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Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae

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Abstract

Several achlorophyllous orchids associate with ectomycorrhizal hymenomycetes deriving carbon from surrounding trees for the plant. However, this has not been shown for achlorophyllous orchids associating with species of *Rhizoctonia*, a complex of basal lineages of hymenomycetes that are the most common orchid partners. We analysed *Neottia nidus-avis*, an achlorophyllous orchid symbiotic with a *Rhizoctonia*, to identify its symbionts by internal transcribed spacer (ITS) sequencing. Analysis of 61 root systems from 23 French populations showed that *N. nidus-avis* associates highly specifically with a group of species of Sebacinaceae. Their diversity emphasizes the need for further investigations in the Sebacinaceae systematics. Sebacinoid ITS sequences were often identical within orchid populations and a trend to regional variation in symbionts was observed. Using ITS and intergenic spacer (IGS) polymorphism, we showed that each root system harboured a single species, but that several genets colonized it. However, no polymorphism of these markers was found among portions of each root: this is consistent with the putative mode of entry of the fungus, i.e. from the rhizome into roots but not repeatedly from the soil. In addition, ectomycorrhizae were always found within the *N. nidus-avis* root systems: 120 of the 144 ectomycorrhizae typed by ITS sequencing were colonized by a sebacinoid fungus identical in ITS sequence to the respective orchid symbiont (even for the IGS polymorphism in some cases). Because sebacinoids were demonstrated recently to be ectomycorrhizal, the orchid is likely to derive its resources from surrounding trees, a mycorrhizal cheating strategy similar to other myco-heterotrophic plants studied to date.

Keywords: ectomycorrhiza, fungal population, ITS, *Neottia nidus-avis*, orchids, *Rhizoctonia*, Sebacinaceae

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Introduction

Orchidaceae, one of the largest plant families (Dressler 1993), share an unusual mode of germination. Their minute-sized seeds are almost devoid of reserves and the undifferentiated embryo relies on a fungus, usually a basidiomycete, not only for its water and mineral needs but also for its carbon supply (Rasmussen 1995). The fungus penetrates the cells and forms hyphal coils that feed the plant, allowing a heterotrophic growth called 'mycotrophy' or 'myco-heterotrophy' (Leake 1994). During further development, seedlings usually become autotrophic. However, some species remain achlorophyllous and myco-heterotrophic at the adult stage. Myco-heterotrophy is also described from some Ericaceae (Bidartondo *et al*. 2000; Bruns & Read 2000; Bidartondo & Bruns 2001) and Gentianaceae (Leake 1994), but there are more myco-heterotrophic species among orchids than in any other family (Furman & Trappe 1971).

Fungal symbionts of some temperate myco-heterotrophic orchids have been identified. *Cephalanthera austinae* and

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Corallorhiza trifida associate with Thelephoraceae (Taylor & Bruns 1997; McKendrick *et al*. 2000a), whereas *Corallorhiza maculata* and *C. mertensiana* harbour symbionts from the Russulaceae family (Taylor & Bruns 1997, 1999). In all cases, the association is specific and implies hymenomycetes that are in other respects mycorrhizal with trees or shrubs on which they form ectomycorrhizae (Smith & Read 1997). Indeed, the presence of the same fungi on surrounding ectomycorrhizae has been assessed (Taylor & Bruns 1997), suggesting that the orchid derives its carbon indirectly from ectomycorrhizal plants via hyphal linkage (Zelmer & Currah 1994; McKendrick *et al*. 2000a,b). However, many achlorophyllous genera remain to be investigated to assess that specific association with ectomycorrhizal basidiomycetes is a general feature of myco-heterotrophic orchids, and the goal of our study was to test the validity of this model in an association implying orchids and fungi that are only distantly related to the previously investigated ones.

Many orchids are associated with basidiomycetes of the *Rhizoctonia* group (Smith & Read 1997), a polyphyletic taxon encompassing various asexual (= anamorphic) fungal stages. In cases where sexual stages are known, *Rhizoctonia* species achieve meiotic sporulation by producing basidia that classify them among teleomorphic heterobasidiomycetous genera such as *Ceratobasidium*, *Thanatephorus*, *Tulasnella* and *Sebacina*, i.e. lineages that are basal to the other hymenomycetes. *Rhizoctonia* species have been isolated from some achlorophyllous orchids, for example *Thanatephorus gardneri* from *Rhizanthella gardneri* (Warcup 1991), or *Sebacina vermifera* from albino mutants of *Microtis rara* (Warcup 1988). Taylor & Bruns (1999) suggested that, during evolution to myco-heterotrophy, mycotrophic orchids often abandoned their *Rhizoctonia* symbionts. The present work aims at confirming by field investigations if some *Rhizoctonia* species associate with myco-heterotrophic orchids in field conditions and, if so, whether they also interact with adjacent tree roots.

Among *Rhizoctonia*-associated plants, *Neottia nidus-avis* (L.) L.C.M. Rich. is an achlorophyllous and leafless orchid that occurs in various European forests (see Rasmussen 1995 for review), which is nonphotosynthetic (Hudak *et al*. 1997). The plant has an underground plagiotropic rhizome that bears thick adventitious roots in a structure roughly reminiscent of a bird's nest (Fig. 1). After some years of subterranean growth, an erected inflorescence emerges. The roots are colonized heavily by a septate, clampless fungus that forms abundant coils in the cells, as already indicated by pioneer studies of Noël Bernard (Bernard 1899, 1901). Infection is restricted to the three cortical cell layers and the meristems are not infected (Nieuwdorp 1972; Barmicheva 1989). Ultrastructural examinations of the septal pore that connects the hyphal cells revealed a dolipore surrounded by a continuous parenthesome

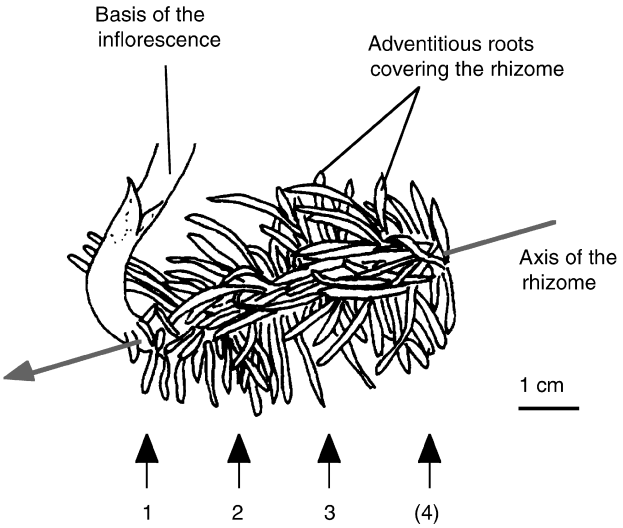

Fig. 1 The *Neottia nidus-avis* root system (= underground rhizome with adventitious roots), with indication of the three to four sections studied in Fig. 5 (reproduced, with permission, from Leake 1994).

(Barmicheva 1989), as in several basal groups of hymenomycetes (Wells & Bandoni 2001), e.g. *Tulasnella* or *Sebacina* (Rasmussen 1995). Consistently, as this study was submitted, McKendrick *et al*. (2002) demonstrated by molecular methods that two sebacinoid species colonized *N. nidus-avis* roots on two forest sites, while another unidentified species, possibly a contaminant, was also present on some root systems.

