Sebacinales are common mycorrhizal associates of Ericaceae

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Summary

• Previous reports of sequences of Sebacinales (basal Hymenomycetes) from ericoid mycorrhizas raised the question as to whether Sebacinales are common mycorrhizal associates of Ericaceae, which are usually considered to associate with ascomycetes.
• Here, we sampled 239 mycorrhizas from 36 ericoid mycorrhizal species across the world (Vaccinioideae and Ericoideae) and 361 mycorrhizas from four species of basal Ericaceae lineages (Arbutoideae and Monotropoideae) that do not form ericoid mycorrhizas, but ectendomycorrhizas. Sebacinales were detected using sebacinoid-specific primers for nuclear 28S ribosomal DNA, and some samples were investigated by transmission electron microscopy (TEM).
• Diverging Sebacinales sequences were recovered from 76 ericoid mycorrhizas, all belonging to Sebacinales clade B. Indeed, some intracellular hyphal coils had ultrastructural TEM features expected for Sebacinales, and occurred in living cells. Sebacinales belonging to clade A were found on 13 investigated roots of the basal Ericaceae, and TEM revealed typical ectendomycorrhizal structures.
• Basal Ericaceae lineages thus form ectendomycorrhizas with clade A Sebacinales, a clade that also harbours ectomycorrhizal fungi. This further supports the proposition that Ericaceae ectendomycorrhizas involve ectomycorrhizal fungal taxa. When ericoid mycorrhizas evolved secondarily in Ericaceae, a shift of mycobionts occurred to ascomycetes and clade B Sebacinales, hitherto not described as ericoid mycorrhizal fungi.

Key words: ectendomycorrhizas, Ericaceae evolution, ericoid mycorrhizas, molecular ecology, Sebacinales.

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Introduction

Recent works on mycorrhizal communities have stimulated considerable interest in a neglected group of fungi related to the genus Sebacina, recently raised to the order Sebacinales (Weiß et al., 2004). This basal order of Hymenomycetes (Basidiomycetes) encompasses fungi with longitudinally septate basidia and imperforate parenthesomes (i.e. the derivatives of the endoplasmic reticulum covering septal pores and allowing communication between cells). They also lack cystidia and structures formed during cytokinesis on some basidiomycetous hyphae, the so-called clamp connections. Cultivable species exhibit monilioid hyphae (i.e. hyphal cells that look like pearls in a chain). This is why sebacinoids with no known sexual stage were placed in the polyphyletic form genus Rhizoctonia.

Although some sebacinoids are described as valid species, and some can grow in pure culture, most of our knowledge on Sebacinales and their diverse host species comes from molecular ecology studies during the last 4 yr, that is, from direct amplification of fungal ribosomal DNA (rDNA) of environmental samples. Phylogenetic analysis, using sequences from cultures, fruitbodies or environmental samples, revealed that...
Sebacinales are divided into two clades, A and B, that differ in their ecology (Weiß et al., 2004). The first clade A sequences were derived from fruitbodies (Weiß & Oberwinkler, 2001), and subsequently from mycorrhizas of the aclorophyllous orchids Neottia nidus-avis (Mckendrick et al., 2002; Selosse et al., 2002a) and Hexalectris spicata (Taylor et al., 2003), as well as of some photosynthetic orchids related to N. nidus-avis that are partly heterotrophic (Selosse et al., 2004; Julou et al., 2005). At the same time, many clade A sebacinaoids, including those from the orchids just mentioned, were demonstrated to form ectomycorrhizas on tree roots (Selosse et al., 2002a,b; Urban et al., 2003; Walker & Parrent, 2004; Moyersoen, 2006). They are among the most common ectomycorrhizal species in temperate and Mediterranean forests (Glen et al., 2002; Avis et al., 2003; Kennedy et al., 2003; Walker et al., 2004; Richard et al., 2005; Tedersoo et al., 2006). Up to now, fruitbodies are only known from clade A sebacinaoids. Clade B displays a larger array of associations: some species are mycorrhizal on green, autotrophic orchids (Warcup, 1988; Bougoure et al., 2005), and others associate with liverwort thalli (Kottke et al., 2003). Clade B also contains the non-specific root endophyte Piriformospora indica (Verma et al., 1998, 2001; Peškan-Berghöfer et al., 2004). Recently, clade B rDNA sequences were amplified from roots of several Ericaceae (Berch et al., 2002; Allen et al., 2003; Bougoure & Cairney, 2005; Setaro et al., 2006a), raising the intriguing possibility that sebacinaoids could be overlooked mycorrhizal fungi of these plants.

