

^{15}N and ^{13}C natural abundance of two mycoheterotrophic and a putative partially mycoheterotrophic species associated with arbuscular mycorrhizal fungi

Vincent Merckx^{1,2}, Marcus Stöckel³, Andreas Fleischmann⁴, Thomas D. Bruns² and Gerhard Gebauer³

¹Laboratory of Plant Systematics, KU Leuven, Institute of Botany and Microbiology, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium; ²Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, CA 94720, USA; ³Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, D-95440 Bayreuth, Germany; ⁴LMU Munich, Systematic Botany, D-80638 Munich, Germany

Summary

Author for correspondence:

Vincent Merckx

Tel: +32 (0)16 328637

Email: vincent.merckx@bio.kuleuven.be

Received: 22 April 2010

Accepted: 31 May 2010

New Phytologist (2010) **188**: 590–596

doi: 10.1111/j.1469-8137.2010.03365.x

Key words: arbuscular mycorrhizal symbiosis, Burmanniaceae, carbon transfer, cheating, Gentianaceae, Glomeromycota, mixotrophy, mycoheterotrophy.

- In contrast to mycoheterotrophs that associate with ectomycorrhizal and saprotrophic fungi, we know little about the ecophysiology of arbuscular mycorrhizal mycoheterotrophs. Here, we identify the mycorrhizal fungi of two unrelated mycoheterotrophs and one putative partial mycoheterotroph that form arbuscular mycorrhizas, and analyse their carbon (C) and nitrogen (N) isotope signatures.
- We used molecular methods to identify the mycorrhizal fungi of *Dictyostega orobanchoides*, *Burmannia capitata* (Burmanniaceae) and *Voyria aphylla* (Gentianaceae). Their C and N sources were investigated by analysing their stable isotope natural abundances ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). In addition, four putative partially mycoheterotrophic *Burmannia* species were grown *ex situ*.
- We found that both mycoheterotrophs and a green *Burmannia* species are associated with nonoverlapping Glomeromycota fungi. The investigated mycoheterotrophs are significantly more enriched in ^{13}C than co-occurring autotrophic plants but lack significant ^{15}N enrichment. The green *Burmannia* species is not significantly enriched in ^{13}C and ^{15}N compared with surrounding plants and can grow fully autotrophically under controlled conditions.
- Our results suggest that mycoheterotrophic Burmanniaceae and Gentianaceae are able to exploit arbuscular mycorrhizal fungi. Green relatives of mycoheterotrophic Burmanniaceae from high-light grassland sites also associate with arbuscular mycorrhizal fungi but we found no evidence that they receive detectable amounts of C from fungi.

Introduction

In deeply shaded forest understoreys, plants are light-limited and to cope with this limitation numerous plants have evolved to cheat mycorrhizal networks, or free-living saprotrophic fungi, by gaining organic carbon (C) and other essential elements from the fungi. These achlorophyllous plants are referred to as 'mycoheterotrophs' (Leake, 1994; Selosse & Cameron, 2010). Analysis of ^{13}C and ^{15}N natural abundances have been extensively used to provide insights

into C and nitrogen (N) sources of mycoheterotrophic plants mycorrhizal with ectomycorrhizal (ECM) fungi (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Bidartondo *et al.*, 2004; Zimmer *et al.*, 2007; Hynson *et al.*, 2009; Roy *et al.*, 2009; Liebel *et al.*, 2010) and mycoheterotrophic plants mycorrhizal with wood-decaying (SAP) fungi (Martos *et al.*, 2009; Ogura-Tsujita *et al.*, 2009). These studies show that mycoheterotrophic plants that associate with ECM and SAP fungi are significantly enriched in ^{13}C and ^{15}N compared with autotrophic understorey plants

(Gebauer & Meyer, 2003; Tedersoo *et al.*, 2007). It has been suggested that these enrichments indicate that mycoheterotrophic plants obtain C and N through distinct pathways compared with those used by autotrophic plants (Gebauer & Meyer, 2003). In addition, the C and N isotope signatures of mycoheterotrophic plants resemble those of their associated fungi providing further evidence that the fungi are the sole nutrient source for these plants. To date, no data on the natural abundances of stable isotopes of arbuscular mycorrhizal (AM) mycoheterotrophs, or AM fungi, has been published.