N. nidus-avis therefore constitutes a suitable model for investigating orchid myco-heterotrophy involving partners of the *Rhizoctonia* complex. In addition, it is phylogenetically remote from the other achlorophyllous orchids mentioned above (Dressler 1993; Cameron *et al*. 1999), suggesting an independant origin of myco-heterotrophy. Our first aim was to assess the systematic position of the *N. nidus-avis* symbionts and to investigate the specificity of the association using a large sampling. Because previous attempts to cultivate the fungus failed (reviewed by Rasmussen 1995) we made use of polymerase chain reaction (PCR) amplifications of the internal transcribed spacer (ITS) sequence of the ribosomal DNA (rDNA), which allows identification of fungal species (Gardes & Bruns 1993) and can be amplified specifically from symbiotic tissues (Selosse 2001). Our second aim was to investigate the distribution of the symbionts at various scales, i.e. among portions of a single root, among roots of a given root system, among plants of a given population and among *N. nidus-avis* populations encountering different ecological conditions. In order to find polymorphism among fungi colonizing a single root system, we also used the rDNA intergenic spacer (IGS), that can be polymorphic at the intraspecific level (Selosse

et al. 1996; Selosse 2001). Our last aim was to understand the carbon source of the association and to test whether *N. nidus-avis* is linked to surrounding tree roots through hyphal linkage. Again using PCR amplifications of ITS and IGS, we investigated the presence of the respective fungal symbionts on ectomycorrhizae growing in the orchid root systems.

Materials and methods

Sampling of orchid root systems

Root systems (Fig. 1) were sampled from 23 *N nidus-avis* populations (2–20 individuals, area $< 8 \times 8$ m) located in eight regions of France (Table 1 and Fig. 2). Sampling was

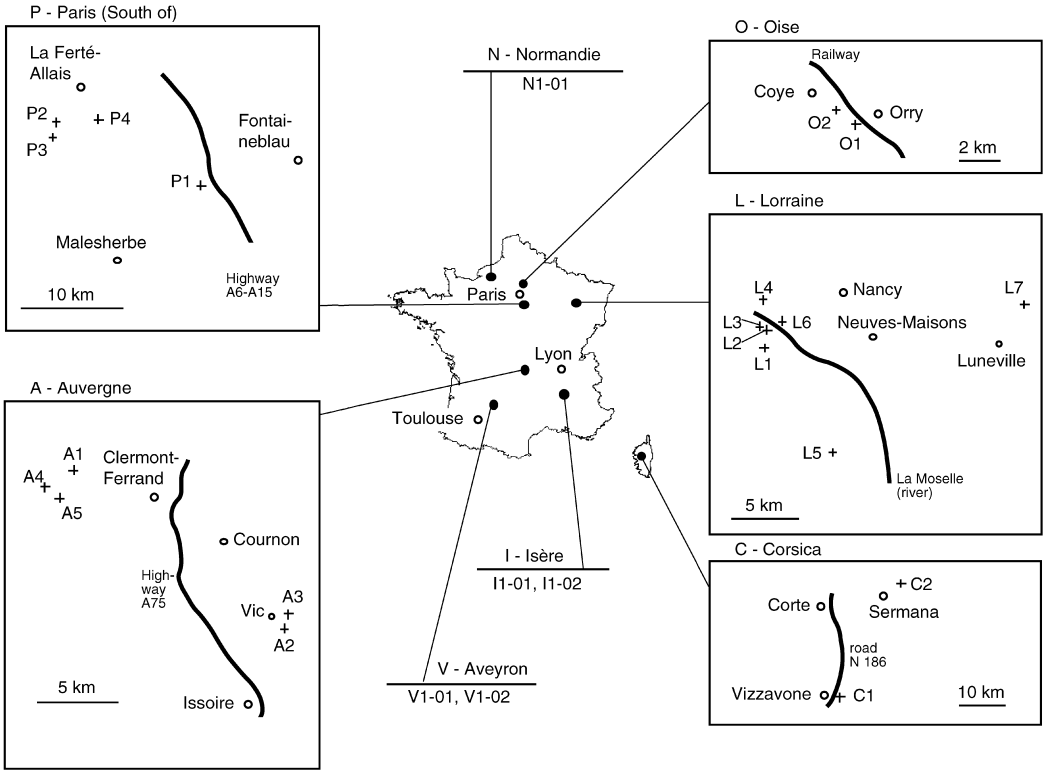

Fig. 2 Location of the various *N. nidus-avis* populations investigated in France. Features of the populations and numbers of cores collected are given in Table 1.

Table 1 Sampling sites and ITS typing of the fungal symbionts of the *N. nidus-avis* roots found in the sampled cores

Orchid population (region and site)*	Elevation (m)	Surrounding tree species†	Core no. (root system number‡)	Date	GenBank Fungal ITS type accession no.§
A — Auvergne, five populations					
A1, Puy de la Goule	1000	F, T	A1-01 (1)	00.05	AF440641
A2, Vic-le Comte — 1	620	Q	A2-02 (1)	00.06	AF440642
			A2-03 (1)	00.06	AF440642
			A2-04 (1)	00.06	AF440642
			A2-05 (1)	00.06	AF440642
			A2-06 (1)	00.06	AF440642
A3, Vic-le Comte — 2	780	Q, Co, Ca, T	A3-07 (2 + 1 h)	00.06	AF440643
			A3-08 (2)	00.06	AF440643
			A3-09 (1)	00.06	AF440643
			A3-10 (1)	00.06	AF440643
			A3-11 (1)	00.06	AF440643
A4, Puy de Comes	950	Co, F, Pin, Po	A4-12 (1)	00.06	AF440644¶
A5, Puy Pariou	1000	F, Q	A5-13 (2)	00.06	AF440645
A1, Puy de la Goule	1000	F, T	A1-14 (1)	01.05	AF440641

