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Ectomycorrhizal *Inocybe* species associate with the mycoheterotrophic orchid *Epipogium aphyllum* but not its asexual propagules

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• *Background and Aims Epipogium aphyllum* is a Eurasian achlorophyllous, mycoheterotrophic forest orchid. Due to its rarity, it is often protected, and its biology is poorly known. The identity and pattern of colonization of fungal associates providing carbon to this orchid have not been studied previously.
• *Methods* Using samples from 34 individuals from 18 populations in Japan, Russia and France, the following were investigated: (*a*) colonization patterns of fungal associates of *E. aphyllum* by microscopy; (*b*) their identity by PCR amplification of nuclear ribosomal ITS carried out on rhizome fragments and hyphal pelotons.
• *Results and Conclusions* Microscopic investigations revealed that thick rhizomes were densely colonized by fungi bearing clamp-connections and dolipores, i.e. basidiomycetes. Molecular analysis identified *Inocybe* species as exclusive symbionts of 75 % of the plants investigated and, more rarely, other basidiomycetes (*Hebeloma*, *Xerocomus*, *Lactarius*, *Thelephora* species). Additionally, ascomycetes, probably endophytes or parasites, were sometimes present. Although *E. aphyllum* associates with diverse species from *Inocybe* subgenera *Mallocybe* and *Inocybe sensu stricto*, no evidence for cryptic speciation in *E. aphyllum* was found. Since basidiomycetes colonizing the orchid are ectomycorrhizal, surrounding trees are probably the ultimate carbon source. Accordingly, in one population, ectomycorrhizae sampled around an individual orchid revealed the same fungus on 11·2 % of tree roots investigated. Conversely, long, thin stolons bearing bulbils indicated active asexual multiplication, but these propagules were not colonized by fungi. These findings are discussed in the framework of ecology and evolution of mycoheterotrophy.

Key words: Asexual multiplication, ectomycorrhizae, *Epipogium*, *Inocybe*, mycoheterotrophy, orchid mycorrhizae, specificity, symbiont transmission.

INTRODUCTION

Orchids depend on their fungal symbionts at germination since their seeds are devoid of food reserves. Soil fungi that colonize orchid seeds provide carbon and mineral resources and allow their development into a heterotrophic, underground protocorm. These fungal symbionts usually belong to a few unrelated basidiomycete taxa collectively called 'rhizoctonias' (mostly Ceratobasidiaceae, Tulasnellaceae and some Sebacinales; Rasmussen, 2002). Adult orchids are often autotrophic and still harbour fungi in their roots, forming typical mycorrhizal associations (Smith and Read, 1997) and perhaps reversing the carbon flow toward fungi (Cameron *et al.*, 2006). However, during orchid evolution, photosynthesis was lost >20 times (Molvray *et al.*, 2000), and non-green, 'mycoheterotrophic' (MH) orchids receiving carbon from their fungal symbionts at adult stage have evolved convergently (Leake, 1994, 2004). Recent research on MH orchids has used molecular techniques to identify fungal symbionts that are often unculturable and thus unidentifiable from their morphology *in vitro* (Taylor *et al.*, 2002; Dearnaley, 2007). MH fungal symbionts turned out to differ from the usual rhizoctonias, both at taxonomic and ecological levels.

Most MH orchids associate with basidiomycetes that also form so-called ectomycorrhizae (ECM) on roots of trees and shrubs (Smith and Read, 1997). Russulaceae were found in *Corallorhiza maculata* (Taylor and Bruns, 1997, 1999), *C. mertensiana* (Taylor and Bruns, 1999), *Limodorum* species (Girlanda *et al.*, 2006), *Dipodium variegatum* (Bougoure and Dearnaley, 2005) and *D. hamiltonianum* (Dearnaley and Le Brocque, 2006). Thelephoraceae occurred in *Cephalanthera austinae* (Taylor and Bruns, 1997), *Corallorhiza trifida* (Taylor and Bruns, 1997; McKendrick *et al.*, 2000) and *C. striata* (Taylor *et al.*, 2002). Sebacinales were found in *Neottia nidus-avis* (McKendrick *et al.*, 2002; Selosse *et al.*, 2002*b*) and *Hexalectris spicata* (Taylor *et al.*, 2003); although rhizoctonias encompass some Sebacinales, the species from MH orchids belonged to a different clade

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with ECM abilities (clade A in Weiss *et al.*, 2004). Using ^{14}C-labelling, McKendrick *et al.* (2000) demonstrated that the shared fungus provided MH orchids with photosynthates from host trees of the fungi. Some rhizoctonias belonging to Ceratobasidiaceae also associate with the MH *Rhizanthella gardneri* (Warcup, 1991) and *Chamaegastrodia sikokiana* (Yagame *et al.*, 2008); in both cases, the isolated fungi were able to form ECM *in vitro*, and these orchids probably also depend on photosynthates from the trees.

However, there are also some reports of saprobic basidiomycetes in MH orchids, such as *Armillaria* species in *Galeola septentrionalis* (Cha and Igarashi, 1996) and *Gastrodia elata* (Lan *et al.*, 1994) or *Erythromyces* species in *Galeola altissima* (Umata *et al.*, 1995) and *Erythrorchis cassythoides* (Dearnaley, 2006). In fact, *Armillaria mellea* colonizes and induces growth of seedlings of *Galeola septentrionalis* (Terashita, 1985), and *Erythromyces crocicreas* induces germination of *Galeola altissima* (Umata, 1995). Campbell (1970) isolated several saprobic fungi from MH orchids. However, these saprobic fungi were identified after *in vitro* isolation, and saprobic contaminants may have overgrown the true mycorrhizal fungi that are often slow growing or unculturable (Taylor *et al.*, 2002). Indeed, fungi isolated by Campbell (1970) were not confirmed by recent molecular studies. Molecular methods are also sensitive to contamination by DNA from endophytic fungi or spores, so that direct observations of fungi (e.g. by electron microscopy; Selosse *et al.*, 2004) or functional tests (such as seed germination using the isolated fungus) are necessary to corroborate the identity of mycorrhizal fungi.

Recently, fungi belonging to Coprinaceae, a group of saprobic basidiomycetes, were found in the Asian MH *Epipogium roseum* (Yamato *et al.*, 2005) and *Eulophia zollingeri* (Ogura-Tsujita and Yukawa, 2008). Appropriately, *E. roseum* grows near tree stumps and fallen logs, and the fungus isolated from *E. roseum* allowed *in vitro* seed germination and development up to flowering stage (Yagame *et al.*, 2007), therefore fulfilling one of the criteria defining a symbiotic fungus. *Epipogium* occurs throughout Eurasia and Africa (Pridgeon *et al.*, 2005) and may thus encompass overlooked associations with saprobic fungi. Alternatively, the other *Epipogium* species associate with ECM fungi, in which case *Epipogium* would illustrate a hitherto unknown variability in ecology of fungal partners.

This study focuses on *E. aphyllum*, which occurs from Europe to Asia (Maekawa, 1971; Rasmussen, 1995). Questions raised by previous studies on *E. roseum* are addressed: (*a*) What are the taxonomic position and ecology of *E. aphyllum* symbionts? (*b*) How much variation is there in symbionts over the range of the species? *Epipogium aphyllum* has a complex vegetative morphology, forming two kinds of rhizomes (Fig. 1A): thick, highly branched rhizomes (the so-called 'coralloid rhizomes') and thin stolons, up to 0·5 m long (Irmisch, 1853; Ziegenspeck, 1936). The latter presumably contribute to asexual reproduction. Although root- or rhizome-sprouting is common among MH plants (Leake, 1994) and sometimes includes transmission of the fungal symbiont (Domínguez *et al.*, 2006), the presence of fungi in propagules has rarely been assessed (Klimešová *et al.*, 2007). Therefore it is also questioned here (*c*) whether the anatomy of hypogeous plant parts has an impact on fungal colonization strategies and (*d*) how transmission to asexual offspring occurs. These data are of relevance to future *in situ* or *ex situ* conservation activities, especially because this species is rare over its range. For example, it has been considered extinct in the United Kingdom since 1987 (Harrap and Harrap, 2005; Kull and Hitchings, 2006) and is highly protected in France (Danton and Baffray, 2005); in Japan, it is listed as an endangered species in the Red Data Book (Environment Agency of Japan, 2000).

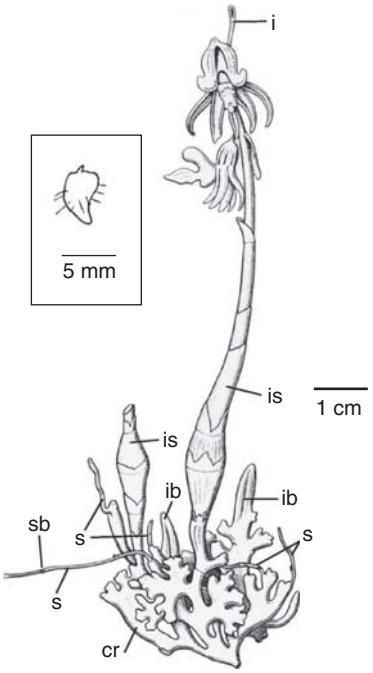

FIG. 1. Morphology of *E. aphyllum* (redrawn from Irmisch, 1853) and rhizoid-bearing protocorm (inset). cr, Coralloid rhizome; i, inflorescence; ib, inflorescence bud; is, inflorescence shoot; s, thin stolon; sb, bulbil on a stolon.

