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 Malloecybe 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 austiniae (Taylor and Bruns, 1997), Corallorhiza trifida (Taylor and Bruns, 1997; McKenzie et al., 2000) and C. striata (Taylor et al., 2002). Sebacinales were found in Neottia nidus-avis (McKenzie et al., 2002; Selosse et al., 2002b) 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 Chamaeleopodium 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 Erythromyces 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; Kul 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).

**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

![Fig. 1. Morphology of E. aphyllum (redrawn from Irmisch, 1853) and rhizoid-bearing protocorm (inset). cr, Coralloid rhizome; i, inflorescence bud; is, inflorescence shoot; s, thin stolon; sb, bulbil on a stolon.](https://academic.oup.com/aob/article-abstract/104/3/595/227055/1004)
TABLE 1. Identification of fungal sequences retrieved from the investigated E. aphyllum individuals, classified by origin

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<td></td>
<td>EM29</td>
<td>EU711220</td>
<td>ITS d¹ + c¹</td>
<td>Inocybe ECM</td>
<td>Inocybe fuscidula (AM882888)</td>
<td>0</td>
<td>99</td>
<td></td>
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<tr>
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<td>EM30</td>
<td>EU711221</td>
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</table>

**Continued**
and isolating fungal pelotons (¼ by collecting one large rhizome fragment in each population. For the seven plants from Japan, the sampling was performed 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.

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)
Molecular investigations

DNA extraction and PCR amplification of fungal internal transcribed spacers of nuclear ribosomal DNA (ITS) were performed as in Selosse et al. (2002b) 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 EcoRI + SacI and HindIII). RFLP were carried out as in Selosse et al. (2002a), 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 (ITS4ul 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. (2002a). An E. roseum ITS sequence was obtained from a plant collected in Java for the Museum National d’Histoire et al. (2001). The below-ground portion of the 28S rDNA was sequenced using primers ITS1F and TW13 as in Selosse et al. (2002b). The sequences were aligned with selected Inocybe sequences from GenBank; Cortinarius odoratus (DQ663360), Galerina autumnalis (AY281020), Hebeloma velutipes (AY818351), L. leucosarx (AB211268), H. pusillum (AB211274) and H. mesophaeum (AB327182) were used as outgroups.

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 ultra-microtomy: 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. (2002b). The sequences were aligned with selected Inocybe sequences from GenBank; Cortinarius odoratus (DQ663360), Galerina autumnalis (AY281020), Hebeloma velutipes (AY818351), L. 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 Orichis militaris (AY699977) were downloaded from Genbank as outgroups. After alignment using Bioedit v7.3.0 (Hall, 1999) and ClustaW (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; Rodriguez 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
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 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
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.
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. dalcamara* 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 Thé’s-entre-Valls, sequences from different clades were retrieved (Fig. 5).

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*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.
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 ($R = 0.174$; $P = 0.007$); more distant plants had
more similar fungal ITS. However, considering data from France only, the correlation was significantly negative ($R = -0.226; P = 0.0001$). On average, similarity between divergent Inocybe ITS sequences from the same plant ($61 \pm 21\%$, mean $\pm$ standard deviation) or the same population ($66 \% \pm 24\%$) was significantly higher than between ITS from different populations ($53 \pm 19\%, P < 0.0001$ 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 (Rasmussen, 1995) and other MH plants (Leake, 1994; Imhoff, 2003; Klimešová, 2007). Absence of roots is a shared feature with MH Corallorhiza species (Füller, 1977; Rasmussen, 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; Ziegen speck, 1936), the plant encompasses two kinds of specialized underground shoots: thin stolons and plagiotropic coraloid rhizomes. Since numerous inflorescence buds were found on some flowering plants, the common idea that ramets die after fruiting (monocarpic development, e.g. Ziegen speck, 1936; Rasmussen, 1995) is questionable. Instead, periods of underground growth could explain the irregular appearance of inflorescences for each ramet. Why this is 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 inves-
tigations 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 (Scruggs et al., 1995). In some MH plants, symbionts are directly transmitted during underground asexual multiplication, such as in root prop-
guages 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 bulbils. This could mean that (a) the fungus would be difficult and perhaps costly to maintain in long, rapidly growing stolons and (b) fungal pre-

cence in bulbils would not ensure that the fungus will form ECM around bulbils (the required ultimate carbon source, see below). Inocybe genets can be <2.5 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 associ-

de 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 Malloctype 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 ascomy-
cetes 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, \( P = 0.33^2 = 0.11 \) 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 paraphyses (Fig. 3F) and clamp connections (Fig. 3G) on intracellular hyphae; clamp connections were also reported by Scruggs et al. (1995) and Rasmussen (1995). Based on peloton morphology, Scruggs et al. (1995) described two kinds of fungi, each in different cell layers. It is unclear whether they rep-
resent different species (perhaps explaining the diverse sequences recovered from some individuals) or different develop-
mental stages of a single Inocybe symbiont. Given the results of direct peloton analysis, we favour the later alterna-
tive. 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 diver-
sity of associated Inocybe species could be explained by the existence, even in sympathy, of several cryptic species differ-
ing 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 Thües-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 (Bidartondo, 2005)). Host jumps are often associated with speciation in MH plants (see also cryptic species

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 (*E. roseum* 1 year (Yagame et al., 2005; Yagame et al., 1994; Umata et al., 2005)), although currently it is unknown whether this is a cause or a consequence of speciation.

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.

**LITERATURE CITED**


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