Table 1 *Continued*

Orchid population (region and site)*	Elevation (m)	Surrounding tree species†	Core no. (root system number‡)	Date	GenBank Fungal ITS type accession no.§
C — Corsica, two populations					
C1, Vizzavone	1000	F, Pin	C1-01 (1)	00.06	AF440646
C2, Monte Muffrage	1400	F	C2-02 (1)	00.06	AF440646
			C2-03 (2)	00.06	AF440647
I — Isère, one population					
I1, St Pierre d'Allevard	1100	P	I1-01 (1)	00.05	AF440648
			I1-02 (1)	00.05	AF440648
L — Lorraine, seven populations					
L1, Fontaine St.-Anne	300	Co, Q, T	L1-01 (1)	00.05	AF440649
L2, Sexey aux Forges — 1	350	Ca	L2-02 (1)	00.05	AF440650
L3, Sexey aux Forges — 2	350	P (plantation)	L3-03 (1)	00.05	AF440650
L4, Vallon Monvaux	250	F, Ca	L4-04 (1)	00.05	AF440651
			L4-05 (1)	00.05	AF440652
L5, Xirocourt	250	Ca	L5-06 (1)	00.06	AF440653
L6, Marron	250	F	L6-07 (1)	00.06	AF440654
			L6-08 (1)	00.06	AF440654
L7, Forêt de Paroy	250	P (plantation)	L7-09 (1)	00.07	AF440655
L1, Fontaine St.-Anne	300	Co, Q, T	L1-10 (1)	01.05	AF440654
			L1-11 (1)	01.06	AF440649
			L1-12 (1)	01.09	AF440649
			L1-13 (1)	01.09	AF440649
N — Normandie, one population					
N1, Elbeuf	90	F, Co, Q	N1-01 (3 h**)	00.06	AF440656
O — Oise (North of Paris), two populations					
O1, Forêt de Coye — 1	100	Co, Q	O1-01 (1)	00.05	AF440657
O2, Forêt de Coye — 2	100	F, Q, T	O2-02 (2 + 3 h)	00.05	AF440658
			O2-03 (2 + 2 h)	00.05	AF440658
			O2-04 (1)	00.05	AF440658
O1, Forêt de Coye — 1	100	Co, Q	O1-05 (1)	00.09	AF440657
			O1-06 (1)	01.05	AF440657
			O1-07 (1)	01.06	AF440657
			O1-08 (1)	01.06	AF440657
			O1-09 (1)	01.07	AF440657
P — Paris (Southern region), four populations					
P1, F. de Fontaineblau	120	Pis	P1-01 (1)	00.04	AF440659
P2, Bouville — 1	100	Pis, Q	P2-02 (1)	00.05	AF440650
P3, Bouville — 2	100	Co, T	P3-03 (1)	00.05	AF440660
P4, Boutigny	120	Co, Q	P4-04 (1)	00.06	AF440661
			P4-05 (1)	00.06	AF440661
V — Aveyron, one population					
V1, Estaing	400	Pis, Q	V1-01 (1)	00.06	AF440662
	400	Pis, Q	V1-02 (1)	00.06	AF440663

*See location on Fig. 2.
†Ca, *Carpinus betulus*; Co, *Corylus avellana*; F, *Fagus sylvatica*; Po, *Populus nigra*; P, *Picea abies*; Pin, *Pinus nigra*; Pis, *Pinus sylvestris*; Q, a species of the *Q. robur — Q. petraea — Q. pubescens* complex.
‡Under brackets, the number of root systems found in the core; 'h' refers to hypogeous individuals, i.e. that did not flower above ground at time of sampling.
§GenBank accession no. of the amplified ITS. Number in italics indicates the cores in which a longer ITS sequence including a part of the 28S rDNA was amplified and sequenced using primer NL4 (see Materials and methods).
¶Variations of this sequence were found on some roots of this individual, see Fig. 5A.
**Hypogeous root systems found under a 1999 dead shoot.

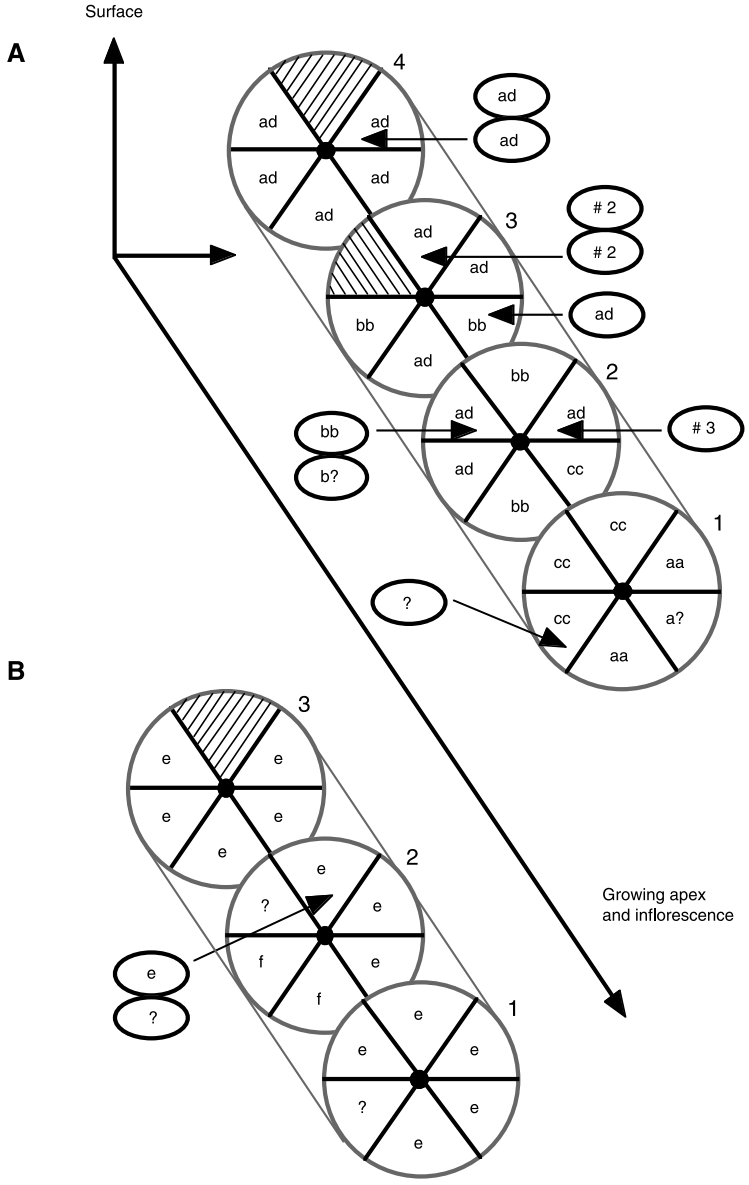

Fig. 3 Colonization of two cores by sebacinoid species. Sections of the *N. nidus-avis* root systems are drawn in three dimensions and numbered as in Fig. 1, with ellipses around the sections symbolizing the ectomycorrhizae (position within the root system indicated by an arrow). No root was growing in hatched sectors and '?' indicates that typing failed. (A) Mapping of the four ribosomal types found in core A4-12: ribosomal type is given according to Fig. 5A–B, with the first letter referring to the ITS type and the second to the IGS1 pattern; 2 and 3 refer to the two ectomycorrhizal basidiomycetes found (see Table 2). (B) Mapping of the two IGS types (*e* and *f*, see Fig. 5C) found in core L3-03.

performed in May–June 2000, as the orchid flowered, as well as during autumn 2000, spring and autumn 2001 for three populations (A1, L1 and O1) in order to follow temporal variations. Sampled orchids were at least 2 m apart from each other. A $20 \times 20 \times 20$ cm soil core was collected under the epigeous inflorescence and dissected in order to extract the intact root system within less than a day after core collection. In some cases (Table 1) more than one root system was found in a core, corresponding to two adjacent inflorescences (e.g. in A5-13 or C2-03) or to hypogeous individuals (i.e. nonflowering rhizomes, e.g. in A3-07 or N1-01). Such additional root systems probably result from vegetative multiplication through roots that, when separated from the main rhizome, transform into new growing rhizomes (Rasmussen 1995). A total of 49 cores accounting for 61 root systems was collected, including seven hypogeous root systems. Three roots were collected on each root system, a young one (position 1 on Fig. 1), an old one (position 3 or 4 on Fig. 1) and a middle-aged one (between the two previous positions). A fine-scale sampling of the root system was performed on four root systems: the whole root system was considered as a cylinder, having the rhizome as axis, and was sliced into three (L6-07 and O1-01) or four (A2-04 and A4-12) blocks (see Fig. 1), depending on its length. Each block was in turn divided into six radial sectors (see Fig. 3). One root per sector was collected and divided into 3-mm long fragments, resulting in 1–10 pieces.

