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

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Received: 1 June 2008 Returned for revision: 22 September 2008 Accepted: 25 November 2008 Published electronically: 19 January 2009

• Background and Aims Epipogium aphyllum is a Eurasian achenloryllous, 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 pellets.

• 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 Malloocybe 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 nongreen, ‘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 australis (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) and Hexalecbris spicata (Taylor et al., 2003); although rhizoctonias encompass some Sebacinales, the species from MH orchids belonged to a different clade.
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 Chamaeagrostidia 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 Erythromchis cassuthoides (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 fungi) 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 (Dominguez 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; Pull 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...
### Table 1. Identification of fungal sequences retrieved from the investigated E. aphyllum individuals, classified by origin

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TABLE 1. Continued

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<td>0</td>
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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
7.2) for 2 h at room temperature and then overnight at 4
preserved for microscopic investigations by quick fixation in
water (3/2, v/v) for transport, and in some populations
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 con-
taining 1 % available chlorine for 30 s. The rhizome was
rinsed and cut with a sterilized scalpel into three pieces,
as in Warcup and Talbot (1967), with modifications as
follows. Surfaces of rhizomes were sterilized by immersion
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taining 1 % available chlorine for 30 s. The rhizome was
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rinsed and cut with a sterilized scalpel into three pieces,
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 (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 relative to E. roseum, plant nuclear and mitochondrial 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. (Table 1) using the plant-specific primer ITS1P as in Selosse et al. (2002b). The sequences were aligned with selected conspecific sequences (Table 1) 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
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
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.

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**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) ¼ 0.5 cm; (C, D) ¼ 50 μm; (E) ¼ 2 μm; (F) ¼ 1 μm; (G) ¼ 2 μm.
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 Thues-entre-Valls, sequences from different clades were retrieved (Fig. 5),

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
FIG. 5. Continued.
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 ± 21%, mean ± standard deviation) or the same population (66 ± 24%) was significantly higher than between ITS from different populations (53 ± 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 (*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; Kliměšová, 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 (Irnisch, 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., 2002b; 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*; Scruggli et al., 1995), for which the adaptive 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 CO₂ 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, Thues-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 adaptive basis for this is unclear (Kliměšová, 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* (Voith, 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 bulbs 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* (Scruggli 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 <2-5 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 *Malloocybe* 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, $P = 0.33^{2} = 0.11$ for two clones, as in EM10 and EM11, assuming a constant probability). This high probability, together with the possible endophytic or saprophic 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. Jolul 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 parentheses (Fig. 3F) and clamp connections (Fig. 3G) on intracellular hyphae; clamp connections were also reported by Scruggli et al. (1995) and Rasmussen (1995). Based on peloton morphology, Scruggli 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 sympathy, 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 *Corallorrhiza 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 cultivable *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 multiplicity 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 in 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* (Irmsch, 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 *Euophria 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; Umatu 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.

**ACKNOWLEDGEMENTS**

We thank Jean-François Bergouignan, Christine Casiez, Laure Civeyrel, Édouard Chas, Hervé Christophe, Jean Dauge, Gérard Joseph, Jean Marc Lewin, Tasuya Nagai, Yuzan Okuyama, Rémy Souche and Irina Tatarenko for help in sampling as well as Marie-Pierre Dubois, Pierre-Henri Fabre, Prune Pellet and Annie Tillier for help in molecular and phylogenetic studies. Thierry Vindolet provided crucial help with light microscopy investigations. We thank Mark Chase and two anonymous referees for excellent suggestions on an earlier version of this article. M.-A. Selosse is funded by the Centre National de la Recherche Scientifique and the Société Française d’Orchidophilie. P. Bonfante is funded by the Biodiversity Project, Comessa ‘Istituto per la Protezione delle Piante’ (CNR, Italy).

**LITERATURE CITED**


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


