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Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees

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In the mycorrhizal symbiosis, plants exchange photosynthates for mineral nutrients acquired by fungi from the soil. This mutualistic arrangement has been subverted by hundreds of mycorrhizal plant species that lack the ability to photosynthesize. The most numerous examples of this behaviour are found in the largest plant family, the Orchidaceae. Although non-photosynthetic orchid species are known to be highly specialized exploiters of the ectomycorrhizal symbiosis, photosynthetic orchids are thought to use free-living saprophytic or pathogenic fungal lineages. However, we present evidence that putatively photosynthetic orchids from five species that grow in the understorey of forests (i) form mycorrhizas with ectomycorrhizal fungi of forest trees and (ii) have stable-isotope signatures indicating distinctive pathways for nitrogen and carbon acquisition approaching those of non-photosynthetic orchids that associate with ectomycorrhizal fungi of forest trees. These findings represent a major shift in our understanding of both orchid ecology and evolution because they explain how orchids can thrive in low-irradiance niches and they show that a shift to exploiting ectomycorrhizal fungi precedes viable losses of photosynthetic ability in orchid lineages.

Keywords: *Epipactis*; *Cephalanthera*; mycorrhizae; partial myco-heterotrophy; symbiosis; *Tuber*

1. INTRODUCTION

The Orchidaceae is the largest and most diverse family of plants on Earth. One of its most distinctive characteristics is the production of minute seeds that contain only minimal reserves of nutrients (Arditti & Ghani 2000). This makes orchids dependent upon mycorrhizal fungi for the provision of the resources necessary for germination and for growth, at least in the early stages of their development (Bernard 1909; Burgeff 1959). Such fungus-dependent modes of nutrition, referred to as myco-heterotrophy, have evolved independently several times during plant evolution (Leake 1994). There is a widespread assumption that in the majority of orchid species, which are photosynthetic in the adult phases of their lives, the ability to photosynthesize will provide a release from the dependence on fungi for carbon supplies (Smith & Read 1997). Nonetheless, a large proportion of green, and hence putatively photosynthetic, orchids grow in such deeply shaded forest habitats that carbon gains from photosynthesis are likely to be minimal. In fact, complete loss of photosynthetic ability, coupled with obligate myco-heterotrophy into the adult phase, may have evolved at least 20 times in the Orchidaceae (Molvray *et al.* 2000). Because terrestrial orchids include some of the most vulnerable components of plant communities worldwide (Batty *et al.* 2002), it is of pressing concern to determine how these orchids are sustained throughout their life cycle under natural conditions. Currently, there are two known mature-orchid nutritional modes: (i) obligate autotrophy (i.e. photosynthetic) with over 17 000 species; and (ii) obligate myco-heterotrophy (i.e. non-photosynthetic) with over 200 species. However, it has been proposed recently that a third nutritional mode in which fungi subsidize the nutrition of putatively photosynthetic orchids ('partial myco-heterotrophy') accounts for a significant number of the forest-understorey species currently considered obligate autotrophs (Gebauer & Meyer 2003).

Since early in the last century, it has been widely accepted that most orchid mycorrhizal fungi are saprophytic or pathogenic rhizoctonia-forming basidiomycete fungi (i.e. a polyphyletic assemblage that includes Ceratobasidiales, Exidiales and Tulasnellales) (Bernard 1909; Roberts 1999). Experimental studies have shown that these easily cultivable fungi can sustain below-ground development of some orchids by transferring carbon from soil organic matter to developing seedlings (Smith & Read 1997). Interest in the possibility that other functional groups of fungi might be symbiotic partners with photosynthetic orchids has recently been aroused by two sets of independent observations: (i) molecular ecological analyses have shown that several wholly non-photosynthetic orchid species form orchid mycorrhizas with hardly cultivable fungi that simultaneously form ectomycorrhizas with the roots of neighbouring trees (Taylor & Bruns 1997; Selosse *et al.* 2002); and (ii) mass-spectrometric analyses of wholly non-photosynthetic orchids, monotropes and putatively photosynthetic orchids (Gebauer & Meyer 2003; Trudell *et al.* 2003) of forest habitats have revealed that

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their tissues carry nitrogen and carbon stable-isotope signatures indicative of alternative pathways for the acquisition of these elements. In fact, Gebauer and Meyer postulated that putatively photosynthetic forest orchids are connected both to typical orchid mycorrhizal fungi (i.e. the rhizoctonia-forming fungi) 'and to basidiomycetes forming ectomycorrhizas with trees' (p. 221).

