Microbial Ecology

Chlorophyllous and Achlorophyllous Specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) Are Associated with Ectomycorrhizal Septomycetes, including Truffles

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Abstract

Mycoheterotrophic species (i.e., achlorophyllous plants obtaining carbon from their mycorrhizal fungi) arose many times in evolution of the Neottieae, an orchid tribe growing in forests. Moreover, chlorophyllous Neottieae species show naturally occurring achlorophyllous individuals. We investigated the fungal associates of such a member of the Neottieae, *Epipactis microphylla*, to understand whether their mycorrhizal fungi predispose the Neottieae to mycoheterotrophy. Root symbionts were identified by sequencing the fungal ITS of 18 individuals from three orchid populations, including achlorophyllous and young, subterranean individuals. No rhizoctonias (the usual orchid symbionts) were recovered, but 78% of investigated root pieces were colonized by *Tuber* spp. Other Pezizales and some Basidiomycetes were also found. Using electron microscopy, we demonstrated for the first time that ascomycetes, especially truffles, form typical orchid mycorrhizae. All identified fungi (but one) belonged to taxa forming ectomycorrhizae on tree roots, and four of them were even shown to colonize surrounding trees. This is reminiscent of mycoheterotrophic orchid species that also associate with ectomycorrhizal fungi, although with higher specificity. Subterranean and achlorophyllous *E. microphylla* individuals thus likely rely on tree photosynthates, and a partial mycoheterotrophy in individuals plants can be predicted. We hypothesize that replacement of rhizoctonias by ectomycorrhizal symbionts in Neottieae entails a predisposition to achlorophylly.

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Introduction

Mycorrhizal fungi form mutualistic associations on plants roots, where they usually exchange plant photosynthates for soil-collected mineral nutrients [45]. However, a considerable variability exists among the various mycorrhizal interactions, and some plants may even recover carbon from their fungal partners. Because of the low specificity of mycorrhizal fungi, coexisting plants can share identical fungal symbionts. Such links have been demonstrated to allow carbon transfer between plants, both *in vitro* [16] and in field conditions [44]. The relevance of this carbon flux at the physiological and ecological levels is still debated [15, 35]. However, some species, namely the mycoheterotrophic plants (MHPs), exclusively rely on this flux to achieve their growth and reproduction.

MHPs are achlorophyllous plants with reduced root systems that obtain both mineral nutrients and carbon from their fungal partners [23]. Molecular methods have showed that MHP species have a strong mycorrhizal specificity to narrow fungal clades, which in most cases form mycorrhizae with surrounding green plants [49]. Carbon labeling has demonstrated that the latter plants constitute the ultimate source of carbon for both the MHP and the fungus [7, 8, 24]. Although the effect on the fitness of the carbon-furnishing plant and the fungus is still unclear, MHPs are therefore considered as "mycorrhizal cheaters" [6]. Species phylogenetically close to MHPs are good candidates for a partial mycoheterotrophy, i.e., for relying not only on their photosynthesis, but also partly on a carbon flux from the fungus [18]. In addition, the study of such species, likely representing a state ancestral to mycoheterotrophy, can clarify how MHPs arose in plant evolution. As a preliminary step,

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identification of their fungal associates is required, to test whether they share mycorrhizal fungi with surrounding plant species.

To address these questions, the Orchidaceae family provide suitable models for two reasons. First, all orchids are transiently mycoheterotrophic (MH) at the germination stage: seeds have few reserves and rely for several years on a basidiomycetous fungus to achieve their initial development as a protocorm [33]. Second, whereas in most species, the protocorm becomes autotrophic during further development, several species remain MH and achlorophyllous at the adult stage [23]. As a matter of fact, there are more MHPs among orchids than in any other family [19]. MH orchids strongly differ in mycorrhizal condition from other orchids [34, 43, 47, 48, 50], since they are not associated with basidiomycetes of the genus *Rhizoctonia*, which encompasses the usual orchid symbionts [33, 34]. By contrast, they associate with other basidiomycetes that form ectomycorrhizae (ECM) with surrounding forest trees [47, 48, 50] or, in few cases, behave as wood-decaying fungi or tree pathogens [49]. In addition, as for other MHPs, each MH orchid species is specifically associated with a very narrow fungal clade.

The forest orchid tribe Neottieae contains green, photosynthetic species in the genera *Epipactis*, *Limodorum*, and *Cephalanthera*. On the other hand, it encompasses several MHPs specifically associated with ECM fungi, namely the American *Cephalanthera austinae*, mycorrhizal with Thelephoraceae [47], the European *Neottia nidus-avis*, mycorrhizal with Sebacinaceae [25, 43], and the tropical *Aphyllorchis* spp. Although the phylogeny of this tribe is poorly known [11, 14], currently available data suggest that chlorophyll loss occurred several times (C. Micheneau, L. Civeyrel and M. Fay, pers. com.). This raises the question whether a preadaptation to mycoheterotrophy exists in Neottieae. Indeed, chlorophyll-free individuals occur in natural populations of chlorophyllous species [22], such as among *Epipactis* [19, 38, 40]. These "albinos", either mutants or environmentally induced phenocopies, obviously rely on the carbon furnished by their fungal associates for their growth and, in some case, seed production [38, 40].

Epipactis microphylla (Ehrh.) Swartz was chosen as model plant to study the identity of fungal symbionts of a green Neottieae species and its albinos. It is a fairly rare orchid from South Europe, with small leaves, which grows in deciduous forests over calcareous soils [22]. In spite of its rarity, it is an interesting model as a population with albino plants has recently been found [40]. Fungal symbionts have not been identified yet in *Epipactis* spp. [33], with the exception of a Fennoscandian *Epipactis helleborine* population, which was associated with an unknown basidiomycete [37, 38]. The aim of this investigation was first to assess the diversity of mycorrhizal fungi associated with *E. microphylla*, combining molecular typing and microscopic analysis. Secondly, we aimed at comparing *E. microphylla* specificity with phylogenetically related MHPs already studied. The last aim was to compare the mycorrhizal diversity in green vs albino plants, as well as in individuals at various development stages (late protocorms and adults).