This investigation aimed to test: whether the mycoheterotrophs *Dictyostegia orobanchoides* and *Voyria aphylla* from a rain forest site in French Guiana are associated with AM fungi as indicated by root anatomical observations (Imhof, 1999, 2001); whether these AM mycoheterotrophs are distinguished by their C and N isotope signatures from co-occurring green plants, similar to what is observed in ECM and SAP mycoheterotrophs; and whether the putative partial mycoheterotroph *Burmanniea capitata*, which is closely related to achlorophyllous species (Merckx *et al.*, 2008), is also associated with AM fungi and can be distinguished from co-occurring plants by its C and N isotope signatures. In addition we test the dependence on fungal carbon of several green *Burmanniea* species in growth experiments.

Materials and Methods

Sample collection and locations

Specimens of *V. aphylla* (Jacq.) Pers. (Gentianaceae), *D. orobanchoides* (Hook.) Miers (Burmanniaceae) and *B. capitata* Mart. (Burmanniaceae) were sampled from two sites (10 × 10 m) in French Guiana. The sites are located c. 50 m from each other on the granitic inselberg 'Savane-Roche Virginie' at 100 m above sea level. The climate is tropically moist, with c. 3000 mm of annual precipitation (Jabiol *et al.*, 2009). The first site (04°11'42.6" N 52°08'58.5" W) consists of rain forest on organic soil (pH 3.6) with only few understorey plants (*Rapatea paludosa* Aubl. (Rapateaceae), *Miconia argyrophylla* DC. (Melastomataceae) and *Matayba inelebensis* Spruce ex Radlk. (Sapindaceae)). The mycoheterotrophic species *V. aphylla* and *D. orobanchoides* co-occurred at this site. The second site (04°11'46.5" N 52°08'56.8" W) consists of wet grassland on organic soil (pH 3.8) that formed in a depression of the inselberg surface and is surrounded by bare granite rock. In addition to *B. capitata* the vegetation consisted of grasses and sedges (including *Rhynchospora* sp. (Cyperaceae)), *Stylosanthes guianensis* (Aubl.) Sw. (Fabaceae), *Ipomoea lepreurii* D.F. Austin (Convolvulaceae), *Chamaecrista diphylla* (L.) Greene (Fabaceae) and the carnivorous plant *Utricularia hispida* Lam (Lentibulariaceae). Light climate data of both plots were obtained with a Twinmate L-208 Lightmeter (Sekonic,

Elmsford, NY, USA) at 20 cm from ground level at three different points in both plots. The rain forest plot received a mean of 160 lux, while in the grassland plot a mean of 57 800 lux was measured.

Molecular identification of mycorrhizal fungi

In the field, five specimens of each species (*V. aphylla*, *D. orobanchoides* and *B. capitata*) were excavated and their entire root systems were thoroughly cleaned with water to remove all soil particles. After cleaning the root systems were separately stored on 2% cetyltrimethylammonium bromide (CTAB) buffer at -80°C. Genomic DNA was extracted from all 15 root systems using methods described by Gardes & Bruns (1993). We carried out PCR amplification of partial 18S rDNA following Schechter & Bruns (2008) with primers NS31 (Simon *et al.*, 1992) and AM1 (Helgason *et al.*, 1998). All 15 PCR products were cloned using the pGEM-T Vector System II (Promega). Of each PCR product eight clones were unidirectionally sequenced using the plasmid primer T7. The resulting 18S rDNA sequences were combined with published sequences from vouchered AM fungi and AM fungi detected in tree seedlings from a tropical forest in Panama (Husband *et al.*, 2002), and aligned with the alignment tool implemented in GENEIOUS PRO (ver. 4.8.5) (Drummond *et al.*, 2009). Identical sequences were defined as a single fungal operational taxonomic unit (OTU) and duplicate sequences were removed from the alignment using RAXML (ver. 7.0.4) (Stamatakis, 2006). Phylogenetic trees were constructed under maximum likelihood (ML) with RAXML and under Bayesian inference (BI) with MRBAYES (ver. 3.1.2) (Ronquist & Huelsenbeck, 2003). Clade support under ML was calculated using 500 nonparametric bootstrap (BS) analyses. Bayesian analyses were run for 5 × 10⁶ generations sampling every 1000th generation. A majority rule consensus tree was calculated on the last 2500 sampled trees to obtain Bayesian posterior probabilities (BPP). Both ML and BI analyses were run using the GTR + Γ + I model of molecular evolution as selected using JMODELTEST (ver. 0.1.1) (Posada, 2008). Voucher details and GenBank accessions are listed in the Supporting Information, Table S1.