MATERIALS AND METHODS

Rhizome and surrounding ECM sampling

Rhizomes were sampled in August 2005 in three countries where official authorizations were obtained (France, Russia and Japan), using a protocol that allows plant survival (data not shown). One to ten independent rhizome fragments were harvested by digging about 20 cm away from shoots and then carefully approaching underground parts of the plant from one side; after sampling, the hole was refilled with the same soil. Up to three coralloid rhizome fragments ('cr' on Fig. 1A) and, when available, thin stolons ('s' on Fig. 1A) were collected from one to three plants per population (Table 1). In a large French population at Saint Clément (Cantal), with >300 shoots, two full plants (EM12 and EM15; Table 1) situated at 150 m from each other were recovered, and all ECM tips of surrounding trees found <15 cm from each of these plants were harvested (in all, $71 + 46$ ECM tips, respectively). In the same population, rhizome fragments were sampled from seven plants to address intrapopulation diversity. Samples were carefully washed with water to

TABLE 1. *Identification of fungal sequences retrieved from the investigated* E. aphyllum *individuals, classified by origin*

Region	Latitude/ longitude	Individual*	Genbank accession number	Gene sequenced[†]	Possible affiliation[‡]	Fungal ecology[§]	Closest sequence(s) found in GenBank by BLAST: name (accession no.)	*E* value[¶]	Maximum identity (%)
Lans en Vercors, Alpes	45°07′47″N 05°35′20″E	EM5	EU711167	28S c^2	*Olpidium*	P	*Olpidium brassicae* (DQ273818)	$8e^{-112}$	95
			EU711168	ITS c^4	*Olpidium*	P	*Olpidium bornovanus* (AB205215)	$4e^{-71}$	100
			EU711169	ITS c^2	*Thelephora*	ECM	Uncultured Thelephoraceae (AY634145)	0	99
			EU711170	ITS c^2	*Inocybe*	ECM	*Inocybe cf. geophylla* (AM882984)	0	98
		EM6	EU711171	ITS + 28S d^1	*Inocybe*	ECM	*Inocybe pudica* (AY228341)	0	87
			EU711172	ITS d^1	*Inocybe*	ECM	*Inocybe cf. geophylla* (AM882984)	0	100
Villard de Lans, Alpes	45°04′11″N 05°33″01″E	EM7	EU711173	ITS d^1	*Inocybe*	ECM	*Inocybe terrigena* (AM882864)	0	99
Luz Saint Sauveur, Pyrénées	42°51′54″N 0°01′13″W	EM1	EU711163	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	96
Thuès-entre-Valls, Pyrénées Orientales	42°30′57″N 2°15′9″E	EM8	EU711174	28S c^2	*Inocybe*	ECM	*Inocybe griseolilacina* (AY380378)	0	98
			EU711175	ITS c^1	*Inocybe*	ECM	*Inocybe cf. glabripes* (AJ889952)	0	90
	42° 30′30″N 2°15′27″ E	EM9	EU711177	ITS + 28S c^1	*Protoventuria*	P	*Protoventuria alpina* (EU035444)	0	96
			EU711176	ITS + 28S c^1	*Inocybe*	ECM	*Inocybe nitidiuscula* (AM882912)	0	85
	42°30′18″N 2°15′29″ E	EM23	EU711209	ITS + 28S d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
			EU711210	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	97
		EM24	EU711211	ITS + 28S d^2	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	100
Cazaux, Pyrénées	43°03′08″N 01°30′35″E	EM17	EU711189	ITS + 28S d^4	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
			EU711190	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	$4e^{-94}$	89
			EU711187	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
			EU711188	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	96
		EM18	EU722336	ITS + 28S d^3	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
Oô, Pyrénées	42°47′49′N 0°30′21″E	EM20	EU711191	ITS + 28S d^1	*Inocybe*	ECM	*Inocybe flocculosa* (AY228354)	0	89
			EU711199	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711200	ITS d^1	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	0	91
			EU711201	ITS d^1	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	0	94
			EU711192	ITS d^1	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	0	91
			EU711193	ITS d^1	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	$2e^{-165}$	89
			EU711194	ITS d^1	*Inocybe*	ECM	*Inocybe nitidiuscula* (AM882912)	$2e^{-124}$	88
			EU711195	ITS d^1	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99

Continued

TABLE 1. *Continued*

Region	Latitude/ longitude	Individual*	Genbank accession number	Gene sequenced†	Possible affiliation‡	Fungal ecology§	Closest sequence(s) found in GenBank by BLAST: name (accession no.)	*E* value¶	Maximum identity (%)
			EU711196	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	100
			EU711197	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711198	ITS + 28S d[1] + c[2]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
Saint Clément, Cantal	44°55′28″N 02°39′41″E	EM2	EU711164	ITS d[1]	*Inocybe*	ECM	*Inocybe dulcamara* (AM882863)	0	96
		EM10	EU711178	28S c[1]	*Neonectria*	E	*Neonectria radicicola* (AY283552)	0	98
			EU711179	28S c[1]	*Protoventuria*	P	*Protoventuria alpina* (EU035444)	0	98
		EM11	EU711180	ITS c[1]	*Metarhizium*	SS	*Metarhizium anisopliae* (AB027383)	0	90
			EU711181	ITS c[1]	*Paecilomyces*	SS	*Paecilomyces carneus* (AB258369)	0	100
		EM12	EU711182	ITS d[1]	*Inocybe*	ECM	*Inocybe dulcamara* (AM882863)	0	99
		EM15	EU711184	ITS d[1]	*Inocybe*	ECM	*Inocybe lanuginosa* (EU525948)	$1e^{-138}$	84
		EM16	EU711185	ITS c[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711186	ITS + 28S c[1]	*Didymella*	P	*Didymella bryoniae* (AB266850)	0	84
Saint Paul de Salers, Cantal	45°08′25″N 02°31′04″E	EM22	EU711202	ITS + 28S c[1]	*Inocybe*	ECM	*Inocybe whitei* (EU486441)	0	86
			EU711203	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	100
			EU711204	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711205	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711206	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	$6e^{-151}$	87
			EU711207	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711208	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	100
Picherande, Puy de Dôme	45°27′50″N 02°46′09″E	EM26	EU711217	ITS + 28S d[1]	*Inocybe*	ECM	*Inocybe whitei* (EU486441)	0	88
			EU711216	ITS d[1]	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	0	92
			EU711213	ITS d[1] + c[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711214	ITS d[1] + c[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711215	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	100
			EU711218	ITS c[1]	*Neonectria*	E	*Neonectria radicicola* (AJ875331)	0	99
		EM27	EU711219	ITS + 28S d[1]	*Inocybe*	ECM	*Inocybe armeniaca* (DQ974803)	0	88
			EU711220	ITS d[1]	*Inocybe*	ECM	*Inocybe rufuloides* (DQ067579)	0	90
			EU711221	ITS c[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
		EM28	EU711222	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	$7e^{-124}$	92

Continued

TABLE 1. *Continued*

Region	Latitude/ longitude	Individual*	Genbank accession number	Gene sequenced†	Possible affiliation‡	Fungal ecology§	Closest sequence(s) found in GenBank by BLAST: name (accession no.)	*E* value¶	Maximum identity (%)
			EU711223	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
			EU711224	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	96
			EU711225	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	97
			EU711226	ITS d[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
Ponteils, Cévennes	44°53′31″N 03°57′32″E	EM3	EU711165	ITS d[1]	*Lactarius*	ECM	*Lactarius scrobiculatus* (EF530942)	0	93
		EM4	EU711166	ITS d[1]	*Inocybe*	ECM	*Inocybe geophylla* (AM882870)	0	96
RUSSIA									
Arshan, Byryatia Ural	51°54′22″N 102°26′02″E	EM14	EU711183	ITS d[1]	*Inocybe*	ECM	*Inocybe lanuginosa* (EU525948)	$1e^{-169}$	85
Murzinka, Lac Baïkal	51°37′51″N 82°32′40″E	EM29	EU711227	28S d[4]	*Inocybe*	ECM	*Inocybe dulcamara* (AY038315)	0	99
JAPAN									
Nagano prefecture	35°58′20″N 138°18′26″E	NA1	EU711233	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	98
			EU711234	ITS p[2]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
		NA2	EU711235	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
	35°58′26″N 138°18′40″E	NB1	EU711236	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711237	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
			EU711238	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
	35°58′20″N 138°20″9″	NC1	EU711238	ITS p[1]	*Inocybe*	ECM	*Inocybe fuscidula* (AM882888)	0	99
Yamanashi prefecture	35°24′01″N 138°41′14″E	YA1	EU711239	ITS p[1]	*Hebeloma*	ECM	*Hebeloma velutipes* (AF430254)	0	99
		YA2	EU711240	ITS p[1]	*Hebeloma*	ECM	*Hebeloma velutipes* (AF430254)	0	99
		YA3	EU711241	ITS p[1]	*Inocybe*	ECM	*Inocybe subnudipes* (AM882809)	0	97
			EU711242	ITS p[1]	*Inocybe*	ECM	*Inocybe subnudipes* (AM882809)	0	97
			EU711243	ITS p[1]	*Inocybe*	ECM	*Inocybe subnudipes* (AM882809)	0	97

*The individuals used in the amplification of plant ITS are underlined.

†Abbreviations: c, from a clone; d, from direct sequencing of a rhizome; p, from direct sequencing of a pool of 20 pelotons. Superscript numbers indicate the number of times a clone or a direct sequence was obtained.

‡Only the closest taxonomically informative accession is reported.

§Putative ecology: ECM, ectomycorrhizal; P, parasite; E, plant endophyte; SS, soil saprobe.

¶The BLAST expected value represents the number of sequence matches expected by random chance (the smaller the value, the better the match between our sample sequences and those in the NCBI database).

eliminate all soil particles. All samples were stored in ethanol/water (3/2, v/v) for transport, and in some populations (Table 1) sub-samples of coralloid rhizome fragments were preserved for microscopic investigations by quick fixation in 2·5 % (v/v) glutaraldehyde in a 0·1 M cacodylate buffer (pH 7·2) for 2 h at room temperature and then overnight at 4 °C. For the seven plants from Japan, the sampling was performed by collecting one large rhizome fragment in each population and isolating fungal pelotons (= intracellular hyphal coils) as in Warcup and Talbot (1967), with modifications as follows. Surfaces of rhizomes were sterilized by immersion in 70 % ethanol for 30 s and sodium hypochlorite solution containing 1 % available chlorine for 30 s. The rhizome was rinsed and cut with a sterilized scalpel into three pieces, each placed in 5 mL of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse the intracellular hyphal pelotons. Twenty fungal pelotons per rhizome piece were harvested and pooled.