Ericaceae form a large, ecologically relevant family of trees and shrubs growing all over the world (Kron et al., 2002a), especially at high altitude and high elevation stands. Most Ericaceae have a particular mycorrhizal association adapting them to the nutrient-poor, acidic soils of such areas (Smith & Read, 1997). In the so-called ericoid mycorrhiza (ERM), fungi colonize fine roots called ‘hair roots’, lacking cortical parenchyma, and form hyphal coils in large epidermal cells. Most cells are independently colonized from the soil (Bergero et al., 2000), with some cell-to-cell hyphal connections (Massicotte et al., 2005), so that each hair root harbours several fungi (Perotto et al., 1996). All ERM fungi reported so far are ascomycetes (Smith & Read, 1997; McLean et al., 1999; Cairney & Ashford, 2002; Midgley et al., 2004). However, the cloning of fungal rDNA sequences amplified from ERM roots suggested that sebacinaoids colonized roots of Gaultheria shallon (Berch et al., 2002; Allen et al., 2003) and Epacris pulchella (Bougoure & Cairney, 2005). These sebacinaoids are likely to have been ignored by classical approaches, as: (i) clampless hyphae of sebacinaoids cannot be distinguished from ascomycetous hyphae by light microscopy; and (ii) in vitro isolation failed to reveal them because they have been difficult to get into culture up until now (Berch et al., 2002). However, electron microscope investigations (Bonfante-Fasolo, 1980; Peterson et al., 1980; Allen et al., 1989; Setaro et al., 2006b) have sometimes revealed that basidiomycetes with imperforate parenthesomes, the type present in Sebacinales, may colonize living roots of ERM Ericaceae. Recently, an unusual mycorrhizal association with clade B sebacinaoids was reported from the neotropical Cavendishia nobilis (Ericaceae), a member of the Vaccinioideae (Kron et al., 2002a,b), with both intracellular colonization, as in ERM, and growth between and around the cortical parenchyma cells (cavendishoid mycorrhizas; Setaro et al., 2006a). These data raise three questions: (i) are sebacoid partners common in other ERM species, especially from the large ERM clade Ericoideae; (ii) if so, do all these ERM sebacinaoids belong to clade B, as suggested by available sequences (Weiß et al., 2004; Setaro et al., 2006a); and (iii) what are the ultrastructural features of sebacoid infection in hair roots, as compared with those of ERM ascomycetes?

ERM association is thought to have arisen once during Ericaceae evolution (Cullings, 1996; Kron et al., 2002a), and basal subfamilies (Pyroleae and Arbutoideae) display totally different mycorrhizas. They form ectendomycorrhizas (EEM), where fungi colonize both the cortical cells and the root surface, forming a sheath, an intercellular network between cortical cells, and intracellular structures that depend on the host taxon (Smith & Read, 1997). Here, a single fungal individual colonizes each root tip in most cases. Ascomycetes and/or basidiomycetes are present on EEM roots in Pyroleae (Robertson & Robertson, 1985; Bidartondo, 2005; Tedersoo et al., 2007) and Arbutoideae (Giovannetti & Lioi, 1990; Münzenberger et al., 1992; Richard et al., 2005). These fungi belong to species that usually form ectomycorrhizas on tree roots. Sebacinaoids were reported to occur on EEM roots of Arbutus unedo (Arbutoideae; Richard et al., 2005) and Orthilia secunda (Pyroleae; Tedersoo et al., 2007), again raising three questions: (i) do sebacinaoids often colonize ERM Ericaceae; (ii) which Sebacinales clade(s) are EEM plants associated with; and (iii) what are the ultrastructural features of their infection?