Methods

Orchid Root Sampling. *Epipactis microphylla* (Ehrh.) Swartz was chosen as a model after preliminary tests (not shown) including three additional *Epipactis* species (*E. muelleri*, *E. helleborine*, and *E. atropurpurea*), as its colonization pattern allows direct typing of the fungus on 2-mm-long root pieces, whereas in other species, such pieces often contain several fungal species (not shown). Three French populations of *Epipactis microphylla* were sampled in July 2002 and June 2003, at the time of their fruiting, when fungal colonization culminates ([41], M.-A. Selosse, personal observations). A limited number of plants were harvested because of the protected status of this species.

Population A is situated at Le Gaizil near Gap (Southern Alps, 3°45′E, 44°35′N [40]) at an elevation of 930 m. It grows under *Quercus pubescens*, *Corylus avellana*, and some *Fagus sylvatica* on a steeply sloping calcareous soil over recent alluvial deposits. In this population, four distant adult plants, namely two green (pA1 and pA2) and two albinos (pA3 and pA4) were harvested in 2002. A late protocorm (i.e., a well-developed protocorm, already visible to the naked eye, but with roots <2 cm) was found in the vicinity of pA4 (Table 1).

Population B is situated in the South of Paris, at Lardy near Arpajon (48°35′N, 1°55′E; elevation 70 m), in ecological conditions similar to population A (a steeply sloping calcareous soil, under a mixed forest including *Quercus pubescens*, *Corylus avellana*, *Fagus sylvatica*, and *Pinus sylvestris*). In this population, two distant adults with a green leafy shoot were harvested in 2002 (Table 1). In addition, two out of five random diggings allowed us to recover a fully subterranean rhizome, devoid of epigeous shoots but larger than the two previous ones, and four young plants, i.e., a late protocorm (pB3) and three young rhizomes already with roots (pB4 to pB7, Table 1).

Population C grows in Mont Maurice near Dieulefit (Southern Alps, 44°35′N, 3°45′E ; elevation 910 m) on a thick brown soil, under a dense, monospecific cover of *Fagus sylvatica*. The reduced size of the population only allowed the sampling of two adult green plants in 2002 (pC1 and pC2, Table 1) and one green plant in 2003 (pC3) which was growing close to a young rhizome and a late protocorm (pC4 and pC5, Table 1).

Table 1. ***E. microphylla*** **sampled in populations A, B and C, and ITS typing of their fungal symbionts**

Population	*Plant*	*Individual description*	*Number of root pieces typed*[a]	*ITSs amplified on the root pieces (and frequency*[b]*)*
A	pA1	With a green epigeous shoot	22/15	Tuberaceae #A1 (15)
	pA2	With a green epigeous shoot	22/18	Tuberaceae #A1 (17) and Cortinariaceae #A1 (1)
	pA3	With an albino epigeous shoot	22/19	Tuberaceae #A1 (17), Sebacinaceae #A(1) and Cortinariaceae #A1 (1)
	pA4	With an albino epigeous shoot	22/20	Tuberaceae #A1 (20) and Trichocomaceae #A1 (1)
	pA5	Late protocorm, hypogeous near pA4	1/1	Tuberaceae #A1 (1)
B	pB1	With a green epigeous shoot	22/19	Tuberaceae #BC2 (19)
	pB2	With a green epigeous shoot	22/20	Tuberaceae #BC2 (18) and Sebacinaceae #B2 (2)
	pB3	Large hypogeous rhizome, no aerial shoot	22/20	Tuberaceae #BC2 (20)
	pB4	Late protocorm, hypogeous near pB3	1/1	Tuberaceaee #BC2 (1)
	pB5	Young rhizome, no aerial shoot near pB3	3/3	Tuberaceae #BC2 (3)
	pB6	Young rhizome, no aerial shoot near pB3	3/2	Tuberaceae #BC2(2)
	pB7	Young rhizome, no aerial shoot near pB8	3/2	Tuberaceae #BC2 (2)
	pB8	Young rhizome, no aerial shoot near pB7	3/1	Tuberaceae #BC2 (1)
C	pC1	With a green epigeous shoot	26/25	Tuberaceae #BC2 (7), Thelephoraceae #C1 (4), Sarcosomataceae #C1 (10), Russulaceae #C1 (4) and Pezizale #C1 (2)
	pC2	With a green epigeous shoot	14/13	Sarcosomataceae #C1 (2), Russulaceae #C1 (4), Pezizale #C1 (2), #C2 (3) and #C3 (1), Hymenogastraceae #C1 (2) and Cortinariaceae #C1 (1)
	pC3	With a green epigeous shoot	22/20	Tuberaceae #C2 (16), Russulaceae #C1 (1), Sarcosomataceae #C1 (2) and Hymenogastraceae #C2 (2).
	pC4	Young rhizome, no aerial shoot near pC3	3/3	Tuberaceae #BC2 (3) and Hymenogastraceae #C2 (1)
	pC5	Late protocorm, hypogeous near pC3	1/1	Tuberaceae #BC2 (1)

[a]Number of root pieces typed/number of pieces allowing amplification of at least one PCR product using the various primer sets.
[b]In parentheses, number of root pieces from which this sequence was amplified (total may exceed the total number of typed pieces as some PCR produced two fragments, separated on gel or by cloning).