Molecular investigations

DNA extraction and PCR amplification of fungal internal transcribed spacers of nuclear ribosomal DNA (ITS) were performed as in Selosse *et al.* (2002*b*) using primers ITS1F and ITS4 on coralloid rhizome fragments and ECM tips. ITS fragments amplified from coralloid rhizomes were directly sequenced as in Selosse *et al.* (2002b), except for the Japanese samples that were handled as in Yamato and Iwase (2008). Whenever direct sequencing was not possible, PCR products were cloned as in Selosse *et al.* (2004), and at least six clones per plant were sequenced. For ECM tips, length polymorphism of ITS was investigated in comparison to the fungal ITS amplified from the nearby orchid, before and after enzymatic digestion (RFLP using *Eco*RI + *Sac*I and *Hin*dIII). RFLP were carried out as in Selosse *et al.* (2002*a*), and only ITS fragments identical in length and RFLP patterns to those from the nearby orchid were sequenced. Further, to ensure absence of usual rhizoctonia orchid symbionts with highly derived rDNA sequences, i.e. tulasnelloid and sebacinoid basidiomycetes, additional PCR amplifications were carried out on coralloid rhizome fragments using specific primers (ITS4tul for Tulasnellaceae and ITS3S for Sebacinales, as in Selosse *et al.*, 2004) and positive controls. Whenever ITS typing failed at the PCR step, sequence was tentatively obtained by amplifying the 28S rDNA using the primers Lr0r and Lr5 (Vilgalys and Hester, 1990). To ensure relatedness of the *E. aphyllum* plants used relative to *E. roseum*, plant nuclear ribosomal ITS sequences were amplified from four populations (Table 1) using the plant-specific primer ITS1P as in Selosse *et al.* (2002*a*). An *E. roseum* ITS sequence was obtained from a plant collected in Java for the Museum National d'Histoire Naturelle of Paris (voucher: Frank, C.W. 729). Sequencing was carried out on an ABI PRISM 3130 XL Genetic analyser (Applied Biosystems, Foster City, CA, USA), using the PCR primers, and sequences from both strands were edited using Sequencher^{TM} 4.6 for MacOS X from Genes Codes (Ann Arbor, MI, USA). Edited sequences (or consensus sequences for similar clones) were deposited in GenBank.

Microscope investigations

After rinsing with fixing buffer (see above), four subsamples of coralloid rhizome fragments were dehydrated in an ascending series of ethanol solutions to 100 %, incubated in two changes of absolute acetone and infiltrated with Epon-Araldite resin (Hoch, 1986). The resin was polymerized for 24 h at 60°C. Embedded samples were processed for ultramicrotomy: semi-thin sections (0·5 μm) were stained with 1 % toluidine blue and ultra-thin (70 nm) sections were counterstained with uranyl acetate and lead citrate (Reynolds, 1963). These were used for TEM analyses under a Philips CM10 transmission electron microscope. Stolon samples were embedded in paraffin before sectioning. Manual transverse sections (10 μm) were cut with a microtome, differentially stained with a mixture of safranin O and fast green FCF (Bryan, 1955), rinsed with distilled water and observed with a light microscope. To look for fungal colonization of stolons, thin sections and gently crushed stolons were stained with Trypan Blue (Koske and Gemma, 1989).

Fungal identification and phylogenetic analyses

A search for similar sequences was conducted with Blast in GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). To confirm the phylogenetic position of the fungal symbionts related to the genus *Inocybe*, the 5' part of the 28S rDNA was sequenced using primers ITS1F and TW13 as in Selosse *et al.* (2002*b*). The sequences were aligned with selected *Inocybe* sequences from GenBank; *Cortinarius odoratus* (DQ663360), *Galerina autumnalis* (AY281020), *Hebeloma velutipes* (AY818351), *H. leucosarx* (AB211268), *H. pusillum* (AB211274) and *H. mesophaeum* (AB327182) were used as outgroups. Similarly, to investigate plant relationships, sequences of *Gastrodia elata* (EF090607), *Nervilia shiriensis* (AF521066), *Cranichis revoluta* (AF391786) and *Orchis militaris* (AY699977) were downloaded from Genbank as outgroups. After alignment using Bioedit v7.3.0 (Hall, 1999) and ClustalW (Thompson, 1994), the result was checked by eye and corrected manually with Bioedit. A bootstrapped neighbor-joining analysis (Saitou and Nei, 1987) was performed with PAUP 4.0 (Phylogenetic Analysis Using Parsimony, version 4.0; Swofford, 2004). Genetic distances were estimated by maximum likelihood using a general time-reversible model (Lanave *et al.*, 1984; Rodriquez *et al.*, 1990), involving unequal base frequencies and six types of substitution. This model of DNA substitution was chosen using a series of hierarchical likelihood-ratio test in Modeltest 3.7 (Posada and Crandall, 1998). Base frequencies were estimated before running the analysis, and 1000 bootstrap replicates were performed. Percentages of sequence identity between fungal ITS sequences were measured with Bioedit and compared with geographical distances and elevations by a Mantel test (using XLstat; Addinsoft, Paris, France) to test whether these two factors were correlated to any differences in fungal associates.

RESULTS

Conspecificity of the sampled orchids

The four populations selected over the range produced ITS sequences (EU711228 to EU711231) that diverged among populations by a maximum of 8·5 %. The four resulting sequences clustered together (100 % bootstrap) as a sister group to *E. roseum* (EU711232) with 80 % support (Fig. 2), supporting the coherence of the investigated taxa in France, Russia and Japan.

Fungal colonization

The below-ground portion of *E. aphyllum* is composed of plagiotropic coralloid rhizomes (Fig. 3A) that at some point become either an ascending inflorescence or a thin stolon (Fig. 3B). Inflorescence buds were filled with starch (not shown). These coralloid rhizomes proved to be densely colonized by fungi, with the exception of the meristematic zone at their apex, characterized by a whitish colour (Fig. 3A). Transverse sections showed that the outer cell layers were usually not colonized (Fig. 3C). Some isolated hyphae running from soil to the more internal cortical cells were

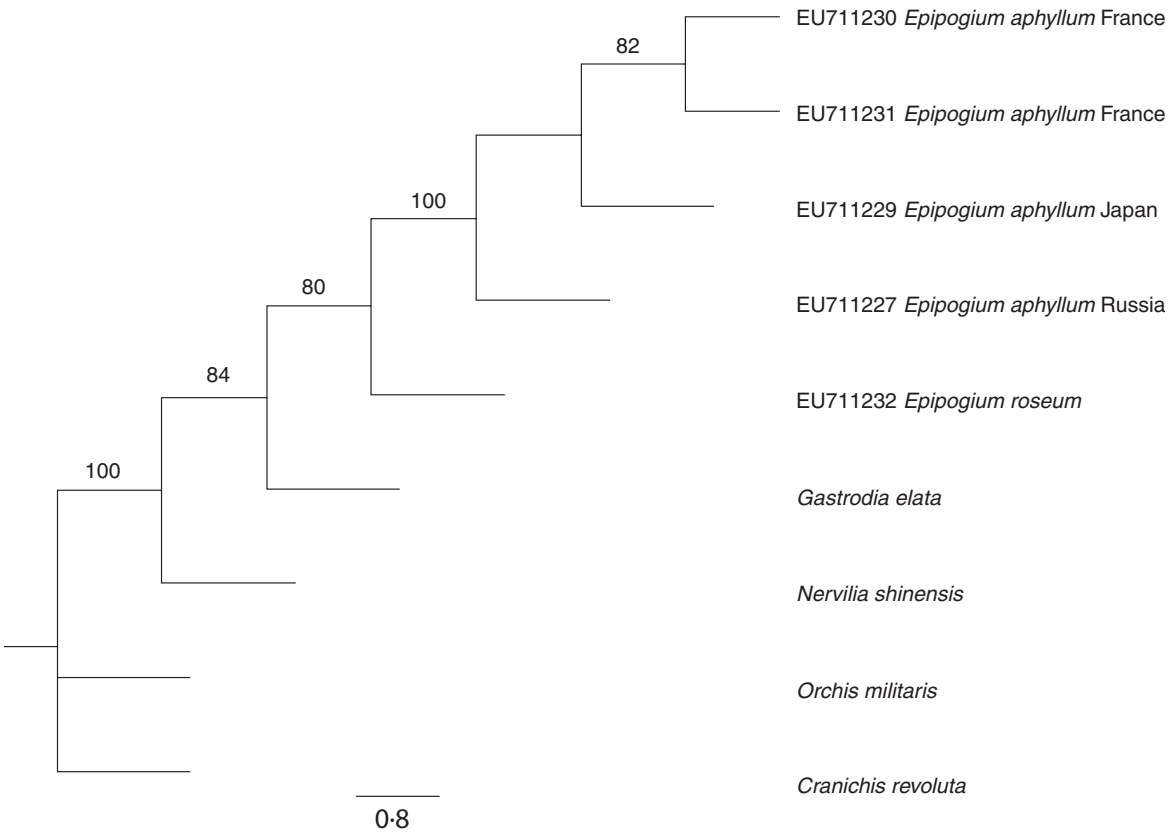

FIG. 2. Clustering of *E. aphyllum* and *E. roseum* individuals with respect to some other genera of Epidendroideae; *Cranichis revoluta* and *Orchis militaris* (Orchidoideae) are outgroups (ML on an alignment of the ITS; GTR model, 1000 bootstrap; only bootstraps >80 % are shown).

occasionally observed (not shown), but their route of penetration could not be followed. The vascular bundle and the air-filled intercellular spaces (Fig. 3C, D) were not infected. The inner cortical cells were filled with pelotons that were often elongated in one direction; these push the enlarged cell nucleus towards the periphery (Fig. 3C, D). Direction of elongation varied from one cell to another, allowing some linear hyphae to be longitudinally cut (Fig. 3D and G). TEM investigations confirmed that hyphae occurred in living cells with intact organelles but without starch (not shown). Hyphae were surrounded by the host plasma membrane (arrowed in Fig. 3E, G). They consistently showed dolipores between cells (Fig. 3E, F) with surrounding perforate reticulum cisternae (the so-called parenthesome; Fig. 3F); clamp connections were also seen (Fig. 3G). On the bases of these cytological features, the fungus is confirmed as a basidiomycete.