To answer these questions on identity, diversity and structural interactions of sebacinaoids associated with Ericaceae, we collected ERM and EEM roots, using worldwide sampling for ERM roots. First, detection of sebacinaoids was achieved using specific PCR primers. Second, the sequences obtained were used to find their phylogenetic position. Third, microscope investigations allowed characterization of the ultrastructural features, and thus the mycorrhizal status, of these sebacinaoids.

Materials and Methods

Root sampling

Ericoid mycorrhiza roots were sampled from various sites in Europe, La Réunion Island (Indian Ocean), and North and South America between 2002 and 2004 (Table 1; Supplementary Material, Table S1). Freshly harvested ERM roots were checked for connection to aerial plant parts to ensure host identity. They were washed carefully under a dissection microscope in order to remove soil and organic matter particles
Table 1  A summary of ericoid mycorrhizal roots of Ericaceae (Vaccinioideae and Ericoideae) investigated and successful PCR amplification and sequencing of a sebacinoid ribosomal DNA sequence, using primers ITS3Seb and TW13 (for more details and GenBank accession numbers, see Supplementary Material)

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Region of origina</th>
<th>No. of samples investigated</th>
<th>No. of successful amplificationsb</th>
<th>No. of sequences recoveredb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinioideae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agauria buxi</td>
<td>Re (1)</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Agauria salicifolia</td>
<td>Re (1)</td>
<td>5</td>
<td>4</td>
<td>3 (all identical)</td>
</tr>
<tr>
<td>Andromeda glauca</td>
<td>Ca (1)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Andromeda polifolia</td>
<td>Eu (2)</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Chamaedaphne calyculata</td>
<td>Ca (2)</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chiogenes hispidula</td>
<td>Ca (2)</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Gaultheria poeppigii</td>
<td>Ar (3)</td>
<td>22</td>
<td>13</td>
<td>8 (6 identical)</td>
</tr>
<tr>
<td>Gaultheria procumbens</td>
<td>Ca (3)</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gaultheria sp.</td>
<td>Ar (1)</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinium angustifolia</td>
<td>Ca (3)</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vaccinium macrocarpum</td>
<td>Ca (1)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vaccinium myrtilloides</td>
<td>Ca (2)</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinium myrtillus</td>
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<td>20</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Vaccinium oxyccocos</td>
<td>Ca (2), Eu (1)</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
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<td>Eu (7)</td>
<td>11</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Vaccinium vitis-idaea</td>
<td>Eu (2)</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Ericoideae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calluna vulgaris</td>
<td>Eu (19)</td>
<td>34</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Empetrum nigrum</td>
<td>Eu (3)</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Erica arborea</td>
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<td>2</td>
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<td>Erica cinerea</td>
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<tr>
<td>Erica multiforma</td>
<td>Eu (2)</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Erica reunionensis</td>
<td>Re (4)</td>
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</tr>
<tr>
<td>Erica vagans</td>
<td>Eu (2)</td>
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<td>1</td>
</tr>
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<td>Kalmia angustifolia</td>
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<td>0</td>
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<td>Kalmia polifolia</td>
<td>Ca (2)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ledum palustre</td>
<td>Ca (1)</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Loiseleuria procumbens</td>
<td>Eu (1)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rhododendron canadensis</td>
<td>Ca (2)</td>
<td>3</td>
<td>1</td>
<td>0</td>
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<td>Rhododendron conicum</td>
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<td>0</td>
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<td>Rhododendron decorum</td>
<td>Eu (1)</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rhododendron ferrugineum</td>
<td>Eu (1)</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Rhododendron fortunei</td>
<td>Eu (1)</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rhododendron groenlandicum</td>
<td>Ca (3)</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rhododendron japonicum</td>
<td>Eu (1)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal for each global regiond</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td>140</td>
<td>81 (57.8%)a</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>53</td>
<td>16 (30.2%)b</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Argentina</td>
<td>24</td>
<td>14 (58.3%)a</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Réunion Island</td>
<td>22</td>
<td>8 (40.9%)a</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>239</td>
<td>119 (49.8%)</td>
<td>76c,e (31.8%)</td>
<td></td>
</tr>
</tbody>
</table>