Ectomycorrhizae were also found within all root systems. In order to test for the presence of the orchid symbiont on these tree roots, they were collected from some cores (Table 2) in order to limit and standardize the sampling,

Table 2 Molecular typing of the ectomycorrhizae found within *N. nidus-avis* root systems according to the fungal ITS sequence. Sampled cores are described in Table 1. For more precise identifications of the ectomycorrhizal fungi differing from the orchid symbiont, see Table 3

Sampling		Result of the ITS typing		
Core	No. of ecm	Type*	No. of ectomycorrhizae†	Total no. of tips‡
A1-01	15	Orchid symbiont	4†	8
		Ascomycete 1	6	6
		Basidiomycete 1	3	4
		Not amplified	2	3
A2-04	7	Orchid symbiont	7	9
A2-05	7	Orchid symbiont	7	7
A3-07	2	Orchid symbiont	2	3
A3-10	4	Orchid symbiont	4	4
A3-11	3	Orchid symbiont	3†	6
A4-12	8	Orchid symbiont	5	7
		Basidiomycete 2	2	3
		Basidiomycete 3	1	1
		Not amplified	1	1
C1-01	3	Ascomycete 2	2	3
		Dual ecm 1	1	3
C2-03	3	Orchid symbiont	2	2
		Not amplified	1	1
I1-01	2	Orchid symbiont	1	1
		Not amplified	1	2
L1-01	11	Orchid symbiont	7†	9
		Ascomycete 3	1	1
		Dual ecm 2	1	2
		Not amplified	2	3
L2-02	6	Orchid symbiont	3	4
		Not amplified	3	3
L3-03	2	Orchid symbiont	2	2
L4-05	10	Orchid symbiont	8†	10
		Not amplified	2	2
L5-06	6	Orchid symbiont	6	6
L6-07	10	Orchid symbiont	9†	10
		Not amplified	1	2
L1-13	13	Orchid symbiont	7	8
		Basidiomycete 4	3	6
		Basidiomycete 5	2	3
		Not amplified	1	2
N1-01	2	Orchid symbiont	2	2
O1-01	11	Orchid symbiont	9	9
		Not amplified	2	3
O2-02	5	Orchid symbiont	4	5
		Dual ecm 3	1	2
O2-03	4	Orchid symbiont	3	3
		Not amplified	1	1
O2-04	6	Orchid symbiont	4	4
		Ascomycete 4	2	2
O1-05	6	Orchid symbiont	6†	7
P1-01	4	Orchid symbiont	4	5
P2-02	3	Not amplified	3	4
P4-04	5	Orchid symbiont	3	4
		Basidiomycete 6	1	1
		Dual ecm 4	1	2
P3-05	5	Orchid symbiont	5	7
Total	164		164	208

*'Orchid symbiont' means that the ITS is identical to that of the fungal symbiont of the orchid roots of the core (Table 1). 'Not amplified': DNA extraction and/or PCR amplification of the ITS failed; 'Dual ecm': case where the ITS of the orchid symbiont was found together with another fungal ITS, suggesting dual colonization (Table 3).

†For one of the ectomycorrhizae from this core, the orchid symbiont was identified based on morphotype similarity and RFLP pattern, but not directly on the ITS sequence (see text).

‡Total number of tips recorded during sampling.

only ectomycorrhizae found between the orchid roots were sampled, excluding those found around the root system. Whenever some roots differed by colour or ramification pattern within a root system, this was recorded (this succinct morphotyping was fully consistent with the molecular analysis and is therefore not reported here). Ectomycorrhizal roots with several tips (= root apices) were considered as a single ectomycorrhiza, leading to a total of 164 ectomycorrhizae, but the number of tips was recorded for each ectomycorrhiza (i.e. 208 tips, Table 2). *N. nidus-avis* roots and ectomycorrhizae were frozen within less than 20 min after core dissection and kept at −80 °C.

DNA extraction and PCR amplifications

DNA was extracted from *N. nidus-avis* roots and ectomycorrhizae using the DNeasy™ Plant Mini Kit (Qiagen SA, Courtaboeuf, France), according to the manufacturer's advice. Total DNA was recovered in 40 µL bidistilled water and kept at −80 °C. The fungal ITS sequence (encompassing the ITS1, 5.8S and ITS2 sequences) was amplified using two sets of primers: ITS1F + ITS4 and ITS1F + ITS4B (Gardes & Bruns 1993). PCR was carried out in 50 µL, with final concentrations of 66 µM for each dNTP, 0.6 µM for each of the primers (Laboratoires Eurobio, Les Ulis, France), 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mgmL^{-1} gelatin, 0.1% (v/v) Triton X100, 5% (v/v) dimethyl sulfoxide and 1.5 units of Taq DNA polymerase (Quantum^R Appligène, Illkirch, France). We used 2.5 µL of the extracted DNA solution but other dilutions were tested when amplification failed. Reactions were performed in a TRIO-Thermoblock (Biometra, Göttingen, Germany) under the following thermoprofile: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. A 2-µL sample of the amplified ITS was then migrated on 1.5% agarose gel in a 0.5X Tris–borate–EDTA buffer to ensure the success of the amplification.

To validate the uniqueness of the symbiotic fungus found by ITS amplification, longer rDNA stretches were amplified from orchids roots and ectomycorrhizae from cores A1-01, N1-01, L1-01, L6-07 and P1-01, using ITS1F with backward primers located at various positions within the 28S sequence, namely starting from its 5′ end: LR21 (5′-ACTTCAAGCGTTTCCCTTT-3′), C2 (5′-TGAACTCTCT-CTTCAAAGTTCTTTTC-3′) and NL4 (5′-GGTCCGTGTT-TCAAGACGG-3′) (O'Donnell 1993).

Samples from fine-scale sampling (*N. nidus-avis* root fragments and ectomycorrhizae from cores A2-04, A4-12, L6-07 and O1-01) were also submitted to amplifications of the rDNA intergenic spacers in order to assess the diversity of genets colonizing them. The 28S/5S spacer (IGS1) and the 5S/18S spacer (IGS2) were amplified under the same conditions as used in the ITS amplification, except for the annealing temperature (50 °C) and elongation time (40 s), using primers and electrophoretic separation on acrylamide gel as described in Selosse *et al*. (1996).

ITS sequencing and sequence analysis

PCR products were purified using the MinElute™ PCR Purification Kit (Qiagen SA, Courtaboeuf, France), using the manufacturer's protocol for PCR purification. Whenever two bands were amplified, the total product was loaded on agarose gel and, after migration, each band was excised from gel and recovered using the MinElute™ PCR Purification Kit (Qiagen SA, Courtaboeuf, France), according to the manufacturer's protocol for gel extraction. PCR products were recovered in 40 µL bidistilled water and sequenced with the CEQ 2000 Dye Terminator Cycle Sequencing Kit using a CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, USA). For each core, the ITS amplified from a *N. nidus-avis* root with primers ITS1F and ITS4 was sequenced using the primers ITS1F and ITS4. Amplified ITS of other roots and ectomycorrhizae from the same core were then sequenced using the primer ITS4. If differences to the previous one occurred, a second sequencing reaction was performed using the primer ITS1F in order to verify the differences. ITS sequences obtained with primers ITS1F and ITS4B or primers located within the 28S rDNA were sequenced with the primers used for their amplification. Sequences were assembled and compared using Sequencher™ 3.11 for MacOS from Genes Codes (Ann Arbor, USA). The consensus sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI); accession nos are given in Fig. 4. Searches for similar sequences (Table 3) were conducted using BLAST (Altschul *et al*. 1997) at the NCBI page http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, using default settings.