Stable isotope signatures

Flowering stalks of eight individuals of each mycoheterotrophic species (*D. orobanchoides* and *V. aphylla*) and leaves of eight individuals of each autotrophic reference species (*R. paludosa*, *M. argyrophylla* and *M. inelebensis*) were collected at the rain forest plot. At the grassland plot flowering stalks of eight individuals of *B. capitata* and leaves of eight or nine individuals of each reference species (*Rhynchospora* sp., *Stylosanthes guianensis*, *I. lepreurii*, *C. diphylla*, and *U. hispida*) were collected. Samples were dried at 105°C,

ground to a fine powder and stored in a desiccator. Relative C and N stable isotope abundances were measured using a dual element analysis mode with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer (Bidartondo *et al.*, 2004). Isotope abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$ (‰), where R is the ratio of heavy to light isotope of the sample or the respective standard). Mean values (± 1 SD) of each species per site were calculated and statistically tested against each other. In cases with normal distribution and homogeneous variances a one-way ANOVA and *post hoc* comparison based on Tukey-HSD test was carried out. Otherwise, a Kruskal–Wallis test followed by a Mann–Whitney U -test with an adjusted significance level (Holm, 1979) was used. The significance level for all statistic tests was 0.05. Enrichment factors, ϵ , were calculated according to $\epsilon_{x_{\text{MH}}} = \delta_{x_{\text{MH}}} - \delta_{x_{\text{R}}}$, with x as ^{13}C or ^{15}N , where $\delta_{x_{\text{MH}}}$ is the δ value of mycoheterotrophic species at a site and $\delta_{x_{\text{R}}}$ is the mean of δ values of the reference plants at the respective site. Statistical analyses were performed with SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA).

Growth experiments

We germinated seeds of *B. capitata*, *Burmannia bicolor*, *Burmannia disticha* and *Burmannia coelestis* in a peat–sand mixture at 25–30°C (daytime) to 15–2°C (night) and kept them continuously wet under bright and humid conditions in a glasshouse. Seedlings were transplanted separately into small plastic pots (using the same substrate) after *c.* 4 months and kept under artificial lights in humid conditions. A liquid fertilizer (WUXAL, NPK-fertilizer 8-8-6; Manna, Ammerbuch-Pfäffigen, Germany) diluted to 0.1% strength with tap water was applied directly on the rosette leaves every 2 wk.

Results

We obtained partial 18S rDNA sequences of 14 fungal OTUs from the root systems of five *D. orobanchoides* specimens. All OTUs are part of the *Glomus* Group A clade (Schüßler *et al.*, 2001), and except for one OTU they form a paraphyletic group that consists of the first two diverging lineages of the included *Glomus* Group A sequences (Fig. 1). In one *D. orobanchoides* specimen an additional OTU was detected that showed affinities with *Glomus proliferum* (AF213462). From the root systems of five specimens of *V. aphylla* a total of 15 OTUs were obtained. All OTUs detected from two specimens form a monophyletic group (100% BPP/84% BS) within Gigasporaceae. In the remaining three specimens *Glomus* Group A OTUs were detected. These OTUs do not form a monophyletic group. Eighteen OTUs were obtained from five *B. capitata*

specimens. All fungal OTUs detected in one *B. capitata* individual form a monophyletic group (97% BPP/86% BS) that is the sister group of all included Acaulosporaceae sequences (Fig. 1). Fungal OTUs obtained from the other three specimens belong to *Glomus* Group A. The majority of sequences belong to OTUs that are placed in a monophyletic group (without significant clade support) with affinities to *Glomus sinuosum* (Gerdemann & Bakshi) Almeida & Schenck (AJ133706). In two specimens somewhat distant *Glomus* Group A OTUs were also detected, each represented by a single sequence only (Fig. 1).

Stable isotope natural abundance analyses show that *D. orobanchoides* and *V. aphylla* are significantly enriched in ^{13}C in comparison with autotrophic reference plants growing at the same site (^{13}C enrichment factor $\epsilon_{D. orobanchoides} = 5.5 \pm 0.6$ ‰, $n = 8$ and $\epsilon_{V. aphylla} = 5.4 \pm 0.8$ ‰, $n = 8$) (Fig. 2a). No statistically significant ^{15}N enrichment was detected in the AM mycoheterotrophs we investigated (^{15}N $\epsilon_{D. orobanchoides} = 1.1 \pm 3.1$ ‰, $n = 8$ and $\epsilon_{V. aphylla} = -1.8 \pm 2.5$ ‰, $n = 8$). The C isotope signatures of *B. capitata* from a grassland site do not show statistically significant enrichment in comparison with all of the reference plants growing at the same site (Fig. 2b). The mean $\delta^{15}\text{N}$ value of *B. capitata* is statistically not distinguished from three of the five reference plant species. Four photosynthetic *Burmannia* species were germinated and grown to flowering stage in separate pots under normal light conditions, physically separated from other plants (Fig. 3). Plants flowered and set seed 7–8 months after germination. To our knowledge this is the first report of cultivation of these plants *ex situ*.