aAr, Argentina; Ca, Canada; Eu, Europe; Re, La Réunion. Brackets contain the number of independent sampled sites (i.e. areas of < 10 m² separated by > 1 km from the other sites).
bPercentages in brackets are related to the total number of samples investigated.
cFive sequences were obtained as a consensus of at least seven clones obtained by cloning the PCR product (namely, EF030919 and EF030867 on Andromeda polifolia, EF030944 on Erica ciliaris, EF030936 on Rhododendron ferrugineum and EF030875 on Vaccinium vitis-idaea).
dValues followed by different letters differ according to a chi-squared test (P < 0.001).
eGiven that some sequences were found on different plants (for Agauria salicifolia and Gaultheria poeppigii), this means a total of 70 different sequences.
as well as old and nonmycorrhizal roots. Then a 0.1–0.5 g (fresh weight) subsample, containing several hair roots, was frozen at −80°C, either directly or after storage in 60% ethanol in water (v/v) for < 5 d before transport. The samples from Argentina were oven-dried at 60°C for 72 h before transport, and stored in silica gel until DNA extraction. Samples from Calluna vulgaris and Erica cinerea from Lanno in Belle-Isle en Mer (France, one sampling of a cluster of co-occurring roots for each species) and Gaultheria poepigii from Pampa de Achala (Argentina, two samplings of a root cluster; see Table S1 for locations) were prepared for transmission electron microscopy (TEM) in a fixating buffer (2% glutaraldehyde in 0.1 M phosphate buffer (0.2 M KH₂PO₄/Na₂HPO₄), pH 7.2) at 4°C.

For EEM roots, each sample was stored separately at −80°C. For Arbutus unedo, mycorrhizal root tips were harvested in the Fango forest (Corsica, France, Table 2). From two soil cores situated 10 m apart, four and three tips, respectively, were selected that fitted the sebacoid morphotype described from the same site by Richard et al. (2005). This is a trichotomic mycorrhiza found in large aggregates, exclusively in the organic soil layer. These mycorrhizas are highly clavate, have no emanating hyphae or rhizomorphs, and are orange in colour and almost yellow at the tips. The seven selected tips were cut in two parts: one was placed in TEM fixating buffer and the other was used for DNA extraction. Four additional sequences from the Fango forest were obtained (GenBank accession numbers EF030880, EF030929, EF030911 and EF030912) by further sequencing A. unedo EEM DNA samples that produced sebacoid internal transcribed spacer (ITS) sequences in a previous study (sebacoid #1 to #4 in Richard et al., 2005). For the Pyroleae, EEM tips or root sections that showed external hyphae were recovered from a 6 m² patch of Orthilia secunda and a 4 m² patch of Pyrola chlorantha (100 EEM per species) at the Chauriat forest (Puy de Dôme, France, Table 2). A set of 49 mycorrhizal roots of Orthilia secunda were collected from a 4 m² patch at Abrahams Lake (Nova Scotia, Canada, Table 2). For Arctoctaphylos uva-ursi, 70 mycorrhizal roots were harvested from a 6 m² patch at Kukka bog reserve (Hiiumaa island, Estonia, Table 2) and 35 other roots from several plants at Crêt de l’Oeillon (Monts du Pilat, France, Table 2). The last four species were not sampled for TEM analysis, as no description of sebacoid morphotypes was available.

### DNA extraction, PCR and sequencing

All roots were submitted to DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions, and DNA was recovered in 40 µl of distilled water. Quality of DNA extract and fungal colonization were tested by PCR amplification of the fungal ITS, using a set of primers universal for fungi (ITS1F + ITS4) as in Selosse et al. (2002a). They were then submitted to amplification of a fragment of partial ITS plus the 5’ part of the 28S rDNA (D1/D2 region), using the primers ITS3Seb (5’-TGAAGTGTCATTGTTAATCTCAC-3’), internal to ITS and specific for Sebacinales, kindly provided by M. Berbee and TW13 (5’-GGTCCGTGTTTCAAGACG-3’), universal for fungi and internal to 28S rDNA; White et al., 1990), using DNA from a sebacoid fruitbody as a positive control. PCR was carried out in 50 µl, with final concentrations of 66 µM for each dNTP, 0.6 µM for each of the primers (Laboratoires Eurobio, Les Ulis, France), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mg ml⁻¹ gelatin, 0.1% (v/v) Triton X100, 5% (v/v) dimethyl sulfoxide and 1.5 units of Taq DNA polymerase (Quantum Appligène, Illkirch, France). We used 1 µl of the extracted DNA solution, but higher