Restriction fragment length polymorphism (RFLP) on ITS amplified from ectomycorrhizae

ITS sequences that entailed sequencing problems, suggesting a possible dual colonization, were submitted to RFLP to compare them with the fungal ITS amplified from other samples of the core. Digestions were performed using *Eco*RI + *Sac*I, both of which cut our sebacinoid ITS sequences at a single position, and *Hin*dIII that does not cut our sebacinoid ITS amplificates. Digestions were carried out at 37 °C over 3 h in a 20-µL solution containing 8 µL of purified PCR product, 10 units of each enzymes (Appligene, Illkirch, France) and the buffer supplied by the provider. Digestion products were separated on a 2% agarose gel in a 1X Tris–borate–EDTA buffer.

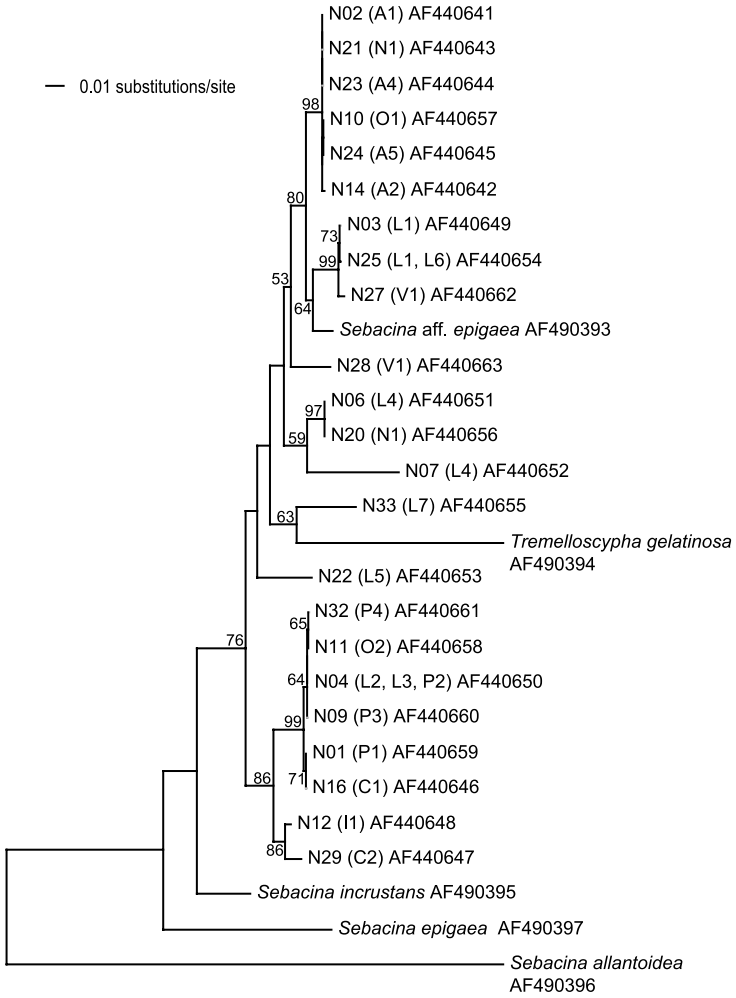

Fig. 4 Neighbour-joining analysis of an alignment of ITS sequences from the *N. nidus-avis* symbionts and other species of Sebacinaceae. Genetic distances were inferred from the Tamura–Nei model of DNA substitution (see Materials and methods for details). Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site, numbers on branches are bootstrap values (1000 replicates, values below 50% not shown; additional bootstrap values: N02, N21: 61%; N10, N24: 64%; N04, N11, N32: 72%). Letters in brackets indicate the population of origin. The topology was rooted with *Sebacina allantoidea*.

Phylogenetic analysis

To estimate phylogenetic relationships among the sebacinoid fungi detected in this study we constructed an ITS alignment including also some sequences derived from herbarium specimens of Sebacinaceae: *Sebacina incrustans*, *S. epigaea*, *S. aff. epigaea* (growing on bark), *Sebacina allantoidea* (a thin resupinate species growing on the base of a *Spiraea* shrub) and *Tremelloscypha gelatinosa* (GenBank accession nos are given in Fig. 4). These specimens had also been included in the molecular phylogenetic study of Weiß & Oberwinkler (2001; *Sebacina allantoidea* as *Sebacina* sp. RoKi 179), where DNA extraction and sequencing are described in detail. For PCR we used the primer combinations ITS1F + NL4 or ITS1F + ITS4 together with the following thermoprofile: 3 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min; PCR was terminated by a final extension step of 72 °C for 7 min. The ITS region was sequenced with sequencing primers ITS1F and ITS4.

An alignment was produced with the MEGALIGN model of the Lasergene package (DNASTAR, Inc., Madison, USA) followed by manual corrections using Se-Al (Rambaut 1996). PAUP* 4.0, version b8a (Swofford 2001) was used to perform a bootstrapped neighbour-joining analysis (Felsenstein 1985; Saitou & Nei 1987). Genetic distances were estimated by maximum likelihood (see Swofford *et al*. 1996 for a review of this method) according to the Tamura–Nei model of DNA substitution (Tamura & Nei 1993) involving the following parameters: base frequencies $\pi_A = 0.2757$, $\pi_C = 0.2072$, $\pi_G = 0.2284$, $\pi_T = 0.2886$, rate matrix coefficients 1 for transversions, 4.3020 for [A-G], 7.3462 for [C-T], and additionally assuming gamma-distributed substitution rates (shape parameter 0.4918) and a proportion of 0.3760 of invariable sites. This model of DNA substitution including parameter values was chosen using a series of hierarchical likelihood-ratio tests as implemented in Modeltest 3.06 (Posada & Crandall 1998).

In our alignment of ITS sequences we did not include a sequence of *Sebacina vermifera sensu* Warcup & Talbot (1967) (GenBank accession no. AF202728) because of too

Table 3 Molecular identification of fungi other than sebacinoids found on ectomycorrhizae growing within *N. nidus-avis* root systems. Dual colonization (dual ecm) represents mycorrhizae giving rise to two ITS, one from a sebacinoid identical to the respective *N. nidus-avis* symbiont and the other analysed here

Name	GenBank accession no.	Supposed affiliation	Closest sequence(s) found in GenBank by BLAST: name (accession no.)
Ascomycete 1	AF440666	Terfeziaceae	*Terfezia claveryi* (AF387648)
Ascomycete 2	AF440667	Sarcoscyphaceae	*Nanoscypha* sp. (AF026311) Ectomycorrhizal isolate (UEC410853)
Ascomycete 3	AF440668	Terfeziaceae	*Terfezia boudieri* (AF301419)
Ascomycete 4	AF440669	Sarcoscyphaceae	*Sarcoscypha vassiljevae* (SVU66017)
Basidiomycete 1	AF440670	Agaricales	*Agaricus arvensis* (AF161015)†
Basidiomycete 2	AF440671	Corticiaceae	*Piloderma fallax* (AY010282)
Basidiomycete 3	AF440672	Russulale	*Russula* sp. R22 (AF350057)
Basidiomycete 4	AF440674	Bolbitiaceae	*Setchelliogaster tenuipes* (AF099363)
Basidiomycete 5	AF440675	*Tomentella*	*Tomentella galzinii* (AF272932)
Basidiomycete 6	AF440665	*Laccaria*	*Laccaria laccata* (AF204814)
Dual ecm 1	AF440667	Ascomycete 2	
Dual ecm 2		unidentified*	
Dual ecm 3	AF440673	Cantharellales	*Clavulina cinerea* (AF335456)
Dual ecm 4	AF440665	Basidiomycete 6	

*RFLP analysis showed that at least 2 other sequences of similar molecular weight were present on this ectomycorrhizae.
†The sequence of the mitochondrial small ribosomal subunit (not shown) suggests a relationship to the genus *Inocybe*.