Molecular Typing of the Fungal Partners. All samples were carefully washed to eliminate all soil particles. For late protocorms, a single 2 × 2 × 2 mm piece was cut and submitted to DNA extraction. For young rhizomes that already bore roots, three 2-mm root pieces were selected from three different roots. For adult rhizomes (pA1-4, pB1-3, pC1-3, Table 1), the following subsampling protocol was used: on all healthy roots, a 2-mm-long piece was cut every 5 mm, starting at the root/rhizome connection. Root pieces were labeled so that samples originating from the same root could be recognized. All pieces of a given plant were then pooled and 22 root pieces were randomly picked up (i.e., 60–100% of the total number), except for plants pC1 (26 pieces) and pC2 (12 pieces because of limited root system size, Table 1), and kept at −80°C until DNA extraction.

DNA was extracted as described in Selosse et al. [43], and the fungal intergenic transcribed spacers of the ribosomal DNA (ITS, encompassing the ITS1, 5.8S rDNA, and ITS2 sequences) were amplified using three sets of primers: ITS1F + ITS4 (a set of primers universal for fungal ITS [17]), ITS1F + ITS4B (the latter primer being specific for some Basidiomycetes [17]), and ITS1F + TW13 (5′-GGTCCGTGTTTCAAGACG-3′; a primer within the 28S rDNA [28]). PCR products were purified and sequenced using the same protocol as in Selosse et al. [43]. All fragments of a given sample were first submitted to a sequencing reaction using the primer ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) internal to the 5.8S rDNA and compared to determine the fragments of identical sequence. For each divergent sequence, a ITS1F-ITS4 PCR product was randomly chosen for sequencing of the whole ITS, using the two amplification primers. Whenever a *Tuber* or a sebacinoid sequence was found, it was sequenced over the whole length of the ITS1F/TW13 following the protocol of Selosse et al. [42], to give access to the phylogenetic signal contained in the 5′ part of the 28S rDNA.

Table 2. Molecular identification of the fungal ITS recovered from *E. microphylla* roots

Population	*Proposed affiliation*	*Two closest sequences found in GenBank by BLAST analysis of the ITS sequence*	*Percent identity*[a]	*GB accession number*
A	Cortinariaceae #B2	*Cortinarius* sp. (ectomycorrhizal isolate) (AJ534713)	95.1% (632 bp)	AY286193
		Cortinarius sp. (ectomycorrhizal isolate) (AJ534712)	95.8% (592 bp)	
A	Sebacinaceae #A1	*Sebacina* endomycorrhiza of *Neottia nidus-avis* (AF440650)	99.9% (1270 bp)[b]	AY286192[b]
		Sebacina endomycorrhiza of *Neottia nidus-avis* (AF440660)	99.9% (1270 bp)[b]	
A	Tuberaceae #A1	*Tuber excavatum* (AF073509)	99.5% (367 bp)	AY286191
		Tuber excavatum (AJ557545)	86.9% (588 bp)	
A	Trichocomaceae #B1	*Talaromyces rotundus* (AF285115)	92.4% (159 bp[c])	AY351631
		Eupenicillium inusitatum (AF033431)	94.5% (145 bp[c])	
B + C	Tuberaceae #BC2	*Tuber uncinatum* (AJ492218)	99.6% (700 bp)	AY286194
		Tuber aestivum (AF106886)	99.0% (705 bp)	
C	Cortinariaceae #C2	*Cortinarius* sp. (ectomycorrhizal isolate) (AJ534713)	94.9% (605 bp)	AY351630
		Ectomycorrhizal root tip (*Cortinarius*) (AF481375)	95.1% (571 bp)	
C	Hymenogastraceae #C1	*Hymenogaster bulliardii* (AF325641)	96.1% (617 bp)	AY351628
		Hymenogaster olivaceus (AF325642)	95.6% (617 bp)	
C	Hymenogastraceae #C2	*Hymenogaster bulliardii* (AF325641)	98.6% (655 bp)	AY351629
		Hymenogaster olivaceus (AF325642)	93.3% (653 bp)	
C	Pezizale #C1	Pezizales sp. (AF266709)	91.6% (644 bp)	AY351625
	Pyronemataceae?	Uncultured ectomycorrhiza (Pezizales) (AY192163)	88.4% (597 bp)	
C	Pezizale #C2	Uncultured ectomycorrhiza (Sarcoscyphaceae) (AF440667)	97.8% (585 bp)	AY351626
	Sarcoscyphaceae?	Pezizales sp. (ectomycorrhizal isolate) (AJ534699)	96.4% (616 bp)	
C	Pezizale #C3	Pezizales sp. (ectomycorrhizal isolate) (AJ534697)	97.1% (173 bp[c])	AY351627
	Pyronemataceae?	*Wilcoxina mikolae* (AY219841)	98.2% (158 bp[c])	
C	Sarcosomataceae #C1	*Strumella coryneoidea* (AF485076)	98.2% (167 bp[c])	AY351623
		Sarcosomataceous endophyte (AF485074)	98.2% (167 bp[c])	
B	Sebacinaceae #B2	Sequence identical to AF440659 (unknown Sebacinaceae)	100% (1276 bp)	(not deposited)
C	Thelephoraceae #C1	*Tomentella* sp. (ectomycorrhizal isolate) (AJ534912)	98.2% (811 bp)	AY351622
		Ectomycorrhizal root tip (AF476987)	92.2% (764 bp)	
C	Russulaceae #C1	*Russula foetens* (AF418613)	99.3% (614 bp)	AY351624
		Russula foetens (AY061677)	96.3% (707 bp)	

[a]Percent identity over the part of the sequences shared by the two GenBank accessions (the length of this alignment is given in parentheses).
[b]The whole ITS + 28S rDNA sequence was used for alignment of sebacinoid sequences, as the 28S rDNA often allows better alignment than the ITS in this taxon [42].
[c]Alignment restricted to the 5.8S, because of low identity in the ITS1 and ITS2 regions.