Discussion

All five *D. orobanchoides* specimens were associated with a narrow range of *Glomus* Group A OTUs, corroborating previous findings of highly specialized AM mycoheterotrophs (Bidartondo *et al.*, 2002; Franke *et al.*, 2006; Merckx & Bidartondo, 2008). However, the use of primer AM1, which has mismatches to *Glomus* Group B, Archaeosporales and Paraglomerales (Redecker *et al.*, 2000; Husband *et al.*, 2002), may have discriminated against these groups (but see Douhan *et al.*, 2005; Schechter & Bruns, 2008). In one specimen of *D. orobanchoides* a *Glomus* Group A OTU was detected that is phylogenetically distinct from the remaining *Glomus* Group A fungi found in this species. Specimens of *V. aphylla* collected at the same site were either associated with a clade of Gigasporaceae or *Glomus* Group A fungi. It remains to be determined whether *V. aphylla* is specialized on multiple fungal lineages – potentially there may exist different mycorrhizal host races within *V. aphylla* (Taylor *et al.*, 2004) – or whether *V. aphylla* lacks mycorrhizal specificity. Interestingly, preferences towards different lineages of mycorrhizal fungi by

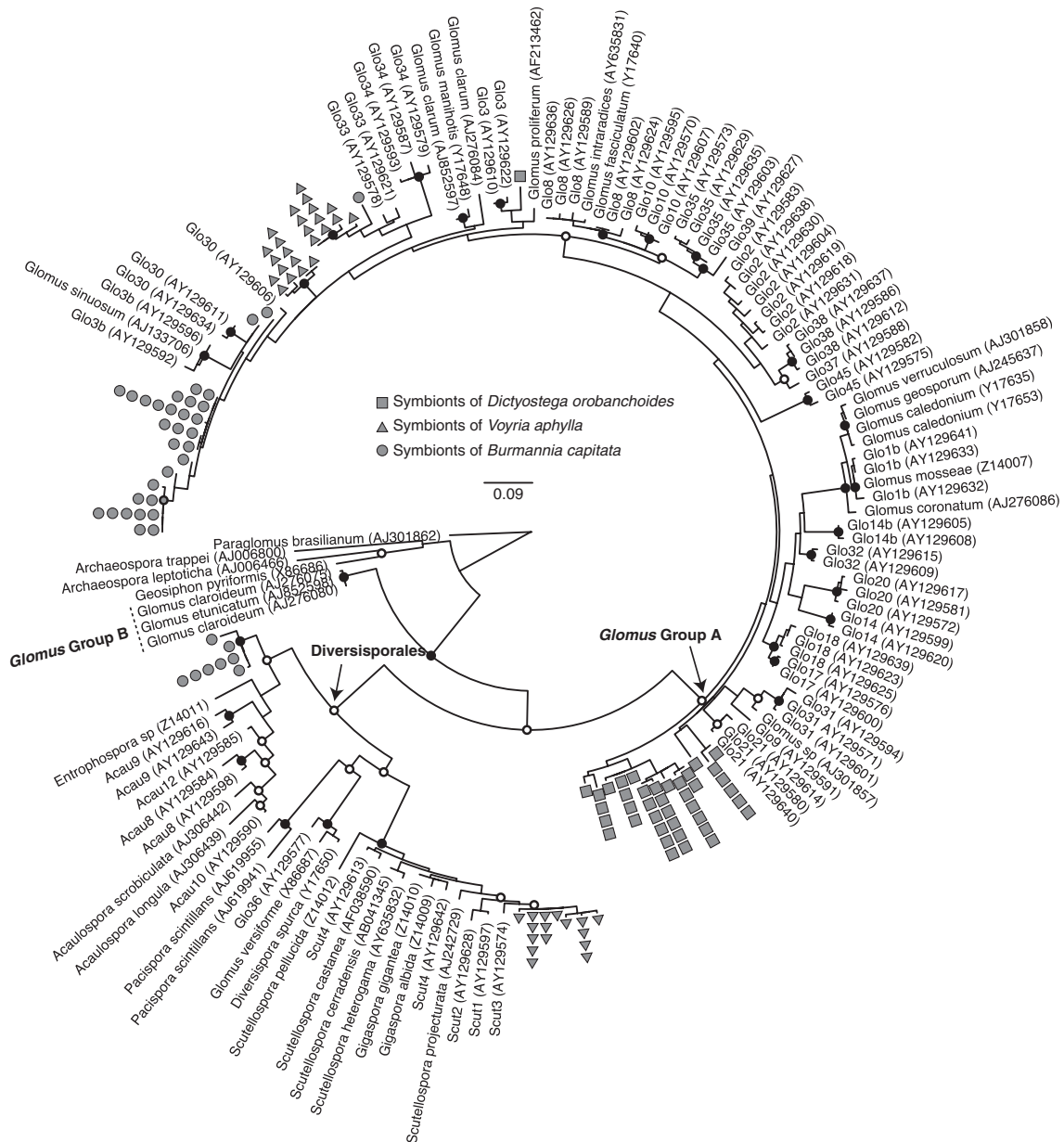


Fig. 1 Phylogenetic relationships of arbuscular mycorrhizal (AM) fungi associated with mycoheterotrophic *Dictyostega orobanchoides* (grey squares) and *Voyria aphylla* (grey triangles) plants, and green *Burmannia capitata* plants (grey circles). Nodes