We combine molecular and mass-spectrometric approaches and apply them to the same individual plants of eight orchid species growing in their natural plant communities at four sites in Germany. These species represent three functional groups: photosynthetic orchids of open habitats, putatively photosynthetic orchids of shaded habitats and wholly non-photosynthetic orchids. We show that the latter two groups are colonized by fungi that are ectomycorrhizal associates of trees and that their shoot nitrogen and carbon signatures are sufficiently distinct from those of orchids colonized by rhizoctonia-forming fungi to indicate reliance upon alternative nutritional pathways. We also surveyed the mycorrhizal fungi of three additional orchid species at 12 locations outside Germany and confirmed that associations between putatively photosynthetic orchids of shaded habitats and ectomycorrhizal fungi are widespread and display relatively narrow fungal preference. This newly revealed interdependence of orchids and the fungal symbionts of neighbouring trees has direct implications for our understanding of the management of rare and endangered orchids, the ecology and evolution of orchid habitat breadth and the evolutionary pathways that are followed by myco-heterotrophic lineages.

2. MATERIAL AND METHODS

(a) *Field sites*

Plant and soil samples were collected from three ectomycorrhizal forest sites and one non-forested wetland site in Nördliche Frankenalb, northeast Bavaria, Germany (49°37′ N–49°48′ N and 11°25′ E–11°38′ E at 438–502 m elevation). The soils have a pH of 7 and they originate from Jurassic dolomite. On the forest sites, the soils are lithic leptosols with a very shallow organic layer. Forest site 1 is an open *Pinus sylvestris* stand, with a small percentage of *Picea abies* and *Fagus sylvatica* trees mainly in the understorey and a species-rich herbaceous ground vegetation. Forest site 2 is a dense *F. sylvatica* stand with a sparse cover of understorey vegetation. At this site samples were collected only from a forest corner receiving unusually high irradiance. Forest site 3 is an open mixed stand dominated by *P. sylvestris* and *Quercus robur* with a species-rich understorey. Non-forest site 4 is a wetland without ectomycorrhizal plants, characterized by a deep water-saturated humic gleysol and by herbaceous vegetation adapted to full-light conditions. Further details of these areas are given elsewhere (Gebauer & Meyer 2003 and references therein).

(b) *Orchid species*

The orchid species were selected on the basis of their known habitat preferences with a particular emphasis being placed upon their typical light requirements according to a scale that ranges from $L = 1$ (lowest irradiance) to $L = 9$ (highest irradiance) (Ellenberg *et al.* 1991). The orchids included one wholly myco-heterotrophic species, *Neottia nidus-avis* (L.) Rich., which characteristically occurs in the most shaded forests ($L = 2$), four species typical of forest habitats *viz*. *Epipactis distans* Arvet-Touvet ($L = 3$), *E. helleborine* (L.) Crantz ($L = 3$), *Cephalanthera damasonium* (Mill.) Druce ($L = 3$) and *C. rubra* (L.) L.C.M. Rich. ($L = 4$), and four species characteristic of open environments, *E. atrorubens* (Hoffm. ex Bernh.) Besser ($L = 6$), *Platanthera chlorantha* (Cust.) Rchb. p. ($L = 6$), *E. palustris* (L.) Crantz ($L = 8$) and *Dactylorhiza majalis* s.l. ($L = 8$). For the current status of Neottiaeae phylogenetics (including *Epipactis* and *Cephalanthera*) see Bateman *et al.* (2004).

(c) *Mass-spectroscopy analysis*

Following a sampling methodology described elsewhere (Gebauer & Meyer 2003), in July 2003 we collected, from each of the four sites, four replicate leaves of between one and four photosynthetic orchid species and leaves of accompanying non-orchid ground vegetation (for species lists see electronic Appendix A). On the three forest sites, the non-orchid plants encompassed four functional types: ectomycorrhizal plants (ECM), plants forming arbuscular mycorrhizas or non-mycorrhizal plants (AM/NM), and leguminous plants potentially living in symbiosis with nitrogen-fixing bacteria and forming arbuscular mycorrhizas (AM/FIX). At the non-forested wetland site, the non-orchid plants included AM/NM and AM/FIX plants only. In addition, we sampled one wholly myco-heterotrophic orchid species from forest site 1. Soil samples from the uppermost 5 cm were collected adjacent to each orchid plant. In total, we collected 32 samples from eight putatively photosynthetic orchid species, four samples from one fully myco-heterotrophic orchid species, 92 samples from 23 non-orchids and 30 soil samples. Leaf samples were cleaned in deionized water. Leaves and soil samples were dried at 105 °C, ground in a ball mill (Retsch Schwingmühle MM2, Haan, Germany) and stored in a desiccator until analysed. Relative nitrogen and carbon isotope abundances of the leaf and soil samples were measured with an elemental analyser in a dual-element analysis mode (Carlo Erba 1108, Milano, Italy) for Dumas combustion followed by gas chromatographic separation of the gaseous combustion products, which were then fed into a gas–isotope ratio mass spectrometer (delta S Finnigan MAT, Bremen, Germany) via a ConFlo III open-split interface (Finnigan MAT). Relative isotope abundances are denoted as δ-values, which were calculated according to the following 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 standards. Standard gases (nitrogen and carbon dioxide, respectively) were calibrated with respect to the international standards (nitrogen in air and Pee Dee Belemnite (PDB), respectively) by use of the reference substances N1 and N2 for the nitrogen isotopes and Australian National University (ANU) sucrose and NBS 19 for the carbon isotopes.