Whenever two fragments were visualized on agarose gel after PCR, and whenever sequencing of apparently unique fragments failed, cloning of the ITS1F/ITS4 PCR products was performed using the Qiagen PCR Cloning^{plus} Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions: namely, PCR products of one piece in pA4, pC2, pC3 and pC4, and two pieces in pC1 were cloned. Positive clones were screened through RFLP typing as described in Selosse et al. [43], and five clones per RFLP type were then sequenced. Since most sequences found by cloning were >99.5% identical to sequences obtained by direct sequencing of PCR products from other roots, we used the latter to edit positions showing divergent bases between clones as a result of PCR errors.

To ensure the absence of tulasnelloid fungi, which are frequent orchid symbionts and have highly derived rDNA sequences, control PCRs were carried out using ITS1 and the specific ITS4Tul primer [7] in PCR conditions identical to the previous ones (except for annealing temperature: 53°C). Positive controls (tulasnelloid DNA samples kindly provided by M. Weiß, Univ. Tübingen, Germany) were used.

Sequences were assembled and compared using Sequencher 3.11 for MacOS from Genes Codes (Ann Arbor, MI, USA). The consensus sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI); accession numbers are given in Tables 1 and 2. Searches for similar sequences (Table 2) were conducted using BLAST [1] at the NCBI [27], using default settings. Entries with highest Blast value were realigned with the query sequence to calculate a homology percentage (Table 2) using Sequencher 3.11.

Ectomycorrhizae Sampling and Typing. Ectomycorrhizae were looked for in the vicinity (<5 cm) of the root systems of eight orchid root systems (Table 3), in order to assess whether symbionts were shared with surrounding trees. All available mycorrhizae were submitted to DNA extraction, PCR amplification, and sequencing using the same protocol as above. A first sequence was obtained using the primer ITS2 and compared to the fungal ITS from the related orchid. In case of similarity, ITS were fully sequenced to ensure full identity. Host trees were identified by PCR amplification and

Table 3. Ectomycorrhizae collected from the vicinity (<10 cm) of *E. microphylla* roots

	Number of neighboring ECM		
Orchid name	*Total number sampled*	*Number identical to an orchid symbiont*	*Identification of the host tree by its ITS sequence:*
pA1	20	5 (Tuberaceae #A1)	100% identical[a] to AF465187 (*Corylus avellana*)
pA2	1	0	—
pA3	15	0	—
pA4	0	—	—
pB1	8	0	—
pB2	7	6 (Sebacinaceae #B2)	98.6% identical to AY226846 (*Quercus pubescens*) over 586 bp[b]; 98.1% identical to AY226839 (*Quercus pubescens*) over 586 bp
pC1	8	1 (Sarcosomataceae C1)	100% identical[a] to AF465188 (*Fagus sylvatica*)
pC3	15	10 (Tuberaceae #BC2)	100% identical[a] to AF465188 (*Fagus sylvatica*)

[a]Identity was checked over the whole sequence obtained from roots, which was identical in size to that from GenBank.
[b]The amplified root sequence was deposited in GenBank under number AY351632.

sequencing of the plant ITS as described in Selosse et al. [42].

Microscopic Investigations and Immunolabeling. A first microscopic investigation was carried out on some adult plants to calculate the percentage of mycorrhization. For each plant, 15 pieces were randomly chosen from among the root fragments discarded for molecular analysis (5 mm in length) by avoiding (i) the 10-mm apical zone and (ii) young roots (<2 cm), that are usually less or not colonized in *Epipactis* spp. [38]. Longitudinal sections were obtained and stained in Trypan Blue by the method of [20]. Percentage of mycorrhizal infection was estimated as the relative length of colonized cortical parenchyma (Table 4).

A second microscopic investigation by light and transmission electron microscopy was performed on roots of plants pB1, pB3, and pC2, selected since ascomycetous ITS were found by molecular typing. Samples immediately adjacent to pieces used for molecular analysis were fixed in 2.5% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.2) at 4°C overnight, rinsed twice, and postfixed for 1 h in 1% (w/v) OsO_4. After rinsing in the same buffer, they were dehydrated in an ethanol series (30%, 50%, 70%, 90%, 100%; 15 min each step) at room temperature and then infiltrated in 2:1 (v/v) ethanol:LR White resin (Polysciences Inc., Warrington, PA, USA) for 1 h, 1:2 (v/v) ethanol:LR White for 2 h, and 100% LR White overnight at 4°C according to [2]. Semithin sections (1 μm) were cut from each sample and the sections were stained with 1% toluidine blue for morphological observations, while thin sections (0.05 μm) were stained using the PATAg method to visualize polysaccharides [36] or counterstained with uranyl acetate and lead citrate. To confirm the nature of the *Tuber* symbionts revealed in samples of plant pB1, sections were treated for immunogold reactions using a rabbit polyclonal antibody (anti-TbSP1) specifically raised against a truffle phospholipase A2 [46]. The immunogold labeling as well as the control experiments were performed according to Bonfante et al. [9].

Results

Mycorrhizal Colonization of E. microphylla. Microscopic analysis of *E. microphylla* roots in populations A and B showed a high colonization level (>70%; Table 4), with a higher mean value for albino plants. However, colonization levels did not significantly differ (as checked by a one-way ANOVA performed on the data set, not shown), because of large standard deviations. Colonization levels were in good agreement with the PCR success (92.8% for albinos vs 75.0% for green plants in population A, 89.3% and 93.9% in green individuals of population B and C respectively, Table 1).