with posterior probability of 1.0 and maximum likelihood bootstrap support of $\geq 85\%$ are marked with closed circles; nodes with posterior probability of ≥ 0.95 and maximum likelihood bootstrap support of $< 85\%$ are marked with open circles.

mycoheterotrophs seem to exist as specimens of *D. orobanchoides* and *V. aphylla* from the same site exhibited no overlap in associated fungal OTUs. This result corroborates morphological observations, which showed structural differences between the mycorrhizas of both species (Imhof, 1999, 2001, 2009) and suggests that mycoheterotroph–fungus associations are not based solely on local availability of fungi.

This is the first report of stable isotope signatures of AM mycoheterotrophs. While it remains to be demonstrated whether the AM fungi we detected in the roots of

mycoheterotrophs are isotopically distinct from their photosynthetic host plants, similar to ECM fungi and associated forest plants (Gebauer & Meyer, 2003; Trudell *et al.*, 2003), we found that both mycoheterotrophs are significantly enriched in ^{13}C in comparison with autotrophic reference plants growing at the same site. We did not investigate the mycorrhizal status of the reference plants but published observations show that *Matayba guianensis* forms arbuscular mycorrhizas (Andrade *et al.*, 2000), and suggest that the two other species are also able to associate with AM fungi (Walker *et al.*, 1998; Wang & Qiu, 2006). Thus, in