Stolons reached up to 50 cm and produced axillary bulbils every 2–3 cm (Figs 3B and 4A, B) more or less deeply protected by a sheathing, scaly leaf. This structure is loose and fragile, and separation from the mother plant occurred easily with disturbance. The oldest bulbils were covered with rhizoids (Fig. 4C). Sections illustrated the contrasting features of cortical cells of stolons (elongated, often empty, separated by air-filled intercellular spaces) and those of bulbils (densely filled with starch and stacked together, Fig. 4D–F). Xylem was poorly differentiated in vascular bundles (not shown). In bulbils, cell nuclei were central, and smaller cells formed an apical meristem (Fig. 4F, G). No fungal colonization was seen in bulbils and stolons after staining with Trypan Blue ($n = 14$ stolons and $n = 33$ bulbils, from $n = 7$ plants; not shown).

Identification of E. aphyllum *mycorrhizal fungi*

From a total of 34 plants in 18 populations (Table 1), 146 coralloid rhizome fragments and 21 peloton pools were investigated. Primers specific for Tulasnellaceae and Sebacinales ITS never produced any amplicon, whereas the general primer pair (ITS1F and ITS4) and/or 28S rDNA primers successfully amplified DNA from 128 plants. Direct sequences were obtained for rhizome fragments from 21 plants and 13 peloton pools from Japanese plants. Among these 79 sequences, 65 (82 %, from 27 plants) were related to *Inocybe* sequences in GenBank (Table 1). Two plants from one Japanese population exhibited a fungus related to *Hebeloma* (Fig. 5), whereas *Xerocomus* and *Lactarius* species were found once each in a plant from two French populations. No fungal fragment was amplified from bulbils or stolons ($n = 24$ and $n = 12$, respectively, from $n = 12$ plants).

To investigate fungal diversity in rhizome fragments for which ITS was not directly sequenced, 12 ITS amplification products were cloned. Ten of these revealed one to four *Inocybe*-related sequences, sometimes associated with

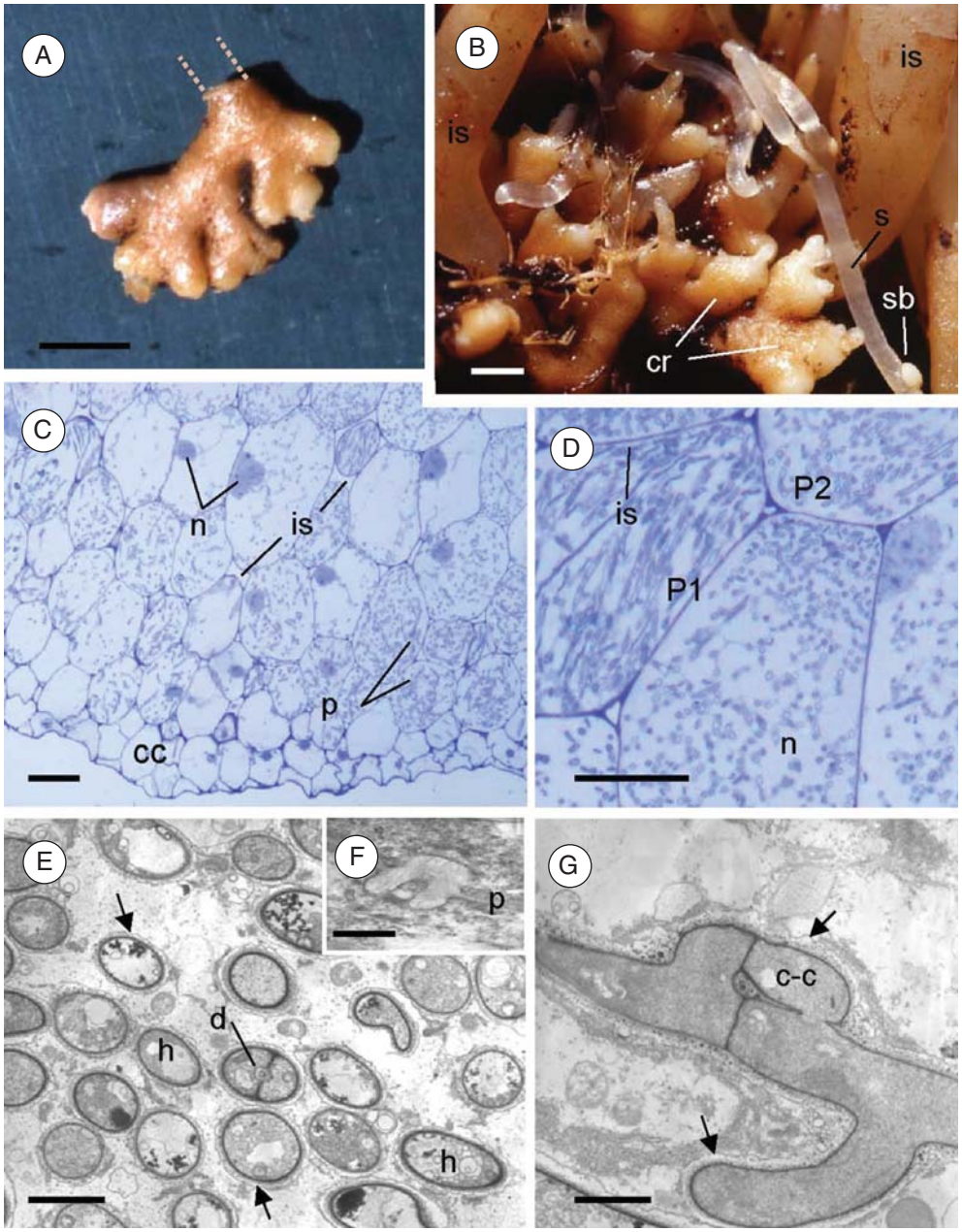

FIG. 3. Morphology and fungal colonization of coralloid rhizomes: (A) fragment of a coralloid dichotomously branching rhizome; (B) close-up of the underground parts with bases of inflorescence shoots (is), the coralloid rhizome (cr) sometimes giving rise to stolon(s) bearing bulbils (sb); (C) section at low magnification with pelotons (p) in the internal cell layers, and absence of hyphae in cortical cells (cc) (is, intercellular spaces; n, orchid cell nucleus); (D) magnification detail of infected cells with view of elongated fungal pelotons, cut transversally in P1 and longitudinally in P2 (C and D optical microscopy stained with Trypan Blue); (E) TEM of hyphae (h) in a peloton cut transversally (as in P1), with a dolipore (d) in one hypha; (F) TEM view of a dolipore with parenthesome (p) in a fungal hypha; (G) TEM detail of a cell with longitudinal cut of the peloton (as in P2), showing a clamp connection (c-c). Note the host membrane surrounding the hyphae (arrows in E and G). Scale bars: (A, B) = 0·5 cm; (C, D) = 50 μm; (E) = 2 μm; (F) = 1 μm; (G) = 2 μm.

sequences of ascomycetes, more rarely zygomycetes or even a basidiomycete (*Thelephora*, Table 1). With the exception of *Thelephora*, an ECM genus, these additional fungi were either soil saprobes (*Paecilomyces* and *Metarhizium* species) or parasites and possible endophytes (*Neonectria*, *Didymella*, *Olpidium* and *Protoventuria* species; see Table 1). Two produced only ascomycetes (EM10 and EM11 from Saint Clément; Table 1).

Inocybe occurred exclusively in 75 % of plants; it was thus the most abundant fungus across the range and at the plant scale (detected in 78 % of root fragments per plant on average). Identical *Inocybe*-related ITS sequences (or >97 % similar, when considering cloned sequences) were most often retrieved from the same plant (e.g. two fragments produced the same ITS in NA1; Table 1). At the population level, only one sequence was shared between two plants