(d) *Statistics and model calculations*

After testing for normal distributions and homogeneity of variances of the isotope-abundance datasets, a one-way ANOVA was used to evaluate differences in $\delta^{15}\mathrm{N}$ or $\delta^{13}\mathrm{C}$ between non-orchids and non-legumes, legumes and the various orchid species separately for each of the four sites. When the effects of groups on the dependent variables were significant ($p < 0.05$), a least-significant difference test ($\mathrm{LSD}_{0.05}$) was used to compare means. The relative contribution of nitrogen or carbon derived from fungal material to the nitrogen or carbon content of the putatively autotrophic orchids ($\%x_{\mathrm{df}}$ with x as nitrogen or carbon, respectively) was calculated for all of those putatively autotrophic orchid species that were significantly distinguished from non-orchids and non-legumes in $\delta^{15}\mathrm{N}$ or from all non-orchids in $\delta^{13}\mathrm{C}$ or from both, using a linear two-source isotopic mixing model as described elsewhere (Gebauer & Meyer 2003). The model is based on the

individual δ-values of each of the putatively autotrophic orchids (δx_{AO}, with x representing ^{15}N and ^{13}C, respectively), mean δ-values of reference plants (non-orchids and non-legumes for $\delta^{15}N$ and all non-orchids for $\delta^{13}C$, respectively) at each site (δx_R) and the mean relative enrichment of the myco-heterotrophic orchid ($\varepsilon_{MHO\text{-}R} = \delta x_{MHO} - \delta x_R$): $\%x_{df} = (\delta x_{AO} - \delta x_R)/\varepsilon_{MHO-R} \times 100$. Mean $\%x_{df}$ values were tested for significant difference from zero (i.e. no nitrogen or carbon gain from fungi) using a Student's *t*-test.

(e) *Molecular analysis*

We excavated two roots from each of the orchid plants selected for study, and kept them cool and moist for up to 24 h until processed. The roots were rinsed thoroughly with distilled water and 10–20 sections (*ca*. 0.5 mm in thickness, 1–2 mm in diameter) were obtained along the length of each root. Root sections were examined for the presence of orchid mycorrhizas (i.e. fungal pelotons) with a light microscope (magnification of ×400), and two to four colonized sections per plant were selected for molecular analysis. Two uncolonized sections from each of two *E. helleborine* and two *D. majalis* plants were selected as negative controls. Axenic isolation was attempted by plating onto dilute agar media the surface-sterilized colonized root sections of two species with contrasting habitat preferences: *D. majalis* (open environments) and *E. distans* (forests). In addition, ectomycorrhizal roots of *P. sylvestris* were sampled adjacent to *E. distans* roots, and individual root tips of different ectomycorrhizal morphotypes were selected for analysis. Roots of *N. nidus-avis* were not sampled because this non-photosynthetic orchid has been repeatedly demonstrated, using molecular phylogenetic placement, to be specifically associated with sebacinoid ectomycorrhizal fungi (Selosse *et al.* 2002; McKendrick *et al.* 2002). We extracted genomic DNA from each individual root section or axenic fungal isolate following methods described elsewhere (Gardes & Bruns 1993) but using GeneClean (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. Each root section's genomic DNA was then used as a template for six PCR reactions with the following primer combinations (target specificities are listed in parentheses): ITS1F/ITS4 (fungal nuclear ribosomal internal transcribed spacer, nrITS), ITS1F/ITS4B (basidiomycete nrITS), ITS1/ITS4-Tul (tulasnelloid nrITS), ML5/ML6, cML5.5/ML6 and Mlin3/ML6 (fungal mitochondrial large subunit, mtLSU). Multiple primer sets were used because no single primer set is universal or specific for the regions targeted for all fungi likely to be encountered. Samples were also amplified using ITS1F/TW14 (fungal nrITS and 5′ nuclear ribosomal large subunit, nrLSU). Oligonucleotide sequences and references are listed in electronic Appendix B. All positive PCR products were sequenced bidirectionally, and multi-template products were cloned using TOPO TA Kits for Sequencing (Invitrogen, Carlsbad, CA, USA) prior to sequencing. PCR products were purified using QIAquick 96 kits (Qiagen, Valencia, CA, USA). DNA sequencing was performed on an ABI3100 Genetic Analyzer using BigDye v. 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. All DNA sequences obtained were compared with those available in GenBank using BLAST. Sequences were then aligned visually to those most similar and analysed using neighbour joining and parsimony with PAUP* v. 4.0beta10 (Swofford 2004) and/or aligned to unpublished sequence databases and analysed in a similar manner. GenBank accession numbers for all unique DNA sequences are AY634111–AY634179.