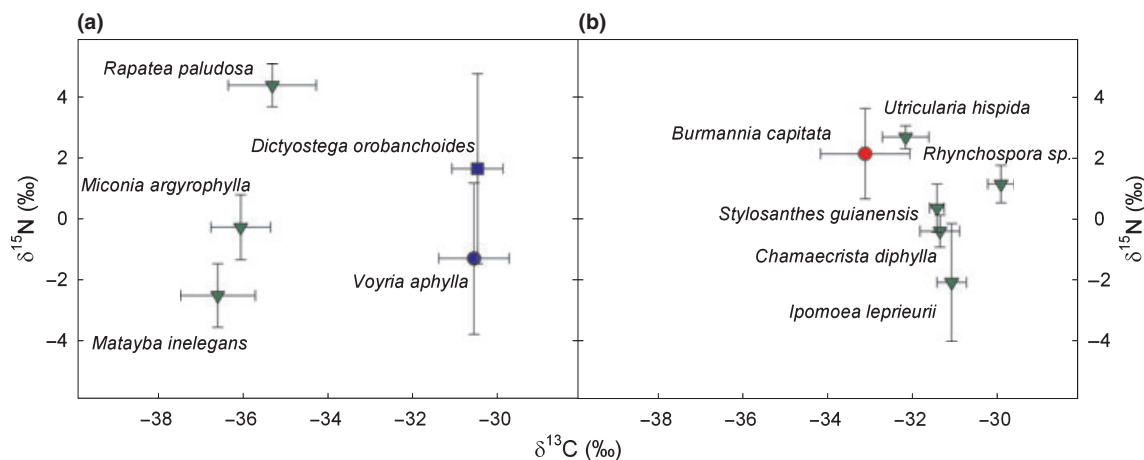


Fig. 2 Isotope signatures show that two achlorophyllous mycoheterotrophic arbuscular mycorrhizal (AM) species growing in the dark shade of a tropical rainforest are enriched in ^{13}C compared with accompanying autotrophic plants, but a related green species, *Burmannia capitata*, growing in the full sunlight of a nearby savanna is lacking ^{13}C enrichment. (a) Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (± 1 SD, $n = 8$) of two mycoheterotrophic AM plants (blue symbols) and three autotrophic reference species (green symbols) from the rainforest site. Mycoheterotrophic species differ significantly in $\delta^{13}\text{C}$ from all reference species ($P < 0.05$), but their respective $\delta^{15}\text{N}$ values show no significant differences. (b) Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (± 1 SD, $n = 8$ or 9) in specimens of *B. capitata* (red symbol) and five autotrophic reference species (green symbols) from the savanna site. Neither $\delta^{13}\text{C}$ nor $\delta^{15}\text{N}$ values of *B. capitata* are consistently distinguished from reference species.

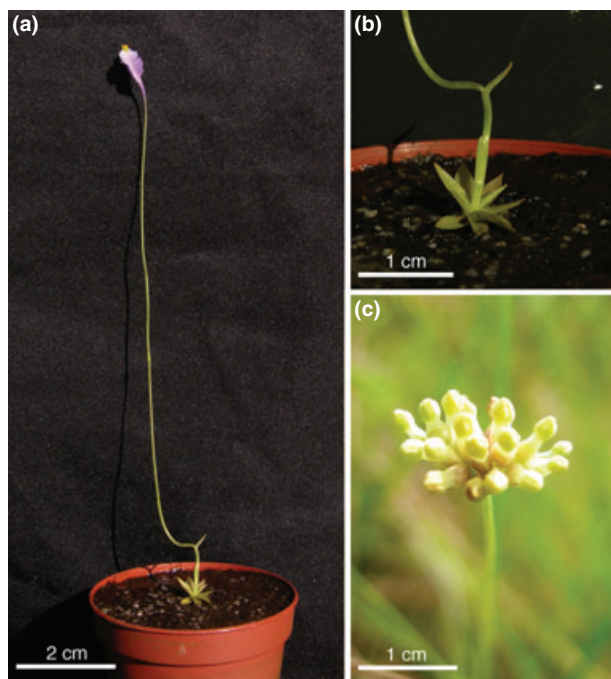


Fig. 3 Green *Burmannia* species *in situ* and in cultivation. (a) Flowering specimen of *B. bicolor* in cultivation. (b) Detail of reduced rosulate leaves of *B. capitata*. (c) Inflorescence of *B. capitata* at a savanna site in French Guiana.

parallel to observations in ECM mycoheterotrophs, the ^{13}C enrichment pattern of mycoheterotrophic plants is most likely the result of the ability of mycoheterotrophs to obtain C compounds from AM fungi. Therefore, this result strongly implies their dependence on fungal C and confirms their

mycoheterotrophic status as ^{13}C enrichment is the hallmark of mycoheterotrophy in plants. Mycoheterotrophs associated with ectomycorrhizal fungi exhibit very similar ^{13}C enrichments ($^{13}\text{C} \epsilon_{\text{ECM-MH}} = 7.2 \pm 1.6 \text{‰}$, $n = 92$) (Preiss & Gebauer, 2008) to the AM associated plants reported here, while mycoheterotrophs that associate with saprotrophic fungi show even greater enrichment ($^{13}\text{C} \epsilon_{\text{SAP-MH}} = 10.2 \pm 1.0 \text{‰}$, $n = 5$) (Ogura-Tsujita *et al.*, 2009). By contrast, ^{15}N enrichment does not occur in the AM mycoheterotrophs we investigated, while it is a pronounced, consistent pattern in ECM mycoheterotrophs ($^{15}\text{N} \epsilon_{\text{ECM-MH}} = 12.0 \pm 1.7 \text{‰}$, $n = 92$) (Gebauer & Meyer, 2003; Preiss & Gebauer, 2008) and a detectable, but less extreme, pattern in mycoheterotrophic plants associated with wood-decaying SAP fungi ($^{15}\text{N} \epsilon_{\text{SAP-MH}} = 2.9 \pm 0.5 \text{‰}$, $n = 5$) (Ogura-Tsujita *et al.*, 2009). The lack of differentiation in ^{15}N natural abundance between AM mycoheterotrophs and reference plants suggests that all of these plants tap isotopically similar N sources, presumably inorganic N compounds obtained through fungal partners, and demonstrates that two species of AM mycoheterotrophs from different taxonomic groups are characterized by a different isotope abundance pattern relative to all previously studied mycoheterotrophs.