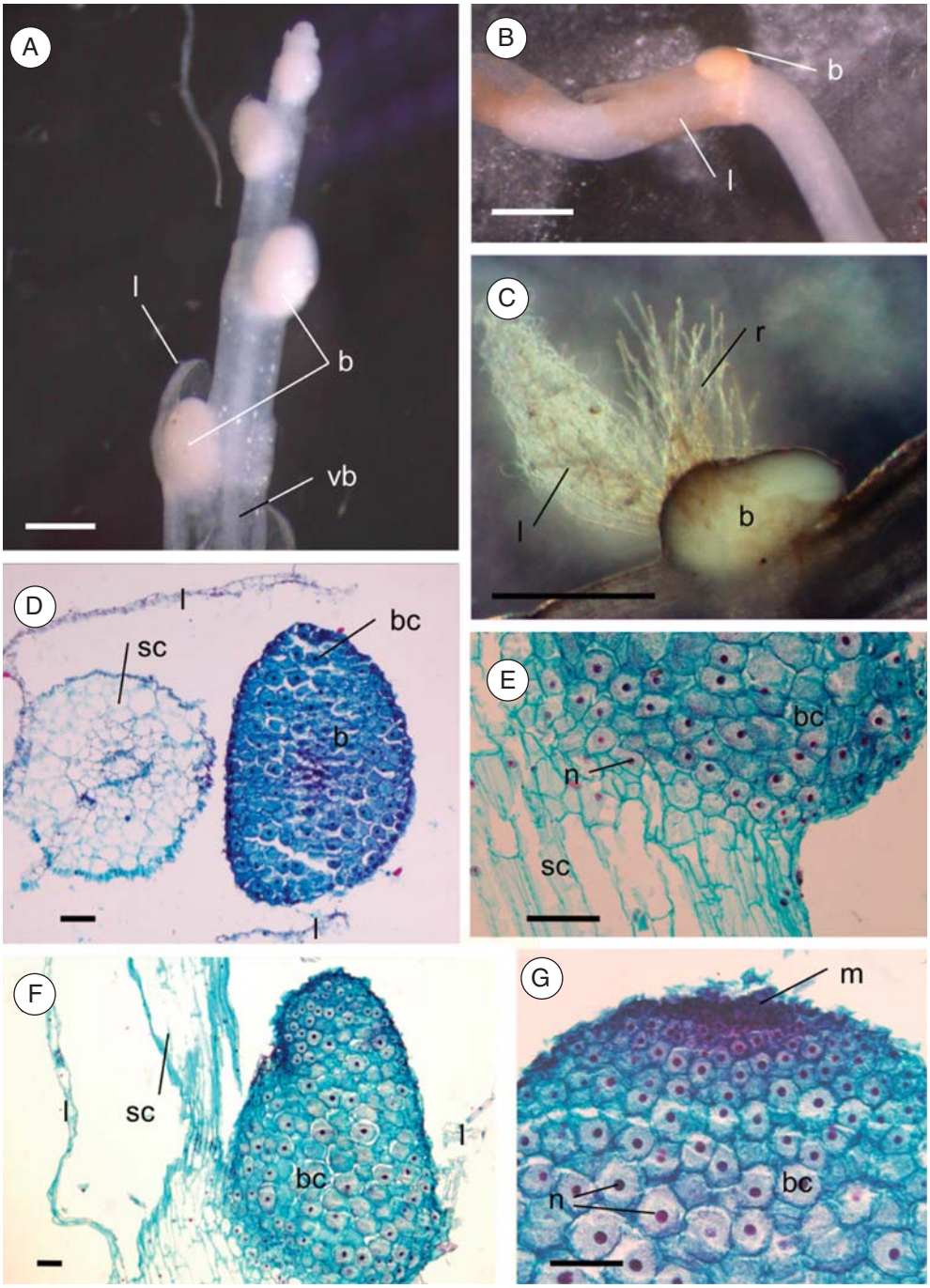

FIG. 4. Morphology and absence of fungal colonization of stolons and lateral bulbils (b): (A) apex of a stolon with bulbils and scaly sheathing leaf (l) and vascular bundle (vb) in transparent stolon tissues; (B) detail of the sheathing leaf protecting a bulbil; (C) sheathing leaf pushed aside to show rhizoids (r) covering a bulbil; (D) transverse section of a stolon with large, empty cells and its lateral bulbil with starch-filled cells (bc); (E) contact between stolon and bulbil (n, cell nucleus); (F) longitudinal section showing the smaller cells in bulbils; (G) detailed view of the meristematic zone (m) at the bulbil apex. (D–G) Optical microscopy stained with safranin O and fast green FCF. Scale bars: (A–C) = 1 mm; (D–G) = 100 μm.

(NB1 and NC1). Conversely, divergent sequences were often found in single plants (up to 11 sequences in EM20; Table 1).

In an analysis of *Inocybe*, the various sequences retrieved clustered into seven well-supported clades (Fig. 5), suggesting that *E. aphyllum* is not highly specific at the intrageneric level. Five clades clustered with identified species (two-thirds of the sequences clustered with *I. fuscidula*, others with *I. subnudipes*, *I. glabripes*, *I. dulcamara* and *I. terrigena*), whereas clades II and V remained unidentified. Sequences obtained from the same plant often clustered together; in three plants (EM20, EM26 and EM27), sequences clustered in the closely related clades I and II (Fig. 5). In well-sampled populations, such as at Saint Clément or Thuès-entre-Valls, sequences from different clades were retrieved (Fig. 5),

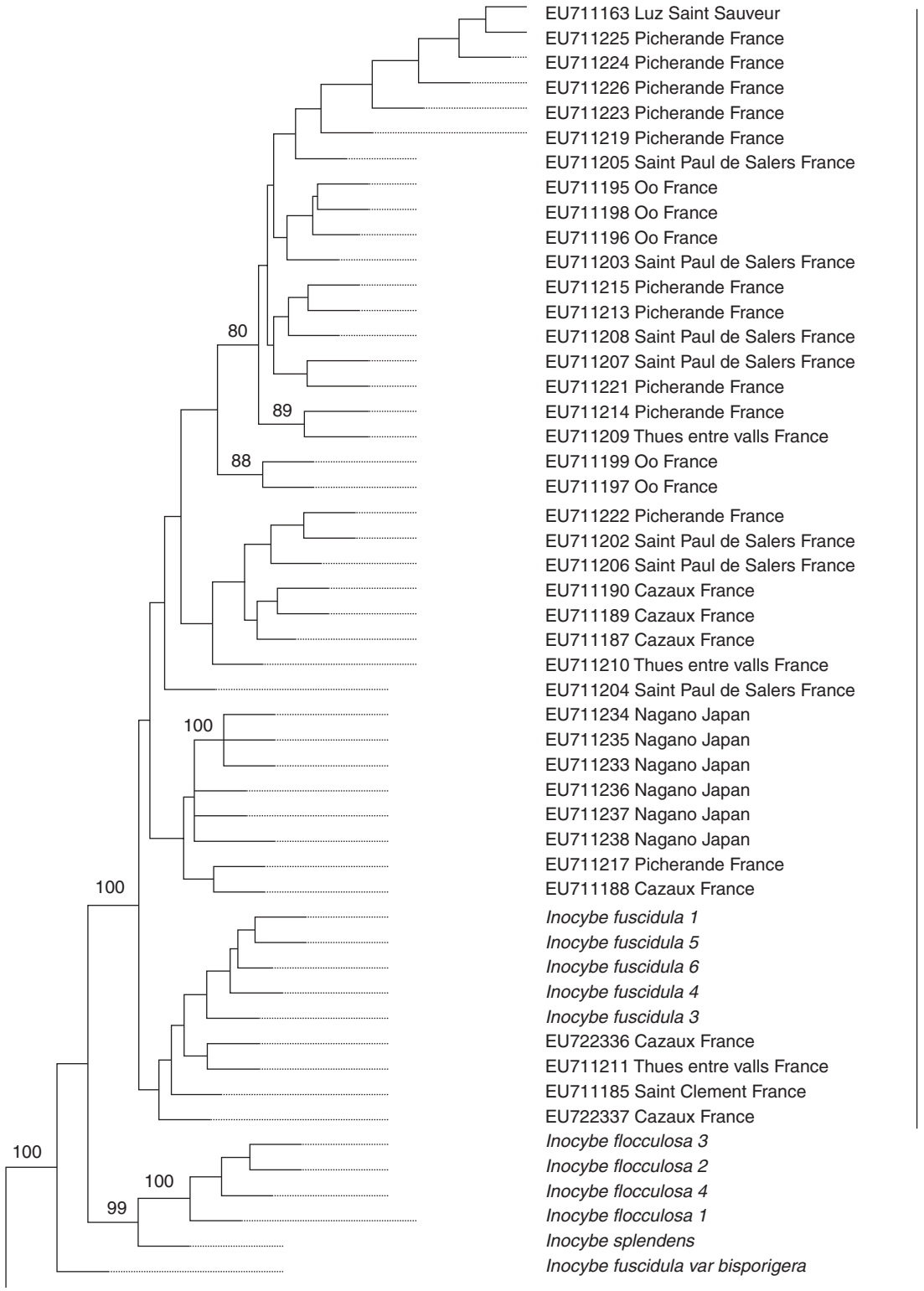

FIG. 5. Phylogenetic position of the *E. aphyllum* mycorrhizal fungi related to other *Inocybe* species (including the subgenus *Mallocybe*) and *Hebeloma* (ML on an alignment of the ITS + 28S rDNA; GTR model, 1000 bootstrap; only bootstraps >80 % are shown). Russian samples for which fungal ITSs were not sequenced are excluded.

further supporting a low specificity within *Inocybe*. With the exception of clades III (from Japan only) and VII (a single sequence from France), each clade was found over large geographic areas (Fig. 5). A Mantel test showed that similarity between *Inocybe*-related sequences and distance was positively correlated ($R = 0{\cdot}174$; $P = 0{\cdot}007$); more distant plants had

