciation in the arbuscular mycorrhizal mutualism. *Proc. R. Soc. B* **275**, 1029–1035. (doi:10.1098/rspb.2007.1622)

Moncalvo, J. M., Lutzoni, F. M., Rehner, S. A., Johnson, J. & Vilgalys, R. 2000 Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Syst. Biol.* **49**, 278–305. (doi:10.1080/10635159950173852)

Moncalvo, J. M. *et al.* 2002 One hundred and seventeen clades of euagarics. *Mol. Phylogenet. Evol.* **23**, 357–400. (doi:10.1016/S1055-7903(02)00027-1)

Ogura-Tsujita, Y. & Yukawa, T. 2008 High mycorrhizal specificity in a widespread mycoheterotrophic plant, *Eulophia zollingeri* (Orchidaceae). *Am. J. Bot.* **95**, 93–97. (doi:10.3732/ajb.95.1.93)

Preiß, K. & Gebauer, G. 2008 A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes Environ. Health Stud.* **44**, 375–383.

Saitou, N. & Nei, M. 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Selosse, M. A., Weiss, M., Jany, J. L. & Tillier, A. 2002 Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Mol. Ecol.* **11**, 1831–1844. (doi:10.1046/j.1365-294X.2002.01553.x)

Smith, S. E. & Read, D. J. 2008 *Mycorrhizal symbiosis*. London, UK: Academic Press.

Swofford, D. L. 2001 *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, v. 4.0.b6. Sunderland, MA: Sinauer Associates.

Tashima, Y., Terashita, T., Umata, H. & Matsumoto, M. 1978 *In vitro* development from seed to flower in *Gastrodia verrucosa* under fungal symbiosis. *Trans. Mycol. Soc. Jpn* **19**, 449–453.

Taylor, D. L. & Bruns, T. D. 1997 Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proc. Natl Acad. Sci. USA* **94**, 4510–4515. (doi:10.1073/pnas.94.9.4510)

Taylor, D. L. & Bruns, T. D. 1999 Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchids *Corallorhiza maculata* and *C. mertensiana*. *Mol. Ecol.* **8**, 1719–1732. (doi:10.1046/j.1365-294x.1999.00760.x)

Thompson, J. N. 2005 *The geographic mosaic of coevolution*. Chicago, IL: The University of Chicago Press.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997 The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882. (doi:10.1093/nar/25.24.4876)

Trappe, J. M. 1987 Phylogenetic and ecologic aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In *Ecophysiology of VA mycorrhizal plants* (ed. G. R. Safir), pp. 5–25. Boca Raton, FL: CRC Press.

Trudell, S. A., Rygiewicz, P. T. & Edmonds, R. L. 2003 Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytol.* **160**, 391–401. (doi:10.1046/j.1469-8137.2003.00876.x)

Trudell, S. A., Rygiewicz, P. T. & Edmonds, R. L. 2004 Patterns of nitrogen and carbon stable isotope ratios in macrofungi, plants and soils in two old-growth conifer forests. *New Phytol.* **164**, 317–335. (doi:10.1111/j.1469-8137.2004.01162.x)

Umata, H. 1995 Seed germination of *Galeola altissima*, an achlorophyllous orchid, with Aphyllophorales fungi. *Mycoscience* **36**, 369–372. (doi:10.1007/BF02268616)

Umata, H. 1997 Formation of endomycorrhizas by an achlorophyllous orchid, *Erythrorchis ochobiensis*, and *Auricularia polytricha*. *Mycoscience* **38**, 335–339. (doi:10.1007/BF02464092)

Umata, H. 1998*a* *In vitro* symbiotic association of an achlorophyllous orchid, *Erythrorchis ochobiensis*, with orchid and non-orchid fungi. *Mem. Fac. Agr. Kagoshima Univ.* **34**, 97–107.

Umata, H. 1998*b* A new biological function of shiitake mushroom, *Lentinula edodes*, in a myco-heterotrophic orchid, *Erythrorchis ochobiensis*. *Mycoscience* **39**, 85–88. (doi:10.1007/BF02461583)

Vilgalys, R. & Hester, M. 1990 Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **172**, 4238–4246.

Walther, G., Garnica, S. & Weiss, M. 2005 The systematic relevance of conidiogenesis modes in the gilled Agaricales. *Mycol. Res.* **109**, 525–544. (doi:10.1017/S0953756205002868)

Wang, B. & Qiu, Y. L. 2006 Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* **16**, 299–363. (doi:10.1007/s00572-005-0033-6)

White, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and application* (eds M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White), pp. 315–322. San Diego, CA: Academic Press.

Xu, J. T. & Fan, L. 2001 Cytodifferentiation of the seeds (protocorms) and vegetative propagation corms colonized by mycorrhizal fungi. *Acta Bot. Sin.* **43**, 1003–1010.

Xu, J. T. & Mu, C. 1990 The relation between growth of *Gastrodia elata* protocorms and fungi. *Acta Bot. Sin.* **32**, 26–31.

Yamato, M., Yagame, T., Suzuki, A. & Iwase, K. 2005 Isolation and identification of mycorrhizal fungi associating with an achlorophyllous plant, *Epipogium roseum* (Orchidaceae). *Mycoscience* **46**, 73–77. (doi:10.1007/s10267-004-0218-4)

Yang, S. & Pfister, D. H. 2006 *Monotropa uniflora* plants of eastern Massachusetts form mycorrhizae with a diversity of russulacean fungi. *Mycologia* **98**, 535–540. (doi:10.3852/mycologia.98.4.535)

Yokoyama, J., Fukuda, T. & Tsukaya, H. 2005 Molecular identification of the mycorrhizal fungi of the epiparasitic plant *Monotropastrum humile* var. *glaberrimum* (Ericaceae). *J. Plant Res.* **118**, 53–56. (doi:10.1007/s10265-004-0188-9)

Young, B. W., Massicotte, H. B., Tackaberry, L. E., Baldwin, Q. F. & Egger, K. N. 2002 *Monotropa uniflora*: morphological and molecular assessment of mycorrhizae retrieved from sites in the sub-boreal spruce biogeoclimatic zone in central British Columbia. *Mycorrhiza* **12**, 75–82.

Zimmer, K., Hynson, N. A., Gebauer, G., Allen, E. B., Allen, M. F. & Read, D. J. 2007 Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. *New Phytol.* **175**, 166–175. (doi:10.1111/j.1469-8137.2007.02065.x)

Zimmer, K., Meyer, C. & Gebauer, G. 2008 The ectomycorrhizal specialist orchid *Corallorhiza trifida* is a partial myco-heterotroph. *New Phytol.* **178**, 395–400. (doi:10.1111/j.1469-8137.2007.02362.x)