The roots of green *B. capitata* specimens are also colonized by AM fungi. One individual collected at the grassland site is associated with a narrow clade of Acaulosporaceae fungi. The other four specimens are associated with a relatively narrow range of *Glomus* Group A fungi. The stable isotope signatures of *B. capitata* do not show enrichment in ^{13}C compared with reference plants growing at the same site. Similar to our observations in AM

mycoheterotrophs there are no significant differences in ^{15}N signatures between *Burmannia* and reference plants (Fig. 2b). In growth experiments, *B. capitata* and three other green *Burmannia* species were successfully germinated from seeds and grown up to flowering stage physically separated from other green plants (Fig. 3). While these data do not reject the potential for partial mycoheterotrophy in these species, they show that partial mycoheterotrophy is not obligate for germination and development of green *Burmannia* species *ex situ* when light levels are high. It remains to be tested whether green *Burmannia* species can obtain any C from surrounding plants through AM fungi in low-light conditions as recently shown for two green orchids associated with ECM fungi (Preiss *et al.*, 2010) or during germination.

Acknowledgements

We thank Martin Bidartondo, Nicole Hynson, Tom Madsen, Shannon Schechter, and Erik Smets for discussion and advice, Steven Janssens, Samuël Fourret, Jeroen Casteels, Merlijn Jocqué and Steven Desein for collecting samples, and Filip Vandeloos, Nathalie Geerts, Anja Vandepierre and Christine Tiroch for technical assistance. This research was supported by grants from the Belgian American Educational Foundation (BAEF), het Van Eeden Fonds, Biodiversity Inventory for Conservation (BINCO vzw), and the Research Foundation–Flanders (FWO) to VM.