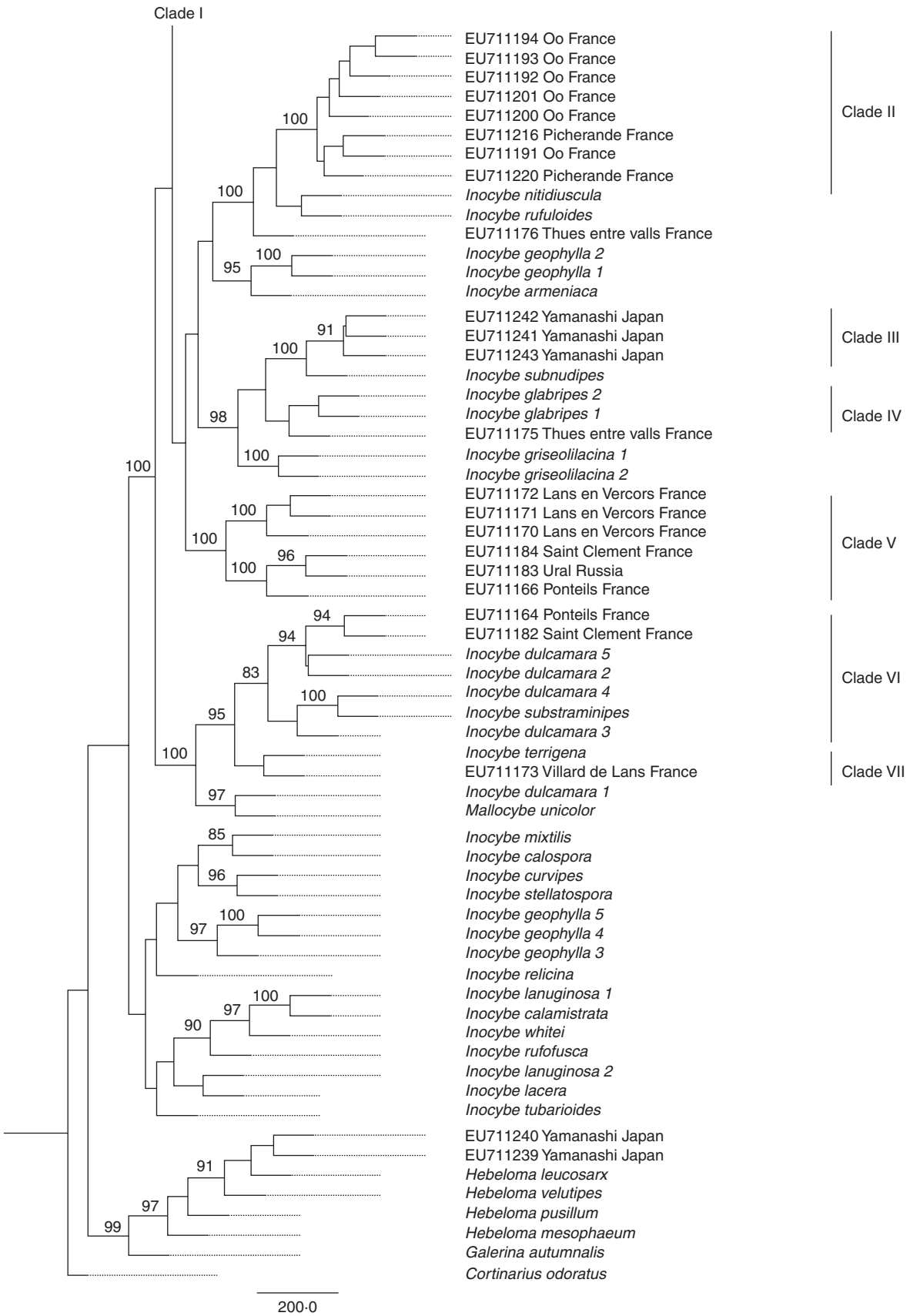

FIG. 5. Continued.

more similar fungal ITS. However, considering data from France only, the correlation was significantly negative ($R = -0{\cdot}226$; $P = 0{\cdot}0001$). On average, similarity between divergent *Inocybe* ITS sequences from the same plant (61 ± 21 %, mean $\pm$ standard deviation) or the same population ($66 \% \pm 24 \%$) was significantly higher than between ITS from different populations (53 ± 19 %, $P < 0{\cdot}0001$ in both cases), suggesting a geographical structure at smaller scale. No significant correlation with elevation was found (not shown).

Investigation of surrounding ECM tips

Since *Inocybe* form ECMs, to find them a search was carried out on tree roots around two plants of the Saint Clément population (Table 1). The 71 ECM tips collected around orchid EM15 exhibited 25 ITS RFLP patterns, one of which was identical to that obtained from EM15 (not shown). Its sequence was identical to that of the orchid (an *Inocybe* ITS, EU711184). It colonized eight tips (11·2 %), and was the second by order of abundance in this ECM sampling. The 46 ECM tips collected around EM12 exhibited ten ITS RFLP patterns, but none of these matched that from the neighbouring orchid.

DISCUSSION

Underground morphology of E. aphyllum

Compared with other orchids, *E. aphyllum* has an unusual, complex subterranean structure (Figs 1, 3 and 4); such a highly derived morphology is common for MH orchids (Ramussen, 1995) and other MH plants (Leake, 1994; Imhoff, 2003; Klimešová, 2007). Absence of roots is a shared feature with MH *Corallorhiza* species (Füller, 1977; Ramussen, 1995). The term 'mycorrhizal' fungus is used here in the enlarged meaning of 'an underground fungal associate, having a nutritional role'. As expected from the literature (Irmisch, 1853; Ziegenspeck, 1936), the plant encompasses two kinds of specialized underground shoots: thin stolons and plagiotropic coralloid rhizomes. Since numerous inflorescence buds were found on some flowering plants, the common idea that ramets die after fruiting (monocarpic development, e.g. Ziegenspeck, 1936; Rasmussen, 1995) is questionable. Instead, periods of underground growth could explain the irregular appearance of inflorescences for each plant (Summerhayes, 1951; Soyrinki, 1987; Robin, 1999). Ramet survival thus deserves further studies.

Coralloid rhizomes are densely branched (Figs 1 and 3) since most axillary buds produce ramifications (Ziegenspeck, 1936), and drastically differ from the simple tuberous rhizome found in *E. roseum* and some other *Epipogium* species (Pridgeon *et al.*, 2005; Yamato *et al.*, 2005). Coralloid rhizomes are densely colonized by fungi (Fig. 3) and therefore constitute a nutritional organ. Fungal colonization closely resembles that in other orchids (Scannerini and Bonfante, 1983; Smith and Read, 1997), with a final lysis of pelotons (not shown) and no colonization of meristems, inflorescence buds and central cylinder. Lack of colonization of outer rhizome cells (Fig. 3C) is convergently reported from the MH orchid *Neottia nidus-avis* (Selosse, 2003) and some other MH plants (Leake, 1994). Another similarity with *N. nidus-avis* is the scarcity of hyphae directly linking root tissues and soil (Selosse *et al.*, 2002*b*; Selosse, 2003).

There is a sharp separation between infection and storage zone, i.e. inflorescence buds, which may act as reserves for flowering (Rasmussen, 1995), and bulbils. MH orchids show variable modalities of separation between storage cells and colonized cells, e.g. between cells from the same tissue (e.g. in *Corallorhiza trifida*; Scrugli *et al.*, 1995), for which the adaptative value is unclear. Xylem was poorly developed (and only limited lignification was observed; Fig. 4), a feature common to many MH plants (Leake, 1994) for which phloem distributes nutrients. Correlatively, inflorescences need wet springs (see Rasmussen, 1995) and appear water-filled, a feature suggesting that turgidity contributes to the erect habit. Another fluid transport system, the aerenchyma, was present in the form of large intercellular spaces (Fig. 3C). It explains the presence of stomata on the underground rhizome shoots (Leake, 1994; our personal observations), a somewhat paradoxical feature since they are usually involved in CO_2 uptake and are therefore absent from most MH plants (Leake, 1994). Intercellular spaces are not devoted to hyphal spread in rhizomes (Fig. 3C, D) but rather may be linked to survival of *E. aphyllum* in wet soils (e.g. the observed Saint Clément population) and valleys near rivers (e.g. populations Oô, Cazaux, Picherande, Ponteils, Thuès-entre-Valls and Nagano Prefecture) by allowing circulation of oxygen in submerged conditions.

Asexual reproduction in E. aphyllum

In contrast to the coralloid rhizomes, the thin stolons (= runners) radiate away from the main rhizome and bear starch-filled bulbils, probably involved in asexual reproduction (Fig. 4). Bulbils are surrounded by rhizoids and thus reminiscent of protocorms (Fig. 4), a heterotrophic stage in orchid seed germination. They may reiterate the same developmental process (Fig. 1), except for the fact that they are initially aposymbiotic (see below). MH plants have often evolved underground asexual multiplication (Leake, 1994; Selosse, 2003), although the adaptative basis for this is unclear (Klimešová, 2007). Their development into adult plants was did not observed, but the existence of similar bulbils in *E. roseum* that develop into new rhizomes (Yagame *et al.*, 2007), together with reserves and a meristem, makes them candidates for asexual reproduction; accordingly, Rasmussen (1995) indicated that they develop into 'small plants' during the autumn. *Epipogium aphyllum* forms few fruits (Füller, 1977; Van der Cingel, 1995; Harrap and Harrap, 2005; our personal observations); despite their strong vanilla smell, flowers are poorly visited, possibly due to a combination of low nectar production and rarity of insects in the dark habitats of *E. aphyllum* (Vöth, 1994). Since animals often eat inflorescences (slugs and deer: Harrap and Harrap, 2005; Kopylov-Gus'kov *et al.*, 2007; our personal observations), sexual reproduction seems of low efficiency. It is unclear whether this facilitated an increase in asexual reproduction or, at the other extreme, low fruit set could be sustained after establishment of an efficient asexual reproduction. Nevertheless, asexual reproduction accounts for the existence of groups of ramets (Kopylov-Gus'kov *et al.*, 2007) and for

the observation that populations often extend downward along valleys (Robin, 1999), as expected if gravity or water disperse bulbils after disturbance.

It is noteworthy that both molecular and microscopic investigations failed to detect fungal colonization in stolons and bulbils, so the fungus is probably independently transmitted. Lack of fungal colonization was also reported in *E. roseum* stolons (Yamato *et al.*, 2005). *Epipogium aphyllum* bulbils have rhizoids that were not described for *E. roseum* and may represent entry points for fungi, as described in protocorms (Rasmussen, 1995) and adults of *E. aphyllum* (Scrugli *et al.*, 1995). In some MH plants, symbionts are directly transmitted during underground asexual multiplication, such as in root propagules of *Arachnitis uniflora* (Domínguez *et al.*, 2006) or in roots separated from rhizomes that develop into new shoots in *Neottia nidus-avis* (Selosse, 2003).