Identity of the E. microphylla Fungal Symbionts. Most samples from population A produced an ITS of a *Tuber* species related to *T. excavatum* (Tables 1 and 2). In addition, two pieces, from an achlorophyllous and a green plant, respectively, produced a *Cortinarius* sp. ITS, while a third piece from achlorophyllous plant pA4 showed a Sebacinaceae ITS. As we were not able to sequence the PCR product of one piece from plant pA4, we

Table 4. Colonization level observed in *E. microphylla* roots from population A

Orchid	*Percentage of the root length colonized by fungi*[a]
pA1 (green)	74.6% (± 25.6%)
pA2 (green)	70.6% (± 33.0%)
pA3 (albino)	92.7% (± 10.3%)
pA4 (albino)	88.0% (± 14.2%)
pB1 (green)	77.3% (± 24.3%)
pB2 (green)	86.6% (± 18.4%)
pB3 (hypogeous)	88.0% (± 17.0%)
pC3 (green)	80.6% (± 14.3%)

[a]Mean of 15 observations (± standard deviation).

cloned it and, in addition to a *Tuber* sequence identical to other pieces, a Trichocomaceae ITS was retrieved (Table 1). Therefore, plants of population A, either albino or green, only differ in the rarest symbionts, but share the *Cortinarius* sp. and the dominant *Tuber* species.

All but one sample from population B produced an ITS of a *Tuber* species related to *T. uncinatum* (Tables 1 and 2). A Sebacinaceae sequence was also found once in plant pB2 (Table 1). The underground plants found in this population therefore had the same symbionts as epigeous plants.

In population C, five basidiomycetes and five ascomycetes (Pezizales) were found (Table 1), and pieces from the same root often produced identical ITS sequences. A *Tuber* sequence identical to that found in population B dominated in roots of plants pC3 and pC4 (Table 1). In all but five cases, a unique PCR fragment per piece was amplified and directly sequenced. In a single case (a pC2 root), the sequence amplified using ITS1F + ITS4B differed from that amplified using ITS1F + ITS4 (revealing the *Cortinarius* #C2 and the Pezizale #C1, respectively). For the five remaining root pieces that could not be directly sequenced, we cloned the PCR products. For two pieces from a pC1 root, sequences of the PCR products suggested a multiple colonization, and in both cases, after cloning, Pezizale #C1 and the Tuberaceae #BC2 were retrieved. Two root pieces from pC3 and pC4, which produced two ITS each, were colonized by both Tuberaceae #BC2 and Hymenogastraceae #C2. Lastly, a root piece from pC2 was simultaneously colonized by Russulaceae #C1 and Pezizale #C2. In conclusion, ITS cloning did not reveal fungal ITS other than those directly sequenced from PCR products.

We ensured the absence of the orchid symbionts *Tulasnella*, using appropriate ITS primers for these species (not shown): although some pieces from population B produced a faint PCR product, its sequence was always shown to be identical to that of Tuberaceae #BC2, the dominant *Tuber* species in population B.

Ectomycorrhizae Surrounding E. microphylla. With the possible exception of the Trichocomaceae sequence found on plant pA4, all fungi found on *E. microphylla* roots belong to ECM taxa. We thus investigated their presence on neighbouring tree roots in the close vicinity of *E. microphylla* plants. Although a low number of such ECM were found, some had common symbionts with the related orchid (Table 3). This suggests that at least some *E. microphylla* symbionts are shared with surrounding trees.

Microscopic Analysis. Since Ascomycetes are not reported as orchid symbionts, we analyzed roots from populations B and C by light and electron microscopy to confirm the identity and mycorrhizal status of the endophytes placed within the Pezizales on the basis of their ITS sequences.

We analyzed root pieces from plant pB1 (three pieces) and pB3 (one piece) that directly flanked a sample exhibiting a *T. uncinatum*-like ITS. One-micron-thick sections consistently revealed an entirely intracellular infection with unclamped fungal hyphae forming coils in most of the cortical cells. Clumped hyphae usually occupied the central part of the cells (Fig. 1a). When seen under the electron microscope, hyphae appeared to be septate and constantly surrounded by the host membrane (Fig. 1b), from which they were separated by an electron-dense interfacial material (Figs. 1c, d). A membrane tightly surrounded the fungus (Fig. 1b), as usual in biotrophic interactions. However, contrasting with orchid mycorrhizae so far described, the fungal septa were of ascomycetous type, with associated Woronin bodies (Fig. 1c). To further corroborate the identity of the fungus, an antibody (anti-Tbsp1) raised against the phospholipase A from *Tuber borchii* [46] was used as a more specific probe in immunogold experiments. As expected, the antibody labelled the fungal longitudinal and cross walls (Fig. 1d). No labeling was observed in the control experiments where the primary antibody was omitted (not shown).

Analysis of a root piece from plant pC2 colonized by the Pezizale #C1 revealed very similar characters both at the anatomical (Fig. 2a) and cytological (Fig. 2b) levels. Hyphae were constantly surrounded by the host membrane and presented ascomycetous septa (Fig. 2c). The fungal wall and the interfacial material were relatively thick and often electron-dense (Fig. 2d). When the anti-Tbsp1 antibody was used in immunogold experiments, the reaction was positive (not shown) and comparable to the labeling observed in the previous samples: since no *Tuber* species were found in molecular analysis of plant pC2, this probably results of the phylogenetic relatedness of Pezizale #C1 with truffles.

In order to look for basidiomycetous pelotons, two other root pieces from plant pC2 were investigated, adjacent to pieces producing ITS of *Hymenogastracea* #C1 in one case and of both *Russulaceae* #C1 and Pezizale #C1 in the other case. They only revealed ascomycetous pelotons (not shown), similar to those previously described. Neither clamped hyphae nor dolipores were observed, suggesting that the basidiomycetes were either missing on the investigated root piece, or poorly represented on it.