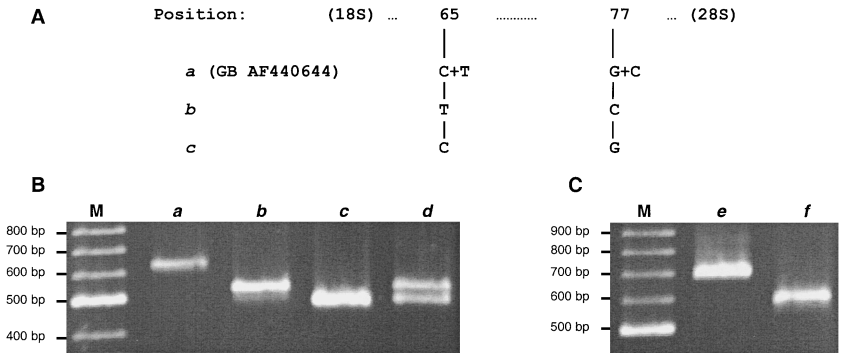

Fig. 5 Ribosomal polymorphism of the sebacinoid symbiont on *N. nidus-avis* root. (A) The three ITS sequences from core A4-12, *a*, *b* and *c*, differing at two ITS1 positions (pattern *a* is a superposition of pattern *b* and *c*; position 0 of the sequence is the 3′ end of the 18S rDNA). (B) The four IGS1 amplification patterns found in core A4-12, differing by their size on agarose gel (pattern *d* is a superposition of patterns *b* and *c*). (C) The two IGS1 amplification patterns found in core L3-03, *e* and *f*. M, size marker.

many ambiguously aligning sites. According to the results of Weiß & Oberwinkler (2001) we used *Sebacina allantoidea* as an outgroup species.

Results

Diversity of N. nidus-avis *symbionts*

Amplification of the fungal ITS from orchid roots (Table 1) was unsuccessful using primers ITS1F and ITS4B, but produced a *c.* 650 bp fragment using primers ITS1F and ITS4 in every case (data not shown), except for a young root from core P2-02. This showed that all roots were infected, independently of their age. The sequences of the *c.* 650 bp fungal ITS were always identical for all roots of a given core (Table 1, with the exception of root system A4-12, see below). We documented several sequence ambiguities, where two bases co-occurred at the same position, e.g. an 'A/C' in ITS2 from core P2-O2 and a 'T/C' in ITS2 from core A2-02 to A2-06. This may result of intranuclear polymorphism, of heterozygosities or of the presence of two closely related genets within the sample. Root system A4-12 produced two such ambiguities (sequence a in Fig. 5A), but other roots from the same root system produced two unambiguous sequences (*b* and *c* in Fig. 5A), whose superposition may produce the sequence a: such variations probably reflect intraspecific polymorphism. Although two very divergent ITS types co-occurred in populations L4 and V1, ITS sequences were most often identical within populations (Table 1).

Orchids from populations A1, L1 and O1, which were also sampled during autumn 2000 and throughout 2001 (Table 1), produced the same ITS type as in 1999 (in population L1, ITS from cores L1-01 and L1-12 produced sequences differing

from cores L1-10 and L1-11 only by an ambiguous position, 'T or C' vs. T, respectively, suggesting closely related strains). No temporal symbiont shift was documented, therefore, in those populations. We detected no other fungi colonizing the orchid roots by performing PCR with DNA extracts from five cores with various primer sets amplifying the ITS and more or less large parts of the 28S rDNA region (see Materials and methods and GenBank accession nos in italic on Table 1). These data also showed that primer ITS4B and the amplified sequences formed four mismatches, two of which are situated at the 3′ end of the primer, explaining why this primer did not amplify the fungal DNA.

Identification of the N. nidus-avis *symbionts*

The 23 ITS sequences recovered (Table 1) were very similar to each other, differing only by point mutations and some < 8 bp indels. They all showed highest BLAST values against the ITS of *Sebacina vermifera* (*sensu* Warcup & Talbot 1967, GenBank accession no. AF202728). Within the 158 bp of the 5.8S DNA, the sequences differ by 1–3 bp among cores and by 2–4 bp with that of *S. vermifera* AF202728. Due to great divergences in the ITS1 and ITS2 regions, however, the ITS sequences obtained from *N. nidus-avis* could not be aligned unambiguously to the ITS sequence of *S. vermifera* AF202728. Our phylogenetic analysis (Fig. 4) showed that the *Neottia* symbionts belong to a subgroup of this family including the type species of *Sebacina*, *S. incrustans*, as well as *S. epigaea* and *Tremelloscypha gelatinosa*.

Ectomycorrhizae from N. nidus-avis *root systems*

Ectomycorrhizae were collected within several root systems (Table 2) and analysed by ITS amplification with two primer sets, ITS1F + ITS4 and ITS1F + ITS4B. A total of 144 ectomycorrhizae (87%) was amplified successfully. One hundred and sixteen ectomycorrhizae produced a single PCR product with primer sets ITS1F + ITS4 but not with ITS1F + ITS4B. Among these, 110 had a sequence identical to that of the sebacinoid symbiont found on orchid roots of the same core (Table 2), whereas the six other ectomycorrhizae shared (i) the morphotype and (ii) the amplification product size and RFLP of the sebacinoid from the same core (not shown), but gave an unclear ITS sequence. However, as no direct evidence of other fungal colonization was found, we considered them as colonized by the sebacinoid species (see Table 2). A total of 24 ectomycorrhizae that produced a single PCR product with both primer sets (ITS1F + ITS4 and ITS1F + ITS4B) were colonized by other basidiomycetes (species 1 to 6, Table 2) or ascomycetes (species 1 to 4, Table 2), related to various ectomycorrhizal taxa (Table 3).

The four remaining ectomycorrhizae produced two amplification products with at least one of the primer sets (dual ecm 1 to 4, Table 2). Separate sequencing of the products suggested dual colonization, i.e. a sebacinoid and another fungus on the same root (Table 3), with exception of dual ecm 1 where the second amplification product was a mix of several other sequences (as proved by RFLP, data not shown) that were not further characterized.