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**Table 2** A summary of sebacoid sequences amplified from ectendomycorrhizal (EEM) Ericaceae using primers ITS3Seb and TW13

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>No. of EEM samples</th>
<th>No. of successful PCRs</th>
<th>Sequences (GenBank accession numbers)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbutus unedo</td>
<td>Fango forest, France (42°20’N; 8°49’E)</td>
<td>7</td>
<td>7b</td>
<td>EF030913 (n = 7)</td>
</tr>
<tr>
<td>Arctoctaphylos uva-ursi</td>
<td>Kukka bog, Estonia (58°14’N; 22°00’E)</td>
<td>70</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Crêt de l’Oeillon, France (45°24’N; 4°37’E)</td>
<td>35</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Pyrola chlorantha</td>
<td>Chauriat forest, France (45°46’N; 3°17’E)</td>
<td>100</td>
<td>1</td>
<td>EF030896 (n = 1)</td>
</tr>
<tr>
<td>Orthilia secunda</td>
<td>Chauriat forest, France (45°46’N; 3°17’E)</td>
<td>100</td>
<td>4</td>
<td>EF030895 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Abrahams Lake, Canada (45°09’N; 62°36’W)</td>
<td>49</td>
<td>1</td>
<td>EF030894 (n = 1)</td>
</tr>
</tbody>
</table>

²With number (n) of samples producing the sequence.

bOnly sebacoid morphotypes after Richard et al. (2005) were harvested and sequenced for A. unedo.

cOne of the sequences obtained from O. secunda at the Chauriat forest (EF030895) was 100% identical to that retrieved from P. chlorantha (EF030896) at the same site.
volumes (up to 10 µl) or dilutes (up to 10×) were tested when amplification failed. Reactions were performed in a TRIO-Thermoblock (Biometra, Göttingen, Germany) under the following thermoprofile: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. After the 35th cycle, a terminal extension of 10 min at 72°C was carried out. PCR products were sequenced as in Selosse et al. (2002a). Five randomly chosen PCR products for which no direct sequencing was possible were submitted to cloning (Table S1), as in Julou et al. (2005), and at least seven clones were sequenced for each of them. Sequences were edited, aligned using Sequencher 4.5 from Genes Codes (Ann Arbor, MI, USA), and confirmed as sebacinoid sequences by BLAST analysis (Altschul et al., 1997) against GenBank (NCBI; www.ncbi.nlm.nih.gov). All sequences (including consensus of cloned sequences) were deposited in GenBank (EF030867 to EF030946).

Transmission electron microscopy

For ERM, only the narrowest roots (hair roots) were used for investigation. About 20 hair roots were selected per glutaraldehyde-fixed sample and processed as in Setaro et al. (2006b) to obtain semithin sections (1 µm) and, when appropriate, ultrathin sections (0.05 µm). For EEM tips, ultrathin sections were obtained directly, by the same procedure as in Selosse et al. (2002b).