Discussion

We demonstrated that *E. microphylla* roots, irrespective of their physiological status (chlorophyllous vs achlorophyllous, mature vs late protocorm individuals), contain several fungi, belonging to both asco- and basidiomycetes (Table 2). In contrast with other green orchids studied to date, no usual rhizoctonia was detected (see the later

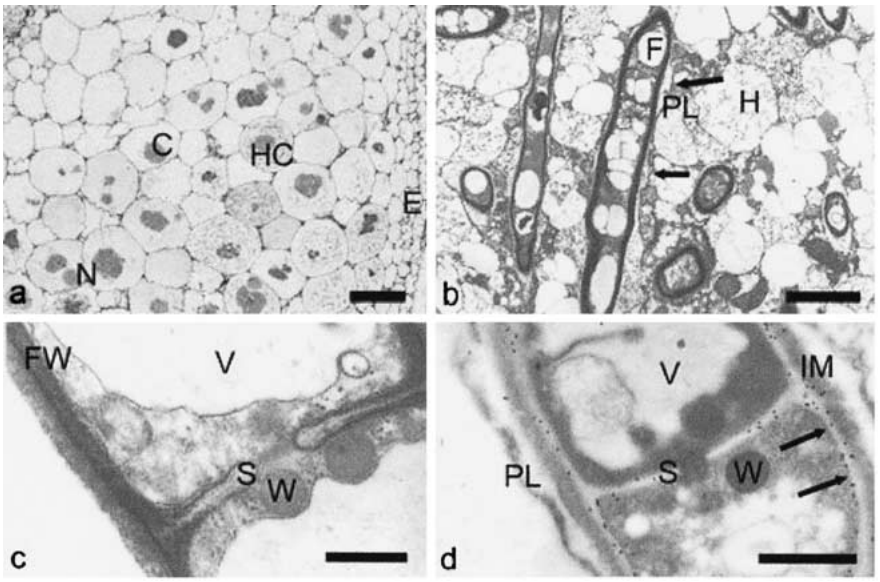

Figure 1. Anatomy and cytology of orchid roots from population B. (a) A light microscopy section reveals intracellular hyphal coils (HC) and fungal clumps (C) resulting from hyphal lysis (E: epidermal root cells; N: plant nucleus; bar: 20 μm). (b) Electron microscopy of truffle septate hyphae (F) inside an orchid host cell (H), where the host plasma membrane (PL, arrow heads) tightly surrounds the fungus (bar: 2 μm). (c) Woronin bodies (W) surrounding the pore of the septum (S), demonstrate the ascomycetous origin of the hyphae (FW: fungal cell wall; V: fungal vacuole; bar: 0.2 μm). (d) Gold granules are regularly distributed (arrows) on the longitudinal and septum wall of the fungus after immunogold reaction with the anti-Tbsp1 antibody specific for a truffle phospholipase (IM: interfacial material; PL: plasma membrane of the host cell; S: septum; V: vacuole; W: Woronin bodies; bar: 0.6 μm).

discussion for Sebacinaceae), while Pezizales dominated. All the identified species are ECM fungi, and some even associate with surrounding tree roots on the same site (Table 3). Taken together, these results indirectly suggest that at least the achlorophyllous and subterranean individuals are MHP using tree photosynthates as ultimate carbon source.

Mycorrhizal Colonization and Specificity. All investigated plants were highly colonized, independently of their physiological status (Table 4). Among *Epipactis* spp., considerable variation in the infection level exists, at both the intra- and interspecific levels [33, 38], ranging from sporadic to regular, dense infections [41]. The high colonization of *E. microphylla* roots explains the success encountered in molecular typing (>86%; Table 1). Since most 2-mm root pieces produced a unique fungal ITS, or no more than two when cloning was performed, each fungus seems to occupy alone large root portions. There is a trend, although not significant, to higher colonization in albino plants (Table 4), as observed for achlorophyllous *E. helleborine* individuals in another study [38], which perhaps allows a better carbon supply by the fungus to these heterotrophic plants (see below).

Another interesting feature is the diversity of fungi found in the molecular analysis (Table 2). The reason why population C (mainly plant pC2) presents more fungi is unclear, but, since all epigeous plants were fruiting, the developmental stage probably does not explain the variable fungal diversity. Interestingly, ecology of population C (pure beech stand on thick brown soil) differs from the two other ones (mixed stand, thin soil). Some partners could have been missed in this study, mainly due to the direct sequencing procedure that can hide weakly amplified ITS sequences. But, even in population C, most fungi have been identified at least twice per population (Table 1): this seems to guarantee that most fungi have been found on the sampled plants.

Formation of pelotons was established by microscopy only for Pezizales (Fig. 1 and 2) and remains questionable for the basidiomycetes and the Trichocomaceae. Trichocomaceae #A1, found with a truffle on a root of the pA4 plant (Table 1), belongs to a family that mostly contains saprobic species. We consider it as either a superficial saprobic contaminant, or as a nonmycorrhizal endophyte, as described for some *Aspergillus* strains [3], but we cannot fully exclude that it forms true orchid mycorrhizae. The basidiomycetes, which were found in all three populations, belong to ECM taxa (see below) that are not frequent contaminants on soil samples. Rare clamped hyphae were seen in intracellular pelotons during the analysis of the infection level on plants pA2 and pC3 (not shown), confirming that basidiomycetes may colonize orchids, as published for the related orchid *Epipactis helleborine* [37, 38]. However, extensive ultrastructural analyses performed on two root pieces of plant