A main difference between asexual reproduction in *Epipogium* species and other MH plants is the distance from the mother plant, which is due to stolons. This could mean that (*a*) the fungus would be difficult and perhaps costly to maintain in long, rapidly growing stolons and (*b*) fungal presence in bulbils would not ensure that the fungus will form ECM around bulbils (the required ultimate carbon source, see below). *Inocybe* genets can be $<2{\cdot}5$ m in diameter (Lilleskov *et al.*, 2004), and ramets are likely to be smaller. It is thus proposed that the ability to produce bulbils meant that no direct transmission was selected in *E. aphyllum*. Accordingly, the aposymbiotic state of *E. aphyllum* propagules correlates to the diversity of fungi retrieved from different plants in a population (Table 1; see below) because independent transmission potentially allows each new ramet to associate with a different fungus. The diverse structures involved in underground sprouting of MH plants, showing variable fungal transmission, probably result from independent evolutionary adaptations of existing structures, with variable trade-offs between exploitation of the fungi and dissemination.

Fungal associates of E. aphyllum *across Eurasia*

The most commonly identified symbionts belonged to *Inocybe* subgenera *Mallocybe* and *Inocybe sensu stricto*; this is a common and worldwide genus of ECM fungi, present throughout the Eurasian range of *E. aphyllum* (Matheny *et al.*, 2005; Ryberg *et al.*, 2008). In two cases, only ascomycetes were recovered in cloning procedures that provided two clones each (EM10 and EM11, Table 1). Among all clones, the average probability of encountering ascomycetes among clones was 0·33 (thus, $P = 0{\cdot}33^2 = 0{\cdot}11$ for two clones, as in EM10 and EM11, assuming a constant probability). This high probability, together with the possible endophytic or saprophytic ecology of these fungi, does not support the hypothesis that ascomycetes were the sole mycorrhizal fungi. Additionally, they were not seen in TEM investigations. Similar ascomycete taxa had already been recovered from other orchids when cloning fungal ITS (e.g. Julou *et al.*, 2005; Abadie *et al.*, 2006). Conversely, in four occurrences, ITS sequences of *Hebeloma*, *Xerocomus* and *Lactarius* species were directly amplified (Table 1). *Hebeloma* sequences were amplified from pelotons, making them likely symbionts (*Hebeloma* and *Inocybe* are closely related; Matheny *et al.*, 2005). Direct amplifications of *Xerocomus* and *Lactarius* species suggest that they are common in investigated rhizomes, but their exact status remains unknown. Similarly, an ECM *Thelephora* species was found when cloning EM5. Such ECM genera are not usual contaminants. Thus, although *Inocybe*-related sequences were recovered from the same populations, it is not possible to rule out that these ECM species are truly mycorrhizal.

The identification of *Inocybe* symbionts is congruent with the presence of dolipores with perforate parenthesomes (Fig. 3F) and clamp connections (Fig. 3G) on intracellular hyphae; clamp connections were also reported by Scrugli *et al.* (1995) and Rasmussen (1995). Based on peloton morphology, Scrugli *et al.* (1995) described two kinds of fungi, each in different cell layers. It is unclear whether they represent different species (perhaps explaining the diverse sequences recovered from some individuals) or different developmental stages of a single *Inocybe* symbiont. Given the results of direct peloton analysis, we favour the later alternative. To our knowledge, no MH orchids were hitherto reported to associate with *Inocybe*. *Inocybe* species have been reported from some partly heterotrophic orchids (*Epipactis* and *Cephalanthera*; Bidartondo *et al.*, 2004), but no evidence was obtained that they actually formed pelotons. *Inocybe* was one of the rare large groups of ECM fungi not shown to have been recruited by MH orchids.

Epipogium aphyllum associates with a great range of *Inocybe* species. Interspecific ITS divergence is considered to be at least 3 % within *Inocybe* species (Matheny *et al.*, 2005); based on this threshold, then at least 22 species were probably encountered here. The exact range of associated fungi in *E. roseum* is unknown because only three Japanese populations of this species were investigated (Yamato *et al.*, 2005). There is no clear geographic pattern of association with *Inocybe* or support for geographical *E. aphyllum* races differing in their fungal partners (Figs 2 and 5). However, the sampling in the present study poorly covers the Eurasian range, because it was not possible to obtain permits to collect in some countries. Although conceived for plant protection, such limitations hinder cross-border movement of scientific samples and thus biological knowledge for protected species such as orchids (Roberts and Solow, 2008). The diversity of associated *Inocybe* species could be explained by the existence, even in sympatry, of several cryptic species differing in *Inocybe* preference. Given the importance of asexual reproduction of *E. aphyllum*, as previously discussed, emergence of local races with diverging specificities would be possible. Subspecies with different fungal preferences among Sebacinales were reported in the MH *Hexalectris spicata* (Taylor *et al.*, 2003), and cryptic species differing in associated Russulaceae exist in the MH *Corallorhiza maculata* (Taylor *et al.*, 2004). Although the great diversity of *E. aphyllum* ITS sequences (Fig. 1) may reflect speciation, the present data rather suggest a single species with a low specificity: first, some individuals harbour different partners (e.g. EM20, 26 or 27; Table 1), supporting the low specificity within *Inocybe*; and secondly, individuals from the same population differ in fungal associates (e.g. Saint Clément or Thuès-entre-Valls; Table 1), although they are likely to result from asexual reproduction (see above).

This work has consequences for conservation of *E. aphyllum*. First, since *Inocybe* species are not now culturable *in vitro* (Matheny *et al.*, 2005), *ex situ* conservation and germination are impossible. However, since some *Hebeloma* species are culturable, their ability to germinate *E. aphyllum* requires further studies. Secondly, the present data show that *Inocybe* symbionts form ECM with nearby trees, making them the most probable carbon source. Thus, trees should be protected around *E. aphyllum* populations. Since *Inocybe* species are generally non-specific ECM associates of trees (Ryberg *et al.*, 2008), there are no obvious tree species to be favoured. Asexual multiplication suggests that soil disturbance may contribute to dispersal at a local scale. Finally, the present observations confirm the existence of large underground rhizomes described by other authors (e.g. Rasmussen, 1995; Kopylov-Gus'kov *et al.*, 2007), so that extinction of populations cannot be assessed by observations of inflorescences only (Harrap and Harrap, 2005).

Evolution of fungal associations in MH orchids

Although a few additional and perhaps questionable symbionts may occur, *E. aphyllum* appears to associate specifically with a single ECM clade. This fits the usual paradigm for MH plants from temperate regions (Taylor *et al.*, 2002; references in the Introduction). It contrasts with the MH sister species *E. roseum* (Fig. 2) convincingly reported to associate with saprobic Coprinaceae (Yamato *et al.*, 2005; Yagame *et al.*, 2007). The host jump is not unexpected in itself, as it has been demonstrated among MH orchids such as *Corallorhiza* (Taylor and Bruns, 1997, 1999; Taylor *et al.*, 2004) and MH Ericaceae (Bidartondo, 2005). Host jumps are often associated with speciation in MH plants (see also cryptic species described above), although currently it is unknown whether this is a cause or a consequence of speciation.

The change in ecology of associated fungi is, however, an unexpected feature, and deserves further study in other *Epipogium* species because it raises many important questions. For example, *E. roseum* has a simpler rhizome morphology and a faster life cycle [$<$1 year (Yagame *et al.*, 2007) versus 10 years for *E. aphyllum* (Irmisch, 1853; Rasmussen, 1995)]. Is this an adaptation to saprobic fungi, perhaps because they persist less well or are active over shorter periods than ECM fungi? Moreover, *E. roseum* is tropical, as is *Eulophia zollingeri* which associates with the same saprobic fungal clade (Ogura-Tsujita and Yukawa, 2008). Other orchids putatively associated with saprobic fungi also tend to be tropical (Terashita, 1985; Lan *et al.*, 1994; Umata *et al.*, 1995; Cha and Igarashi, 1996; Dearnaley, 2006). It is probable that higher decay rates due to hot and wet tropical climates make saprobic fungi better able to obtain carbon and support growth of MH plants. *Epipogium aphyllum* also occurs in tropical regions (Pridgeon *et al.*, 2005); although some ECM fungi also occur in these regions, the fungal associates of such plants, if conspecific, deserve further study. To address these issues and better understand MH biology on a global scale, investigations on other tropical MH orchids and comparison with related MH species from temperate regions are now required.

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LITERATURE CITED

Abadie JC, Püttsepp Ü, Gebauer G, Faccio A, Bonfante P, Selosse MA. 2006. *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and non-photosynthetic individuals. *Canadian Journal of Botany* **84**: 1462–1477.

Bidartondo MI. 2005. The evolutionary ecology of mycoheterotrophy. *New Phytologist* **167**: 335–352.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London, Series B, Biological Sciences* **271**: 1799–1806.

Bougoure JJ, Dearnaley JDW. 2005. The fungal endophytes of *Dipodium variegatum* (Orchidaceae). *Australasian Mycologist* **24**:15–19.

Bryan JH. 1955. Differential staining with a mixture of safranin and fast green FCF. *Staining Technology* **30**: 153–157.

Cameron DD, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytologist* **171**: 405–416.

Campbell EO. 1970. Morphology of the fungal association in three species of *Corallorhiza* in Michigan. *Michigan Botanist* **9**: 108–113.

Cha JY, Igarashi T. 1996. *Armillaria jezoensis*, a new symbiont of *Galeola septentrionalis* (Orchidaceae) in Hokkaido. *Mycoscience* **37**: 21–24.

Danton P, Baffray M. 2005. Inventaire des plantes protégées en France. Paris: Nathan Ed.

Dearnaley JDW. 2006. The fungal endophytes of *Erythrorchis cassythoides*—is this orchid saprophytic or parasitic? *Australasian Mycologist* **25**: 51–57.

Dearnaley JWD. 2007. Further advances in orchid mycorrhizal research. *Mycorrhiza* **17**: 475–486.

Dearnaley JDW, Le Brocque AF. 2006. Molecular identification of the primary root fungal endophytes of *Dipodium hamiltonianum* (yellow hyacinth orchid). *Australian Journal of Botany* **54**: 487–491.