(f) *Additional* Epipactis *species survey*

We sampled and analysed mycorrhizal root sections from three *Epipactis* species at 12 additional locations outside Germany using all methods described in § 2e, but we screened with only the primer combinations ITS1F/ITS4 and ITS1/ITS4-Tul. We did not generate mass-spectroscopy data for these plants. The species were: *E. dunensis* (T. & T. A. Stephenson) Godfery (seven plants), an endemic inhabitant of *Pinus*-forested dunes in northwest England and Wales; *E. gigantea* Douglas ex Hooker (18 plants and one underground seedling), a native inhabitant of unshaded riparian areas, where it often grows adjacent to *Alnus* or *Salix*, in California and Oregon; and *E. helleborine* (29 plants, including one non-photosynthetic individual), a native European forest species that is an introduced invasive in urban and native North American ectomycorrhizal forests (Squirrell *et al.* 2001).

3. RESULTS

Based on a one-way ANOVA significant effects of plant species on $\delta^{15}N$ were found at all German sites (F between 17.1 and 41.3, $p < 0.0001$). For the $\delta^{13}C$ values the one-way ANOVA showed significant species effects only for forest site 1 ($F = 54.3$, $p < 0.0001$). Based on subsequent $LSD_{0.05}$ tests four groups of orchids were distinguished by their stable-isotope signatures. The wholly myco-heterotrophic orchid *N. nidus-avis*, which associates with ectomycorrhizal fungi, was the plant species significantly most enriched in ^{15}N ($\varepsilon_{MHO\text{-}R} = 11.6‰$) and least depleted in ^{13}C ($\varepsilon_{MHO\text{-}R} = 8.1‰$) of all plant species from forest site 1 (group 1). The putatively autotrophic orchids without ectomycorrhizal fungi formed two significantly different groups. They either had signatures that were not significantly different from those of the non-orchids in the respective sites (*P. chlorantha*, group 2), or were not different in $\delta^{13}C$, but were significantly different from the non-orchids in $\delta^{15}N$ (*E. palustris*, *D. majalis*, group 3). However, in the latter group the difference in $\delta^{15}N$ between non-orchids and orchids was small (figure 1). Another group was formed by the putatively autotrophic orchids with ectomycorrhizal fungi (*C. damasonium*, *C. rubra*, *E. atrorubens*, *E. helleborine*, *E. distans*, group 4). They were significantly more enriched in ^{15}N and had a consistent tendency to be less depleted in ^{13}C than the non-orchids from their respective sites. The difference in $\delta^{15}N$ between non-orchids and orchids was clearly more pronounced for all species of this group than for the representatives of group 3 (figure 1). Thus, the putatively autotrophic orchids with ectomycorrhizal fungi had an isotopic position intermediate between the wholly myco-heterotrophic orchid and the putatively autotrophic orchids without ectomycorrhizal fungi (figure 1). Within the non-orchids, the plants associated with nitrogen-fixing bacteria and forming arbuscular mycorrhizas (AM/FIX) had consistently and significantly less negative $\delta^{15}N$ values than the ectomycorrhizal, arbuscular mycorrhizal or non-mycorrhizal plants from the respective sites. This indicates nitrogen gain from atmospheric nitrogen fixation by all AM/FIX plants sampled. The soil samples from the forest sites had remarkably high $\delta^{13}C$ values, owing to a very shallow organic layer and mixing of organic carbon with limestone carbon from the bedrock material. Based on a linear two-source isotopic-mixing model, the putatively autotrophic orchids associated with ectomycorrhizal fungi gain