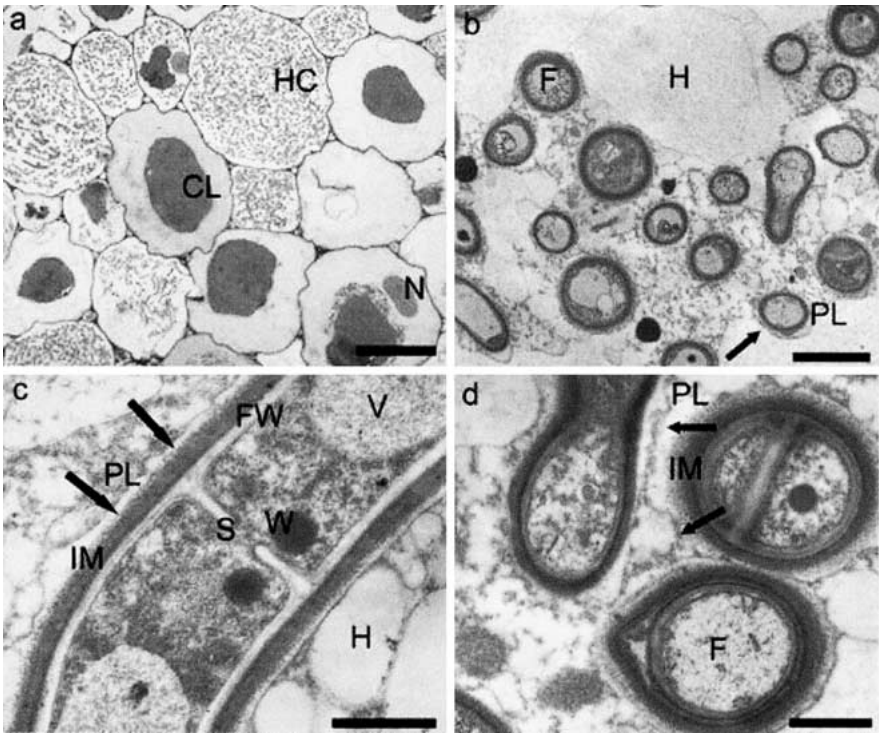

Figure 2. Anatomy and cytology of an orchid root from plant pC2, putatively colonized by Pezizale #C1. (a) Intracellular hyphal coils (HC) surround fungal clumps (CL) inside the cortical host cells (N: plant nucleus; bar: 20 μm). (b) Electron microscopy of hyphae (F) inside an orchid cell (H), where the host plasma membrane (PL, arrow heads) tightly surrounds the fungus (bar: 2 μm). (c) Woronin bodies (W) surrounding the pore of the septum (S) demonstrate the ascomycetous origin of the hyphae (FW: fungal cell wall; V: fungal vacuole; PL (and arrow heads): host plasma membrane; IM: interfacial material; bar: 0.2 μm). (d) An electron-dense material is laid down between the fungal wall and the host membrane (same abbreviations as for c, bar: 1 μm).

pC2 failed to detect any basidiomycetous hyphae. We suggest that these basidiomycetes may be symbionts on restricted portions of *E. microphylla* roots, perhaps overrepresented in molecular abalysis, but, as an alternative, they could behave as rhizoplane colonisers.

E. microphylla presents a trend toward specificity, since *Tuber* spp. dominated in all populations (78% of all occurrences, Table 1) and at all developmental stages, being exclusive symbionts of late protocorms and present on all plants but pC2. In all, Pezizales dominated by species number. However, *E. microphylla* is not as specific as related MHPs, i.e., *Cephalanthera austiniae* [47] and *Neottia nidus-avis* [25, 43], where only ITS from a single clade can be amplified by PCR. Our data also differ from the unique, unclamped fungus cultivated by Salmia [37] from green and albino *E. helleborine*, but additional, minor or noncultivable fungi may have been missed in the latter study. In conclusion, *E. microphylla* resembles other green orchids where low to intermediate specificity is commonly reported [13, 34, 45], and where a fungal species sometimes dominates among others [21].

E. microphylla Symbionts Are not Rhizoctonia, but ECM Fungi. *E. microphylla* symbionts strongly differ from those of other green orchids, as no rhizoctonia was detected, even using primers specially designed for tulasnelloid fungi. From a phylogenetic point of view, the two identified *Sebacinaceae* spp. (#A1 and #B2, Table 2) can be considered as related to rhizoctonias, since some *Sebacinaceae* have rhizoctonial asexual stages. However, these *Sebacinaceae* spp. are close (or identical) to species forming ECM and endomycorrhizae in MHPs [42, 43, 50], but are very distantly related to the *Sebacinaceae* spp. colonizing green orchids (Weiß and Selosse, in preparation). ITS of these two clades are so divergent that they cannot be confidently aligned [43].

Contrasting with other green orchids, all the identified fungi from *E. microphylla*, with the possible exception of *Trichocomaceae* #A1 discussed above, belong to ECM taxa, another feature hitherto unexpected in photosynthetic orchids, whose rhizoctonias are considered as saprotrophic [34]. (However, a recent study [7] questions this ecology at least for several tulasnelloids, a group many orchid rhizoctonia belong to). All other fungi belong to ECM taxa, and the three Pezizales of uncertain familial affiliation (Pezizales #C1-3) had ITS similar to those amplified from ectomycorrhizae in other studies (Table 2). At least four *E. microphylla* symbionts formed ECM on surrounding trees (Table 3). *E. microphylla* therefore resembles MH orchids, as far as the ECM status of its fungal symbionts is concerned.