References

- Andrade ACS, Queiroz MH, Hermes RAL, Oliveira VL. 2000. Mycorrhizal status of some of the Araucaria forest and the Atlantic rainforest in Santa Catarina, Brazil. *Mycorrhiza* 10: 131–136.
- Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society Series B, Biological Sciences* 271: 1799–1806.
- Bidartondo MI, Redecker D, Hijri I, Wiemken A, Bruns TD, Dominguez LS, Sérsic A, Leake JR, Read DJ. 2002. Epiparasitic plants specialized on mycorrhizal fungi. *Nature* 419: 389–392.
- Douhan GW, Petersen C, Bledsoe CS, Rizzo DM. 2005. Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification? *Mycorrhiza* 15: 365–372.
- Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A. 2009. *Geneious v4.8*. URL <http://www.geneious.com/> [last accessed 5 July 2010].
- Franke T, Beenken L, Döring M, Kocyan A, Agerer R. 2006. Arbuscular mycorrhizal fungi of the *Glomus*-group A lineage (Glomerales; Glomeromycota) detected in mycoheterotrophic plants from tropical Africa. *Mycological Progress* 5: 24–31.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.
- Gebauer G, Meyer M. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist* 160: 209–223.
- Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW. 1998. Ploughing up the wood-wide web? *Nature* 394: 431–431.
- Holm S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 65–70.
- Husband R, Herre EA, Turner SL, Gallery R, Young JP. 2002. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Molecular Ecology* 11: 2669–2678.
- Hynson NA, Preiss K, Gebauer G, Bruns TD. 2009. Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae). *New Phytologist* 182: 719–726.
- Imhof S. 1999. Root morphology, anatomy and mycotrophy of the achlorophyllous *Voyria aphylla* (Jacq.) Pers. (Gentianaceae). *Mycorrhiza* 9: 33–39.
- Imhof S. 2001. Subterranean structures and mycotrophy of the achlorophyllous *Dictyostegia orobanchoides* (Hook.) Miers (Burmanniaceae). *Revista de Biología Tropical* 49: 239–247.
- Imhof S. 2009. Arbuscular, ecto-related, orchid mycorrhizas – three independent structural lineages towards mycoheterotrophy: implications for classification? *Mycorrhiza* 19: 357–363.
- Jabiou J, Corbara B, Dejean A, Céréghino R. 2009. Structure of aquatic insect communities in tank-bromeliads in a East-Amazonian rainforest in French Guiana. *Forest Ecology and Management* 257: 351–360.
- Leake JR. 1994. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist* 127: 171–216.
- Liebel HT, Bidartondo MI, Preiss K, Segreto R, Stöckel M, Rodda M, Gebauer G. 2010. C and N stable isotope signatures reveal constraints to nutritional modes on orchids from the Mediterranean and Macaronesia. *American Journal of Botany* 97: 1–10.
- Martos F, Dulormne M, Pailler T, Bonfante P, Faccio A, Fournel J, Dubois M-P, Selosse M-A. 2009. Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous orchids. *New Phytologist* 184: 668–681.
- Merckx V, Bidartondo MI. 2008. Breakdown and delayed cospeciation in the arbuscular mycorrhizal mutualism. *Proceedings of the Royal Society Series B, Biological Sciences* 275: 1029–1035.
- Merckx V, Chatrou LW, Lemaire B, Sainge MN, Huysmans S, Smets EF. 2008. Diversification of myco-heterotrophic angiosperms: evidence from Burmanniaceae. *BMC Evolutionary Biology* 8: 178.
- Ogura-Tsujita Y, Gebauer G, Hashimoto T, Umata H, Yukawa T. 2009. Evidence for novel and specialized mycorrhizal parasitism: the orchid *Gastrodia confusa* gains carbon from saprotrophic *Mycena*. *Proceedings of the Royal Society Series B, Biological Sciences* 276: 761–767.
- Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* 25: 1253–1256.
- Preiss K, Adam IKU, Gebauer G. 2010. Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially myco-heterotrophic orchids. *Proceedings of the Royal Society Series B, Biological Sciences* 277: 1333–1336.
- Preiss K, Gebauer G. 2008. A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes in Environmental and Health Studies* 44: 375–383.
- Redecker D, Morton JB, Bruns TD. 2000. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Molecular Phylogenetics and Evolution* 14: 276–284.
- Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.
- Roy M, Watthana S, Stier A, Richard F, Vessabutr S, Selosse M-A. 2009. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. *BMC Biology* 7: 51.

- Schechter SP, Bruns TD. 2008. Serpentine and non-serpentine ecotypes of *Collinsia sparsiflora* associate with distinct arbuscular mycorrhizal fungal assemblages. *Molecular Ecology* 17: 3198–3210.
- Schüßler A, Gehrig H, Schwarzott D, Walker C. 2001. Analysis of partial Glomales SSU rRNA genes: implications for primer design and phylogeny. *Mycological Research* 105: 5–15.
- Selosse M-A, Cameron DD. 2010. Introduction to a Virtual Special Issue on mycoheterotrophy: *New Phytologist* sheds light on nongreen plants. *New Phytologist* 185: 591–592.
- Simon L, Lalonde M, Bruns TD. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular–arbuscular endomycorrhizal fungi colonizing roots. *Applied and Environmental Microbiology* 58: 291–295.
- Stamatakis A. 2006. RAxML-VI-HPC: maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.
- Taylor DL, Bruns TD, Hodges SA. 2004. Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society Series B, Biological Sciences* 271: 35–43.
- Tedersoo L, Pellet P, Kõljalg U, Selosse M-A. 2007. Parallel evolutionary paths to mycoheterotrophy in understory Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. *Oecologia* 151: 206–217.
- Trudell SA, Rygielwicz PT, Edmonds RL. 2003. Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytologist* 160: 391–401.
- Walker C, Cuenca G, Sanchez F. 1998. *Scutellospora spinosissima* sp. nov., a newly described glomalean fungus from acidic, low nutrient plant communities in Venezuela. *Annals of Botany* 82: 721–725.
- Wang B, Qiu YL. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16: 299–363.
- Zimmer KA, Hynson A, Gebauer G, Allen EB, Allen MF, Read DJ. 2007. Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. *New Phytologist* 175: 166–175.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Voucher information and GenBank accessions of specimens analysed

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.



About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at www.newphytologist.org.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *Early View* – our average submission to decision time is just 29 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £151 in Europe/\$279 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (newphytol@lancaster.ac.uk; tel +44 1524 594691) or, for a local contact in North America, the US Office (newphytol@ornl.gov; tel +1 865 576 5261).