As a final control, ectomycorrhizae from cores A1-01, A5-13, L1-01, L6-07 and O1-01 were amplified with various backward primers located within the 28S rDNA gene (see Materials and methods): all amplified sequences overlapped to 100% with those obtained using other primer sets. A sebacinoid fungus identical to the respective orchid symbiont was therefore found on 120 of 144 (83.3%) successfully typed ectomycorrhizae. Such ectomycorrhizae were present in all sampled cores (except P2-02, where all DNA extraction failed), together with some other ectomycorrhizal fungi (Table 3).

Fungal populations of four N. nidus-avis *root systems*

Amplification of the fungal IGS2 sequences from cores A2-04, A4-12, L6-07 and O1-01 often failed or gave unreproducible results, whereas the amplification of the IGS1 was successful on most (> 80%) samples, including ectomycorrhizae. The amplified pattern was monomorphic in cores A2-04 and L6-07 (data not shown) but polymorphic in cores A4-12, where three different patterns occurred (Fig. 5B), and O1-01 (Fig. 5C). In every case, the pattern amplified from the various fragments of a given root were all identical (not shown). Since the samples from core A4-12 also showed variations in the ITS regions (Fig. 5A), a total of four different patterns of ribosomal DNA were found in this core (Fig. 3A): one ribosomal type (*'ad'*) colonized half of the samples whereas the others occupied more restricted areas. Similarly, on core O1-01, pattern *'e'* was found on most samples (Fig. 3B). This suggests that several genotypes can occur on the orchid root system and intermingled ectomycorrhizae.

Discussion

N. nidus-avis *symbionts belong to the Sebacinaceae*

Based on a sampling of 61 root systems throughout France, we conclude that *N. nidus-avis* associates at adult stage with sebacinoid species (Fig. 4). As this study was submitted, McKendrick *et al.* (2002) reported similar fungal partners, using ITS and partial 28S rDNA sequencing, on *N. nidus-avis* found in two more northern forests, one in Germany and the other in the United Kingdom. This is in good agreement with previous ultrastructural analyses of *N. nidus-avis* roots, demonstrating that the fungal symbiont has dolipores with imperforate parenthesomes (Barmicheva 1989), the septal pore type found in *Sebacina*

(Wells & Bandoni 2001). Bernard (1899) also observed that various non-*Rhizoctonia* species could be found in germinating *N. nidus-avis*, but they were never seen on adult plants, nor in germinating *N. nidus-avis* investigated by McKendrick *et al*. (2002).

Among Sebacinaceae, *Sebacina vermifera sensu* Warcup & Talbot has been isolated from various orchids, either chlorophyllous (e.g. Warcup & Talbot 1967) or not, such as albino mutants of *Microtis rara* (Warcup 1988). A sebacinoid ITS sequence has also been amplified by D.L. Taylor from an American achlorophyllous orchid, *Hexalectris spicata* (McKendrick *et al*. 2002). Interestingly, *Hexalectris* and *Neottia* are only distantly related, belonging to the Arethuseae and Neottieae subfamilies, respectively (Dressler 1993; Cameron *et al*. 1999). Their symbiont relatedness could result either from a convergence driven by evolution to mycoheterotrophy, or recapitulate a plesiomorphic association of orchids with basal Hymenomycetes: this alternative suggests the need for further investigations on orchid associates and their analysis in the framework of orchids phylogeny.

Our observations, as well as the isolation of a *Thanatephorus* sp. from an Australian achlorophyllous orchid (Warcup 1985, 1991), show that species of the *Rhizoctonia* complex can support the myco-heterotrophic growth of achlorophyllous orchids. Moreover, the large set of orchid root systems analysed demonstrates that *N. nidus-avis* associated highly specifically with sebacinoid species, in spite of the various fungi potentially present in their rhizosphere (Table 3). No trace of other fungi were found, even by changing the primer set used for ITS amplification, suggesting that the additional, unidentified fungus detected by McKendrick *et al*. (2002) could be a contaminant, as supposed by the authors. Mycorrhizal specificity, already reported for some achlorophyllous orchids (Taylor & Bruns 1997 and 1999) and several achlorophyllous Ericales (Bidartondo & Bruns 2001), is thus confirmed for *N. nidus-avis*.

Unexpected ITS diversity among sebacinoid species

A total of 23 ITS sequences were distinguished among our samples (Table 1): sequencing of the ITS proved necessary, as the differences would not have been revealed by RFLP analysis due to low sequence divergence. Our phylogenetic analysis suggests that the *N. nidus-avis* symbionts belong to a subclade of the Sebacinaceae (Fig. 4) that also encompasses *Tremelloscypha gelatinosa* and a *Sebacina* species similar to *S. epigaea* growing on wood. Some ITS variations are limited to point mutations and probably represent intraspecific polymorphism, such as in core A4-12 (Fig. 5A): intraspecific variability of ITS was already described in some hymenomycetes (e.g. Aanen *et al*. 2001). The other larger variations, extending into the highly conserved 5.8S rDNA, probably relate to different species (Fig. 4). Ectomycorrhizae, from which various sebacinoid fungi were detected (Table 2), often had morphotypes differing slightly from one core to another as the ITS sequence differed (data not shown). Additional data on the 28S rDNA (Weiß & Selosse, unpublished data) also suggested that different species are present within our samples. Because a given ITS sequence was rarely found more than once among our populations, numerous other sebacinoid species are likely to associate with *N. nidus-avis*. Considering the small number of characters that can be used to distinguish species (Jülich 1983) and even to delimit genera (Wells & Oberwinkler 1982) in this well-founded monophyletic group (Weiß & Oberwinkler 2001), Sebacinaceae may be a larger taxon than thought previously, where many still undescribed species exist. In addition, the position of *Tremelloscypha gelatinosa* on Fig. 4 (consistently with the phylogenetic analysis on the 28S rDNA by Weiß & Oberwinkler 2001) shows that the genus *Sebacina*, as currently defined, is not monophyletic. More research focused on diversity and phylogeny of the Sebacinaceae that integrates morphological, anatomical and DNA data will therefore be necessary in the near future.

Patterns of fungal colonization at the population level

Populations often shared the same or very closely related fungi (Table 1 and Fig. 4), resulting in a limited symbiont diversity. Together with the limited core number due to the scarcity of the orchid, this limits the discussion of the effect of the environmental conditions on symbiont choice by the orchid. Adjacent populations sometimes exhibited identical or closely related sebacinoid ITS sequences, such as L2 and L3 or L6 and L1 (Table 1). Similarly, sebacinoid ITS sequences from Aveyron, Corsica and Paris, respectively, clustered together in phylogenetic analysis (Fig. 4), suggesting a trend to a regional specialization in fungal symbionts such as described for the achlorophyllous *Corallorhiza maculata* (Taylor & Bruns 1999). However, in one case, the same ITS sequence was found in two different sampling regions, namely Paris and the Lorraine region (populations P2, L2 and L3, Table 1) and, on the other hand, different symbionts were found within the same region, e.g. in Orry and Aveyron (Fig. 4), so this trend suffers exceptions. However, in contrast to *C. maculata*, most *N. nidus-avis* symbionts seem to be dependent neither on the altitude of sampling nor on the surrounding tree species: the same sebacinoid ITS sequence was found in populations L2, L3 and P2, where tree species differ strongly (Table 1), and different populations under the same tree species showed various symbionts (e.g. *Fagus sylvatica* in populations A1, C2, L6 and O2, Table 1). A sebacinoid related closely to that found in Auvergne (populations A1, A2 and A4, elevations: 620–1000 m)

occurs in Oise (population O1, elevation: 100 m) (Fig. 4). However, a better delineation of sebacinoid species than with rDNA is required before further discussing the symbiont choice.