Ascomycetes (Truffles Particularly) Are Unexpected Orchid Symbionts. Intriguingly, one-half of the symbionts (89% of all occurrences) are ascomycetes (Table 2). This was unexpected, as ascomycetes have never been rigorously demonstrated to colonize orchid roots. Some rhizoctonias are asexual stages of ascomycetes,

from the *Tricharina* and *Wilcoxina* genera [52], but they have never been isolated from orchids. Some ascomycetes have been isolated from orchid roots [13], but their mycorrhizal status is debated [34], and other ascomycetes behave as systemic endophytes of orchids [4]. Our morphological data unambiguously demonstrate that ascomycetes are mycorrhizal on *E. microphylla*, forming typical pelotons in living host cells. They closely resemble field-collected orchid mycorrhizae hosting basidiomycetous partners [39]. Cell-to-cell interactions between plant and fungus mirror features usually found in orchid protocorms (i.e., surrounding membrane, interface material [30]) as well as in all endomycorrhizae [9].

The coil organization shown by ascomycetous symbionts suggests that the morphogenetic potentialities of ectomycorrhizal ascomycetes are wider than expected and are controlled by the host plant. A given fungus produces divergent symbiotic structures when associated with different plants, as already exemplified for basidiomycetes that penetrate the cells of MHP but form ECM on trees [42, 47]. Similarly, some ascomycetes species form both intracellular coils on *Ericaceae* and ECMs or endophytic colonizations on tree roots [5, 31, 51]. Our data extend the host range and colonization capabilities of truffles, usually considered as extracellular symbionts of tree roots. Truffles sometimes develop intracellular structures during *in vitro* colonization [26] or in old root cap cells [29], but in the latter conditions, they mostly grow in senescent host cells. Similarly, parasitism by truffles has been claimed to cause the brulé [32], i.e., the plantless zone developed around trees mycorrhizal with truffles, suggesting an ability to penetrate cell walls.

This is, to our knowledge, the first direct evidence for ascomycetes forming endomycorrhizae in orchids, but this may not be an exception, at least among *Neottieae*, since *E. helleborine* is also colonized by *Tuber* spp. and other ascomycetes (M.-A. Selosse, unpublished data; M. Bidartondo, personal communication). Ascomycetes can easily be mistaken for rhizoctonias, since both have clampless hyphae. Morphological approaches based only on light microscopy can overlook their presence in orchids: for example, Scrugli et al. [41] considered that *E. microphylla* was colonized by rhizoctonias.

E. microphylla Mycoheterotrophic? Our data do not clarify the origin of achlorophylly in albino plants, but suggests explanations for their survival. Albino plants, as well as subterranean rhizomes and late protocorms, are heterotrophic. Since albino plants never formed green shoots in previous years [40], they are unlikely to rely on reserves from the previous season and thus receive their carbon from the fungi. Subterranean rhizomes can be either dormant individuals (relying on reserves accumulated in previous years, when they formed green shoots), or fungus-fed plants. Late protocorms do not form shoots for some years [33], and therefore also receive carbon from their fungi. Two sources exist for the fungal carbon: (i) neighboring green *E. microphylla*, which share the same symbionts (Table 1), and (ii) surrounding trees sharing the same associates (Table 3). Although these alternatives are not mutually exclusive, we favor the latter one, as some albino plants were observed to grow more than 7 m away from the nearest green orchid. Photosynthate labeling could help to establish the carbon source of heterotrophic plants. However, *E. microphylla* albinos provide the first reported case of a mycoheterotrophic orchid partnered by an ascomycete, to our knowledge.

Moreover, the ecology of their fungal symbionts raises the possibility of a partial mycoheterotrophy for green *E. microphylla*. A carbon flux from the fungus could explain two features of this species. First, it has reduced leaf size; second, whereas in other *Epipactis* spp. shoots are devoid of flowers during the first years and probably only contribute to carbon accumulation [33], the first aerial shoot bears flowers in *E. microphylla*. More generally, a partial mycoheterotrophy can be questioned for all Neottieae: a recent study demonstrated that isotopic abundances (^{15}N and ^{13}C) of some green species (including two *Epipactis* spp.) differed from these of other green plants growing on the same sites, but were closer to these of MHPs [18]. It was suggested that, depending on the species, 7 to 85% of the carbon could be derived from the mycorrhizal fungi. Again, these plants await further experimental studies by labeling tree photosynthates, or by investigating CO_2 exchanges *in situ* to measure the ratio of respiration to photosynthesis.

Fungal Symbionts and Emergence of MHP Among Neottieae. We propose that the acquisition of ECM symbionts by Neottieae, at some stage of their evolution, entailed a predisposition to achlorophylly. The replacement of the usual rhizoctonias in Neottieae may have been selectively driven (i) by the low availability of rhizoctonias in the forest niches where they currently grow, or (ii) by the fact that ECM symbionts give indirect access to tree photosynthates. As far as the latter feature is concerned, tree canopy limits available light and probably selects for carbon sources alternative to photosynthesis. It has been argued that ECM fungi could represent a more stable carbon source over the year than saprophytic ones [34]. Such fungal links to the trees allow the survival of albino and underground adult plants, and maybe even contribute to the carbon budget of green species. During evolution, several species specialized in the exclusive use of carbon provided by the fungi, giving rise to MHPs, perhaps again as a result of a selection driven by the dense tree cover. In Neottieae, evolutionary transition to

full mycoheterotrophy seems only to imply a narrower fungal specificity.

Testing this scenario will require a more detailed analysis of the phylogeny, metabolism, and fungal partners of green Neottieae, e.g., for the genera *Epipactis*, *Limodorum*, and *Cephalanthera*. *E. microphylla* is a good model, because it presents albino plants and a colonization pattern allowing rapid fungal typing by direct sequencing in most cases. Nevertheless, its rarity limits its use for establishing a detailed symbiont spectrum, as well as for performing carbon labeling experiments. In our opinion, other Neottieae will provide useful models to understand how fungi contribute to the evolutionary emergence of MHPs, and their role in carbon transfer between plants.

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