Domínguez L, Sérsic A, Melville L, Peterson RL. 2006. 'Prepackaged symbioses': propagules on roots of the myco-heterotrophic plant *Arachnitis uniflora*. *New Phytologist* **169**: 191–198.

Environment Agency of Japan. 2000. *Threatened wildlife of Japan, red data book*, 2nd edn. Vol. 8. *Vascular plants*. Tokyo: Japan Wildlife Research Center.

Füller F. 1977. Limodorum, Epipogium, Neottia, Corallorhiza. Wittenberg Lytherstadt: A. Ziemsen Verlag.

Girlanda M, Selosse MA, Cafasso D, *et al.* 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology* **15**: 491–504.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Harrap A, Harrap S. 2005. *Orchids of Britain and Ireland*. London: C. Helm.

Hoch HC. 1986. Freeze-substitution of fungi. In: Aldrich HC, Todd WJ, eds. *Ultrastructure techniques of microorganisms*, New York, NT: Plenum Press, 183–211.

Imhof S. 2003. A dorsiventral mycorrhizal root in the achlorophyllous *Sciaphila polygyna* (Triuridaceae). *Mycorrhiza* **13**: 327–332.

Irmisch T. 1853. *Beiträge zur Morphologie und Biologie der Orchideen*. Leipzig.

Julou T, Burhardt B, Gebauer G, Berviller D, Damesin C, Selosse MA. 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and non-photosynthetic mutants of *Cephalanthera damasonium*. *New Phytologist* **166**: 639–653.

Klimešová J. 2007. Root-sprouting in myco-heterotrophic plants: prepackaged symbioses or overcoming meristem limitation? *New Phytologist* **173**: 8–10.

Kopylov-Gus'kov YO, Vinogradova TN, Lyskov DF, Volkova PA. 2007. Population of *Epipogium aphyllum* (F.W. Schmidt) Sw., Orchidaceae Juss.) on the Kem'-Ludskij archipelago (White Sea). In: *Tverskoj gosudarstvennyj universitet*, Tver.

Koske RE, Gemma JN. 1989. A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research* **92**: 486–505.

Kull T, Hutchings MJ. 2006. A comparative analysis of decline in the distribution ranges of orchid species in Estonia and the United Kingdom. *Biological Conservation* **129**: 31–39.

Lan J, Xu JT, Li JS. 1994. Study on symbiotic relation between *Gastrodia elata* and *Armillariella mellea* by autoradiography. *Acta Mycologica Sinica* **13**: 219–222.

Lanave C, Preparata G, Saccone C, Serio G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution* **20**: 86–93.

Leake JR. 1994. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist* **127**: 171–216.

Leake JR. 2004. Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. *Current Opinion in Plant Biology* **7**: 422–428.

Lilleskov EA, Bruns TD, Horton TR, Taylor DL, Grogan P. 2004. Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities. *FEMS Microbiology Ecology* **49**: 319–332.

McKendrick SL, Leake DJ, Taylor DL, Read DJ. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corallorhiza trifida* and characterization of its mycorrhizal fungi. *New Phytologist* **145**: 523–537.

McKendrick SL, Leake JR, Taylor DL, Read DJ. 2002. Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. *New Phytologist* **154**: 233–247.

Maekawa F. 1971. *The wild orchids of Japan in color*. Tokyo: Seibundo-shinkosha [in Japanese with English summary].

Matheny PB. 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (Inocybe, Agaricales). *Molecular Phylogenetics and Evolution* **35**: 1–20.

Molvray M, Kores PJ, Chase MW. 2000. Polyphyly of mycoheterotrophic orchids and functional influences on floral and molecular characters. In: Wilson KL, Morrison DA, eds. *Monocots: systematics and evolution*. Melbourne: CSIRO, 441–447.

Ogura-Tsujita Y, Yukawa T. 2008. High mycorrhizal specificity in a widespread mycoheterotrophic plant, *Eulophia zollingeri* (Orhcidaceae). *American Journal of Botany* **95**: 93–97.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN. 2005. *Genera orchidacearum*. Vol. 4. *Epidendroidea* (part one). Oxford: Oxford University Press.

Rasmussen HN. 1995. *Terrestrial orchids – from seed to mycotrophic plant*. Cambridge: Cambridge University Press.

Rasmussen HN. 2002. Recent developments in the study of orchid mycorrhiza. *Plant and Soil* **244**: 149–163.

Reynolds EW. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cellular Biology* **17**: 208–212.

Roberts DL, Solow AR. 2008. The effect of the convention on international trade in endangered species on scientific collections. *Proceedings of the Royal Society* **275**: 987–989.

Robin M. 1999. Evolution d'une population d'*Epipogium aphyllum*: une énigme dévoilée ? *Cahiers de la Société Française d'Orchidophilie* **6**: 254–260.

Rodriguez R, Oliver JL, Marin A, Medina JR. 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142**: 485–501.

Ryberg M, Nilsson RH, Kristiansson E, Töpel M, Jacobsson S, Larsson E. 2008. Mining metadata from unidentified ITS sequences in GenBank: a case study in *Inocybe* (Basidiomycota). *BMC Evolutionary Biology* **8**: 50.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Scannerini S, Bonfante P. 1983. Comparative ultrastructural analysis of mycorrhizal associations. *Canadian Journal of Botany* **61**: 917–943.

Scrugli A, Cogoni A, Riess S. 1995. Mycorrhizal endophytes of the chlorophyll-free orchids, *Corallorhiza trifida* Chatelain and *Epipogium aphyllum* Swartz, under light microscopy and true confocal scanner microscopy. *Caesiana* **5**: 29–38.

Selosse MA. 2003. La néottie, une "mangeuse" d'arbres. *L'Orchidophile* **155**: 21–31.

Selosse MA, Bauer R, Moyersoen B. 2002*a*. Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees in silva: microscopic and molecular evidence. *New Phytologist* **155**: 183–195.

Selosse MA, Weiß M, Jany JL, Tillier A. 2002*b*. Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. *Molecular Ecology* **11**: 1831–1844.

Selosse MA, Faccio A, Scappaticci G, Bonfante P. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. *Microbial Ecology* **47**: 416–426.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis*, 2nd edn. London: Academic Press.

Soyrinki N. 1987. On the periodicity in the flowering of *Epipogium aphyllum* (Orchidaceae). *Memoranda Societas Pro Fauna et Flora Fennica* **63**: 63–72.

Summerhayes VS. 1951. *Wild orchids of Britain with a key to the species*. London: Collins.

Swofford DL. 2004. *PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0*. Sunderland, MA: Sinauer.

Taylor DL, Bruns TD. 1997. Independent, specialized invasions of ectomycorrhizal mutualism by two non photosynthetic orchids. *Proceedings of the National Academy of Sciences of the USA* **94**: 4510–4515.

Taylor DL, Bruns TD. 1999. Population, habitat and genetic correlates of mycorrhizal specialization in the cheating orchids *Corallorhiza maculata* and *C. mertensiana*. *Molecular Ecology* **8**: 1719–1732.

Taylor DL, Bruns TD, Leake JR, Read D. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. In: Van der Heijden MGA, Sanders I, eds. *Mycorrhizal ecology*. Berlin: Springer Verlag, 375–413.

Taylor DL, Bruns TD, Szaro TM, Hodges SA. 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *American Journal of Botany* **90**: 1168–1179.

Taylor DL, Bruns TD, Hodges SA. 2004. Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society of London, Series B, Biological Sciences* **271**: 35–43.

Terashita T. 1985. Fungi inhabiting orchids in Japan (III). A symbiotic experiment with *Armillaria mellea* and *Galeola septentrionalis*. *Transactions of the Mycological Society of Japan* **26**: 47–53.

Thompson JD, Higgins DG, Gibson TG. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Umata H. 1995. Seed germination of *Galeola altissima*, an achlorophyllous orchid, with aphyllophorales fungi. *Mycoscience* **36**: 369–372.

Van der Cingel NA. 1995. *An atlas of orchid pollination, European orchids*. Rotterdam: A. A. Balkema.

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

Vöth W. 1994. Sind Blüten von *Epipogium aphyllum* Sw. entomogam oder autogam? *Die Orchideen* **45**: 248–251.

Warcup JH. 1991. The *Rhizoctonia* endophytes of *Rhizanthella* (Orchidaceae). *Mycological Research* **95**: 656–664.

Warcup JH, Talbot PHB. 1967. Perfect states of rhizoctonias associated with orchids. *New Phytologist* **66**: 631–641.

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F. 2004. Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycological Research* **108**: 1003–1010.

Yagame T, Yamato M, Mii M, Suzuki A, Iwase K. 2007. Developmental process of an achlorophyllous orchid, *Epipogium roseum* (D.Don) Lindl. from seed germination to flowering under symbiotic cultivation with a mycorrhizal fungus. *Journal of Plant Research* **120**: 229–236.

Yagame T, Yamato M, Suzuki A, Iwase K. 2008. Ceratobasidiaceae mycorrhizal fungi isolated from nonphotosynthetic orchid *Chamaegastrodia sikokiana*. *Mycorrhiza* **18**: 97–101.

Yamato M, Iwase K. 2008. Introduction of asymbiotically propagated seedlings of *Cephalanthera falcata* (Orchidaceae) into natural habitat and investigation of colonized mycorrhizal fungi. *Ecological Research* **23**: 329–337.

Yamato M, Yagame T, Suzuki A, Iwase K. 2005. Isolation and identification of mycorrhizal fungi associating with an achlorophyllous plant, *Epipogium roseum* (Orchidaceae). *Mycoscience* **46**: 73–77.

Ziegenspeck H. 1936. Orchidaceae. In: Wangerin W, Schröter C, eds. *Lebensgeschichte der Blütenpflanzen Mitteleuropas*, Vol. 4. Stuttgart: Eugen Ulmer, 1–840.