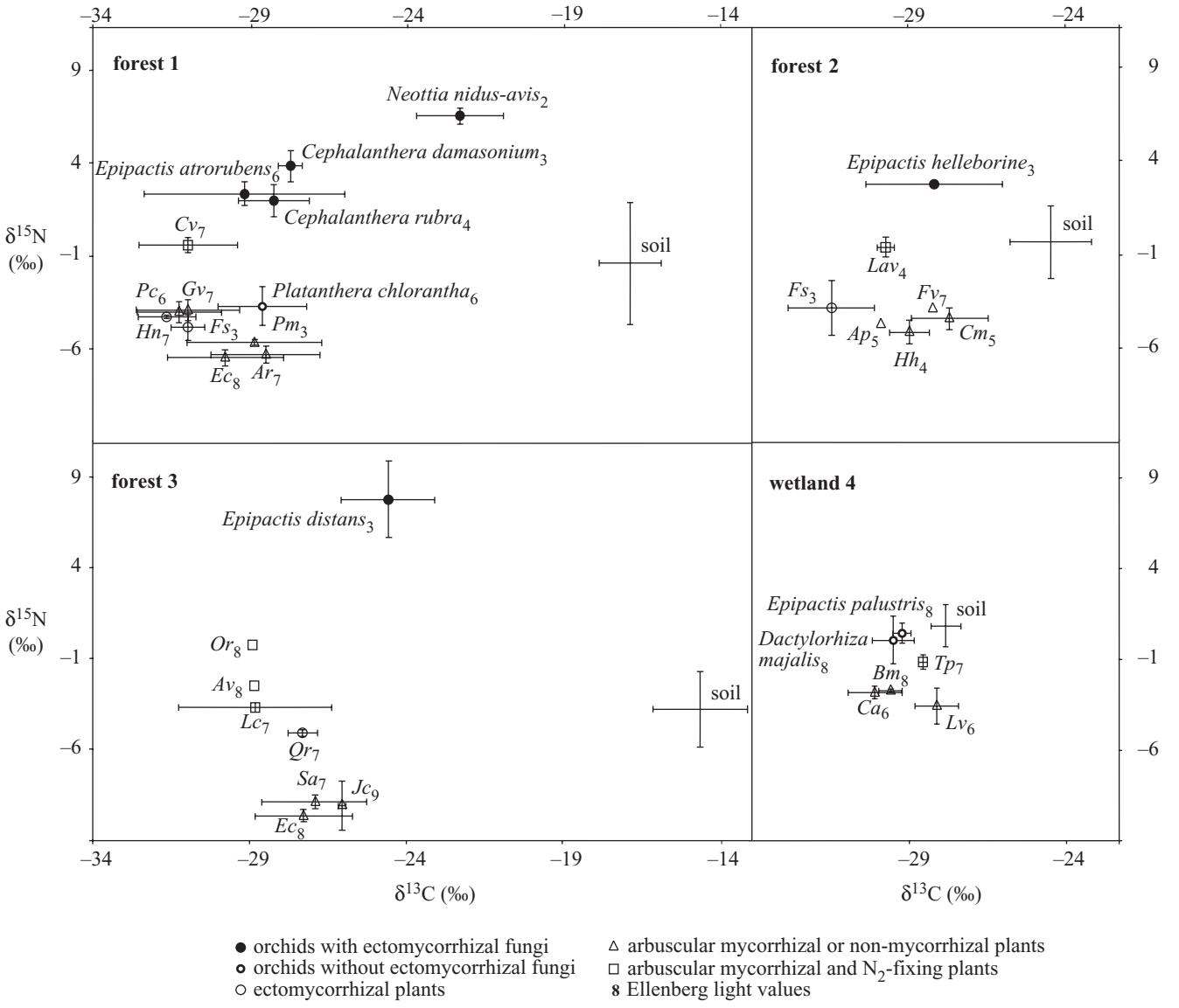

Figure 1. Mean ±1 s.d. values of $\delta^{13}C$ and $\delta^{15}N$ in leaves of eight putatively autotrophic orchid species, one myco-heterotrophic orchid species and 23 non-orchid species and in 30 soil samples collected from three forest sites and one non-forested wetland site. The non-orchids include ectomycorrhizal, arbuscular or non-mycorrhizal, and arbuscular mycorrhizal nitrogen-fixing plants. For the numbers of replicates see electronic Appendix A. Error bars are missing if smaller than the symbols or if $n < 3$. Plant species abbreviations: *Ap*, *Aegopodium podagraria*; *Ar*, *Anthericum ramosum*; *Av*, *Anthyllis vulneraria*; *Bm*, *Briza media*; *Ca*, *Colchicum autumnale*; *Cm*, *Convallaria majalis*; *Cv*, *Coronilla varia*; *Ec*, *Euphorbia cyparissias*; *Fs*, *Fagus sylvatica*; *Fv*, *Fragaria vesca*; *Gv*, *Galium verum*; *Hh*, *Hedera helix*; *Hn*, *Helianthemum nummularium*; *Jc*, *Juniperus communis*; *Lav*, *Lathyrus vernus*; *Lc*, *Lotus corniculatus*; *Lv*, *Lysimachia vulgaris*; *Or*, *Ononis repens*; *Pc*, *Polygala chamaebuxus*; *Pm*, *Polygonatum multiflorum*; *Qr*, *Quercus robur*; *Sa*, *Sesleria albicans*; *Tp*, *Trifolium pratense*.

between 77% and 61% of their nitrogen from the fungal host, whereas the autotrophic orchids not associated with ectomycorrhizal fungi gain no significant amounts (*P. chlorantha*) or only between 30% and 26% of their nitrogen from the fungal host (table 1). Based on the mixing-model calculations, the carbon gained from their fungal hosts by the orchids associated with ectomycorrhizal fungi ranges between 36% and 14% and it is significantly different from the no fungal carbon gain for four out of the five orchid species in this group (table 1). For the autotrophic orchids that are not associated with ectomycorrhizal fungi the model calculation indicates slightly negative carbon gains from the fungal hosts (table 1), but these are not significantly different from zero (i.e. no carbon gain).