Patterns of fungal colonization at the root system level

The pattern of orchid root colonization by the fungus was analysed using ITS and IGS polymorphism. Such a polymorphism was found in only two of four cores investigated (Figs 3 and 5) and showed the presence of several infecting genets. However, polymorphism was never found among the various pieces recovered from a single root. Assuming that sebacinoids have dicaryotic hyphae (Moore 1987), the amplified patterns of core A4-12 can be interpreted in different ways (Fig. 3A). One possibility is colonization by a single diploid genet, either homozygous (patterns *bb* and *cc*, see Fig. 3A) or heterozygous (patterns *ad* and *aa*) at the rDNA locus. The observed patterns could also arise by co-amplification of several genets colonizing the same root; in this case, one would expect one of the genets to be absent from some root portions: however, all portions of the investigated roots showed the same ribosomal pattern. The number of detected genets per orchid root is low, as the total number of alleles per root is low even in polymorphic populations, such as in core A4-12 (Fig. 5). Microscopic analysis of the surface of *N. nidus-avis* roots by many authors failed to detect any hyphal linkage with the soil (for review, see Leake 1994). Hyphal connections with the soil could be ephemeral, with multiple hyphal penetrations in the root: the pattern of colonization would then reflect the structure of the sebacinoid population in the *N. nidus-avis* rhizosphere: this would explain the almost congruent repartition of genets on surrounding ectomycorrhizae (Fig. 3). However, it is often suspected that the fungus enters via some epidermal cells in the oldest parts of the rhizome (Nieuwdorp 1972; Barmicheva 1989) and propagates internally during root growth (Nieuwdorp 1972). The number of founding hyphae penetrating each young root would thus be low, explaining that few genotypes (or a single one) are present. This contrasts strongly with the multiple colonizations and numerous hyphal linkages between soil and root reported in other endomycorrhizal types, such as the ericoid mycorrhizae (Monreal *et al*. 1999).

N. nidus-avis *is a mycorrhizal cheater*

How do the sebacinoids obtain carbon resources for the growth of *N. nidus-avis*? While not giving a definitive physiological evidence, our work demonstrates that a sebacinoid fungus of identical ITS fingerprint to the orchid symbiont was present on ectomycorrhizae collected in all orchid root systems sampled (but one that did not allow any typing: P2-02, Table 2). They account for *c.* 83% of all successfully typed ectomycorrhizae (i.e. 68.2% of the tips, Table 2). In some cases, identical IGS alleles were found in sebacinoids from neighbouring orchids roots and ectomycorrhizae (Fig. 3). The latter result suggests strongly that the same genets interact with the tree roots and the orchid, thus creating mycelial linkages between the two plants. In this way, the surrounding trees could provide the carbon resources necessary to *N. nidus-avis* growth.

Various works suggest that some sebacinoids are ectomycorrhizal: this was claimed in a brief report by Warcup (1988), who obtained *in vitro Sebacina* ectomycorrhizae on *Melaleuca uncinata* and Eucalypts (Myrtaceae) as well as on *Casuarina* and annual herbs. A separate study on site L1 (Selosse *et al*. 2002) showed that two sebacinoids, including the species found in population A1, formed true ectomycorrhizae with various host trees, and the same is currently suspected for various other sebacinoid species (A. Urban, personal communication; M. Gardes, personal communication). It can therefore be inferred that the roots from which a sebacinoid ITS was amplified were sebacinoid ectomycorrhizae. The absence of other fungi on those ectomycorrhizae is supported by the fact that we did not succeed in amplifying other fungal ITS using several primer combinations.

The infrequent dual ectomycorrhizae (< 3%; the six *Sebacina* ectomycorrhizae identified only by RFLP may also represent such cases, see Table 2) can be interpreted in two ways. First, it could result from a mycoparasitism of the sebacinoids. Some reports are consistent with this hypothesis: (i) some sebacinoids were isolated from ascomycete ectomycorrhizae (Warcup 1988) or vesicular–arbuscular mycorrhizae (Williams 1984) and (ii) some *Sebacina* species develop in the hymenium of various corticioid fungi (Oberwinkler 1964; Jülich 1983). So far, no anatomical data support the hypothesis of mycoparasitism in Sebacinaceae, and intrahymenial growth may simply indicate a suitable microhabitat, without involving parasitism (Oberwinkler 1964; Roberts 1993). Thus, we favour a second explanation: dual ectomycorrhizae (Table 3) have their various tips colonized independently by different fungi, as in our sampling design DNA is extracted from all tips of a given mycorrhiza (note that dual ectomycorrhizae 1, 3 and 4 have two to three tips, Table 2). Such a dual colonization on non-overlapping root areas was described by Selosse *et al*. (2002) for a sebacinoid species with a thelephoroid. A last explanation would be that sebacinoid species form ectomycorrhizae by replacing an ectomycorrhizal fungus that they outcompete: such substitutions of fungal symbionts occur in lichens, e.g. in the Teloschistaceae ('algal take over', Richardson 1999), and probably often in mycorrhizae.

N. nidus-avis is thus likely to derive its carbon from the tree via ectomycorrhizae, exactly as described for other achlorophyllous orchids (Taylor & Bruns 1997, 1999), as

well as for all other myco-heterotrophic plants studied to date, ranging from Ericaceae (Bidartondo & Bruns 2001) to achlorophyllous hepatics (Read *et al*. 2000). The *Rhizoctonia* complex, i.e. basal lineages of the hymenomycetes, can be involved in such relationships: a similar mechanism may account for the survival of albino mutants of *Microtis rara* associated with ectomycorrhizal *Sebacina vermifera* (Warcup 1988), as well as of the achlorophyllous orchid *Rhizantella gardneri* associated with *Thanatephorus gardneri*, also thought to be ectomycorrhizal (Warcup 1985 and 1991). The exact nature of the association between the fungus and the orchid is unclear: it could be a purely parasitic relationship. Alternatively, the fungus may increase its fitness, either by deriving some vitamins from the orchid, or even by enjoying a greater ectomycorrhizal success in the *N. nidus-avis* rhizosphere. The high frequency of sebacinoid ectomycorrhizae in the *N. nidus-avis* root systems (Table 2) may in fact be enhanced by the orchid, reminiscently of the stimulation of ectomycorrhiza development induced by some myco-heterotrophic Ericales (Bidartondo *et al*. 2000). This aspect of the tripartite association deserves further analysis.

Conclusion

Our analysis confirms, in *N. nidus-avis*, the general features of mycoheterotrophic orchids studied to date: (i) they associate specifically with closely related fungal species, belonging here to the Sebacinaceae; (ii) the same species are involved specifically in the germination (McKendrick *et al*. 2002); (iii) the fungus is also present on ectomycorrhizae growing around the orchid roots (Taylor & Bruns 1997), suggesting that (iiii) the achlorophyllous plant ultimately derives its carbon from the surrounding trees. Our study sheds light on the ecological importance of Sebacinaceae and, because they also associate with chlorophyllous orchids, this raises the possibility that even some green orchids could in part behave as mycorrhizal cheaters.

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