We examined roots from 28 German orchid plants, 25 of which had orchid mycorrhizas. These were either dense coils of distinguishable, but often translucent, fungal hyphae, dense and coloured aggregations of collapsed hyphae, or both. We obtained PCR products with at least one of the six fungal primer combinations from every one of the plants where mycorrhizas were found, and from all mycorrhizal roots analysed. Approximately 50% of the PCR products generated could be sequenced directly and the rest were cloned and sequenced. The fungi detected in orchid mycorrhizal root sections belong to diverse fungal lineages: orchid mycorrhizal rhizoctonia-forming basidiomycetes (*Ceratobasidium*, *Sebacina*, *Tulasnella*), orchid mycorrhizal ascomycetes (*Leptodontidium*), obligate ectomycorrhizal basidiomycetes (*Cortinarius*, *Hymenogaster*,

Table 1. Percentages of nitrogen (mean $\%N_{df} \pm 1$ s.e., $n = 4$) and carbon (mean $\%C_{df} \pm 1$ s.e., $n = 4$) derived from fungi in the leaves of putatively autotrophic orchid species that were statistically different in their $\delta^{15}N$ values from the non-orchids of the respective sites.
(The data were calculated based on a linear two-source isotopic-mixing model. Asterisks indicate significance levels for deviations from zero, based on a Student's *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: AO+, putatively autotrophic orchid associated with ectomycorrhizal fungi; AO−, putatively autotrophic orchid not associated with ectomycorrhizal fungi.)

orchid species	category	site	$\%N_{df}$	$\%C_{df}$
Cephalanthera damasonium	AO+	forest 1	$77 \pm 3^{***}$	$33 \pm 10^{***}$
Cephalanthera rubra	AO+	forest 1	$61 \pm 10^{***}$	$26 \pm 11^{***}$
Epipactis atrorubens	AO+	forest 1	$64 \pm 27^{***}$	15 ± 8
Epipactis helleborine	AO+	forest 2	$61 \pm 19^{***}$	$14 \pm 1^{***}$
Epipactis distans	AO+	forest 3	$67 \pm 38^{**}$	$36 \pm 19^{**}$
Epipactis palustris	AO−	wetland	$30 \pm 2^{***}$	-2 ± 7
Dactylorhiza majalis	AO−	wetland	$26 \pm 6^{***}$	-8 ± 16

Table 2. Orchid mycorrhizal fungi detected in orchid roots at four German sites.
(Obligate ectomycorrhizal lineages are shown in bold. Lineages that contain some ectomycorrhizal strains are indicated by an asterisk. *L* is Ellenberg's light indicator value, and *n* is the number of plants sampled. No ectomycorrhizal plants were present at site 4. See electronic Appendix C for detailed information.)

orchid species	*L*	site	*n*	mycorrhizal fungi
Cephalanthera damasonium	3	forest 1	4	***Cortinarius***, ***Hymenogaster***, ***Inocybe***, ***Thelephora***, ***Tomentella***
Cephalanthera rubra	4	forest 1	4	*Leptodontidium*, *Phialophora**, ***Tomentella***
Epipactis atrorubens	6	forest 1	4	***Inocybe***, *Leptodontidium*, *Phialophora**, sebacinoid*, ***Tuber***, *Tulasnella**, ***Wilcoxina***
Platanthera chlorantha	6	forest 1	2	*Ceratobasidium*, *Leptodontidium*, *Phialophora**, *Tulasnella**
Neottia nidus-avis	2	forest 1	4	***Sebacina***
Epipactis helleborine	3	forest 2	4	*Ceratobasidium*, sebacinoid*, ***Tuber***
Epipactis distans	3	forest 3	2	***Wilcoxina***
Dactylorhiza majalis	8	wetland 4	4	*Ceratobasidium*, *Tulasnella**
Epipactis palustris	8	wetland 4	4	*Ceratobasidium*, *Leptodontidium*, sebacinoid*, tulasnelloid*

Inocybe, *Thelephora*, *Tomentella*) and obligate ectomycorrhizal ascomycetes (*Tuber*, *Wilcoxina*) (table 2). *Sebacina*, *Phialophora* and *Tulasnella* contain some ectomycorrhizal species (Selosse *et al.* 2002; Vrålstad *et al.* 2002; Bidartondo *et al.* 2003) and *Ceratobasidium* includes some *P. sylvestris*-endophytic strains (Sen *et al.* 1999). Detailed results from the molecular analysis of mycorrhizal fungi can be found in electronic Appendix C.

We also obtained PCR products from four out of the eight non-mycorrhizal orchid root sections analysed. In these sections we detected fungi from ascomycete and zygomycete lineages not known to form mycorrhizas (i.e. *Verticillium*, *Endothia*, *Cylindrocarpon*, *Trichosporon*, *Mortierella*) and which were probably present as minor hyphal endophytes, spores or rhizosphere-associated hyphae. These and other non-mycorrhizal endophytes and soil fungi were also commonly detected, in addition to mycorrhizal fungi, in multitemplate PCR products generated from orchid mycorrhizal roots (data not shown).

Culturability biases were clearly observed: rhizoctonia-forming fungi could be readily isolated from orchid mycorrhizas but ectomycorrhizal fungi could not. From the *D. majalis* mycorrhizal root sections we isolated a *Ceratobasidium* strain with an identical nrITS DNA sequence to that obtained directly from mycorrhizal roots of the same plants. However, of the sections taken from *E. distans*, few yielded isolates, these being invariably fast-growing soil fungi rather than the *Wilcoxina* sp. detected in all *E. distans* root sections using molecular methods. An identical *Wilcoxina* sp. ITS sequence was detected in *P. sylvestris* ectomycorrhizal roots collected adjacent to roots of *E. distans* (see electronic Appendix C).

Ascomycete fungi dominated the roots of the two additional forest-dwelling *Epipactis* species, *E. dunensis* and *E. helleborine*, sampled in Britain and North America, respectively (35 out of 36 plants). The ectomycorrhizal ascomycete *Tuber* was the most common of these fungi (20 out of 36 plants). Basidiomycete fungi, including typical rhizoctonia-forming orchid mycorrhizal fungi, dominated the roots of the stream-dwelling *E. gigantea* (12 out of 19 plants). See electronic Appendix D for detailed information.

4. DISCUSSION

Whereas recent studies have demonstrated that wholly non-photosynthetic orchid species of three genera, *Cephalanthera* (Taylor & Bruns 1997), *Neottia* (Selosse *et al.* 2002; McKendrick *et al.* 2002) and *Corallorhiza* (Zelmer &

Currah 1995; Taylor & Bruns 1997; McKendrick *et al.* 2000), are colonized by ectomycorrhizal fungi, this study is the first to demonstrate clearly that putatively autotrophic orchids associate with diverse ectomycorrhizal fungi. Indeed, this appears to be an unrecognized but widespread phenomenon: we have detected ectomycorrhizal fungi forming orchid mycorrhizas with *Epipactis* species in Germany, Wales, Québec, Massachusetts, New York and California. Our findings provide a missing link in the evolution of myco-heterotrophy. Previously, it had been thought that the non-photosynthetic orchid *Cephalanthera austinae* achieved full myco-heterotrophy in conjunction with its mycorrhizal switch from rhizoctonia-forming saprotrophic fungi to a thelephoroid ectomycorrhizal fungus (Taylor & Bruns 1997). This requires three major changes to be accomplished in the transition from photosynthetic to non-photosynthetic plants: (i) switch from orchid fungi to ectomycorrhizal fungi; (ii) exploit these fungi for carbon and nutrients; and (iii) specialize on particular ectomycorrhizal fungi. Our data demonstrate that the first two steps have already been made by several photosynthetic orchids that inhabit shaded settings including putatively photosynthetic *Cephalanthera* species; these *Cephalanthera* are also associated with thelephoroid and cortinarioid ectomycorrhizal fungi. Thus, contrary to the accepted theory, the switch to ectomycorrhizal fungi preceded the viable loss of photosynthesis in a manner analogous to that in the distantly related dicot family Ericaceae (Bidartondo & Bruns 2001). This switch may be the mechanism by which orchids such as *C. damasonium* have been able to thrive, often as the only green plants present, in the deepest woodland shade.

The differences in stable-isotope signatures between the three functional groups of orchids (i.e. photosynthetic orchids of open habitats, putatively photosynthetic orchids of shaded habitats and wholly non-photosynthetic orchids) predicted in a recent report by Gebauer & Meyer (2003). Subsequently, Trudell *et al.* (2003) showed that the stable-isotope signatures of several wholly myco-heterotrophic Ericaceae and one wholly myco-heterotrophic orchid are most similar to those of ectomycorrhizal fungi, least like those of photosynthetic plants and strongly positively correlated with those of their specific fungal symbiont species. The isotopic differences reported here show a clear separation in δ^{15}N between non-orchids, a wholly myco-heterotrophic orchid and putatively autotrophic orchid species associated with ectomycorrhizal fungi in low-irradiance habitats (figure 1). The δ^{15}N signatures of the orchid species from high-irradiance habitats (i.e. high Ellenberg light values), which do not associate with obligately ectomycorrhizal fungi, may cluster with those of non-orchid plants, or they are only slightly distinguished in δ^{15}N and not at all in δ^{13}C. It has been suggested that the relative ^{15}N enrichment detected in wholly myco-heterotrophic and putatively autotrophic orchids arises as a result of receiving nitrogen from sources mobilized and assimilated by fungi that are themselves distinctive (Gebauer & Meyer 2003). It is evident from the current analysis not only that putatively autotrophic orchids do associate with unique fungi but also that these symbionts are in most cases ectomycorrhizal (table 2). In view of the evidence that ectomycorrhizal fungi provide their host plants with access to organic sources of nitrogen (Read & Pérez-Moreno 2003), it is probable that the differences in δ^{15}N between putatively and fully autotrophic orchids are based upon the exploitation of these distinctive nitrogen sources.

As would be expected if the orchids associated with ectomycorrhizal fungi received some or all of their nitrogen in organic form, their δ^{13}C signatures are also enriched. However, these values are not as distinct as those for nitrogen. The explanation for the lower carbon than nitrogen gain of the putatively autotrophic orchids associated with ectomycorrhizal fungi may lie in the greater complexity of the pathways for carbon gain and loss. Partially photosynthetic orchids have the potential to carry the signatures of carbon derived from autotrophic and heterotrophic sources; thus, the carbon signature of the photosynthetic above-ground phase will be influenced by photosynthetic carbon assimilation depending upon irradiance and the processes associated with respiratory loss. As a result, the gross carbon gain from the fungal source and the net carbon gain measured by our analyses would be substantially different. Our results indicate that under high-irradiance conditions the signature derived from heterotrophically gained carbon becomes undetectable. However, enriched δ^{13}C signatures are evident in the orchids of low-irradiance habitats (i.e. *E. distans*, *E. helleborine*, *carbon. damasonium* and *carbon. rubra*), indicating that carbon from heterotrophic sources does indeed contribute to their carbon budget. The δ^{13}C signatures previously reported for some of these orchid species were more enriched, probably because the plants were collected from lower-irradiance habitats (Gebauer & Meyer 2003). The carbon budgets of the putatively photosynthetic shade-tolerant orchids are more heavily subsidized by heterotrophic carbon gain than are those of the orchids characteristic of open habitats. Precise carbon budgets will require direct measurements of photosynthesis and heterotrophic carbon transfer under controlled-irradiance conditions.

One of the key features to emerge from this study is that the requirement for ectomycorrhizal fungi will impose strict spatial constraints upon the establishment and distribution of partially photosynthetic orchids. In contrast to most, if not all, of the rhizoctonia-forming fungi, which are cosmopolitan soil saprophytes, the ectomycorrhizal fungi are entirely dependent upon supplies of photosynthate derived from the roots of their host trees or shrubs. Although there is evidence that some ectomycorrhizal fungi can survive as spores in soil at some distance from their host plants, the colonization by fungal hyphae of the underground stages of orchids would be dependent upon carbon fluxes from colonized ectomycorrhizal roots. Consequently, such orchids will be spatially restricted to locations adjacent to plant roots that are already supporting ectomycorrhizal fungi.

In addition to spatial constraints, a further limitation upon the germination, establishment and/or growth of an orchid species may be that imposed by specificity for a subset of the ectomycorrhizal fungal lineages available at a site. Whether the extreme specificity to narrow lineages of mycorrhizal fungi that characterizes wholly myco-heterotrophic plants and their seedlings (Cullings *et al.* 1996; Taylor & Bruns 1997; Bidartondo & Bruns 2001, 2002; Bidartondo *et al.* 2002, 2003; McKendrick *et al.* 2002; Selosse *et al.* 2002) is to be found in partially photosynthetic orchids remains to be determined, but it does not appear to be a rule (e.g. *Cephalanthera damasonium*, *E.*

atrorubens). However, unlike all photosynthetic ectomycorrhizal plants, several *Epipactis* species display surprisingly consistent associations with one clade of fungi: ascomycetes formed mycorrhizas with nearly every plant we sampled of *E. distans*, *E. dunensis* and *E. helleborine*. In fact, for *E. helleborine*, the orchid species that we sampled most broadly geographically, nearly half of the plants were associated with the obligately ectomycorrhizal ascomycete *Tuber*. It is unknown whether the phylogenetic breadth of orchid mycorrhizal fungi changes, perhaps by expanding, during the development of strictly myco-heterotrophic seedlings into partially myco-heterotrophic mature plants. However, if found, specificity for a narrow phylogenetic range of mycorrhizal associates at any stage of the life cycle will impose strong constraints on the distribution of any plant species (McKendrick *et al.* 2000).

In summary, we have shown that there is a widespread and hitherto unrecognized nutritional mode in the Orchidaceae, most appropriately termed partial myco-heterotrophy. This has been achieved by species that grow in the dark understorey of forests through an association with several previously unrecognized lineages of orchid mycorrhizal fungi that simultaneously form ectomycorrhizas with forest trees.

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