Phylogenetic reconstruction

The nucLSU portions of the retrieved DNA sequences were aligned with representative reference sequences taken from GenBank using MAFFT, version 5.850 (Katoh et al., 2002). To estimate phylogenetic relationships, the alignment was analysed using heuristic maximum likelihood (ML) as implemented in the PHYLML software, version 2.4.4 (Guindon & Gascuel, 2003), starting from a BIONJ tree (Gascuel, 1997), with a general time-reversible model of nucleotide substitution and additionally assuming a percentage of invariant sites and gamma-distributed substitution rates at the remaining sites (GTR + I + G). The gamma distribution was approximated with four discrete rate categories. All model parameters were estimated using ML. Branch support was inferred from 1000 replicates of nonparametric bootstrapping (Felsenstein, 1985), with model parameters estimated via ML individually for each bootstrapped alignment. Additionally, we performed a Bayesian Markov chain Monte Carlo (MCMC) analysis using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). We ran two independent MCMC analyses, each involving four incrementally heated chains over five million generations, using the GTR + I + G model of nucleotide substitution and starting from random trees. Model parameters were not fixed but sampled during MCMC. Trees were sampled every 100 generations, resulting in an overall sampling of 50 000 trees per run, from which the first 20 000 trees of each run were discarded (burn in). The remaining 30 000 trees sampled from each run were pooled and used to compute a majority rule consensus tree to get estimates for the posterior probabilities. Stationarity of the process was assessed using the Tracer software (Rambaut & Drummond, 2003).

Results

Sebacinoids from ERM samples

In all, 239 ERM root samples were recovered from 36 Ericaceae species (Table 1), that is, from 50 sites worldwide (Table S1). ITS amplification using the universal fungal primers ITS1F and ITS4 produced multiple PCR fragments for all samples, demonstrating that several fungal species were present on these roots (data not shown). The sebacinoid-specific primer set ITS3Seb and TW13 produced either a single 900 bp fragment (in 49.8% of the samples, i.e. in 29 out of the 36 investigated species, Table 1), or no PCR product. Although variable percentages of successful PCR amplification were obtained, none of these differences was significant (using pairwise chi-squared tests; \( P > 0.05 \), not shown) among Vaccinoideae and Ericoideae, nor among the most extensively sampled species (\( V. \) myrtillus, \( C. \) vulgaris and \( E. \) cinerea), nor even among the most extensively sampled genera (Gaultheria, Vaccinium, Erica and Rhododendron). Only samples from Canada yielded significantly fewer PCR products than those from Europe and Argentina according to chi-squared tests (\( P < 0.001 \), Table 1).

Sequences were obtained by direct sequencing of the 900 bp fragment from 71 samples (i.e. 59.6% of samples producing a PCR product – Table S1). BLAST analysis confirmed that they were closely related to sebacinoid sequences from GenBank. In some cases (e.g. EF030869, EF030888 or EF030899), two bases were present at some positions, suggesting that two close sequences were sequenced as a result of heterozygosities or the presence of two closely related sebacinoids. All other PCR products yielded a mix of different sebacinoid sequences, so that the conserved areas were the same but many polymorphic sites were detected in the variable regions (not shown). Five of these PCR products were cloned and sequenced (Table 1). In all cases, a unique sequence (with the exception of a few PCR and cloning errors, not shown) was found among clones, suggesting that at least one sebacinoid was present in these samples. (Perhaps other minor sequences were present that were not seen because only seven clones were sequenced). In all, sebacinoid sequences were obtained in 31.8% of the samples (Table 1) and no nonsebacinoid sequences were recovered.

All sequences differed by at least some base pairs, even for plants growing on the same site: as the only exceptions, identical sequences were found in five co-occurring Gaultheria poepigii samples from Argentina (EF030889) and three co-occurring Agaria salicifolia samples from La Réunion.
As a result, 70 different sequences were found. Up to five different sequences were found from the same site, that is from < 10 m² (Table S1), and different sequences were recovered from the same host at the same site (e.g. Vaccinium myrtillus, V. uliginosum or Calluna vulgaris produced two different sebacoid sequences at some sites; Table S1).

**Light and transmission electron microscopy of fungal colonization in ERMs**

Semithin sections of clusters of hair roots from C. vulgaris (n = 7), Erica cinerea (n = 2) and Gaultheria poeppigii (n = 8) displayed typical ERM colonization, with intracellular hyphal colonization (Fig. 1). TEM revealed both ascomycetes and hymenomycetous basidiomycetes as associated fungi (Fig. 2), with ultrastructure clearly distinguishing them. Ascomycetes had simple septal pores with Woronin bodies (Fig. 2b,c) and electron-light cell walls (Fig. 2b,c,e), whereas basidiomycetes had imperforate parenthesomes (i.e. the parenthesomal type also present in Sebacinales; Fig. 2d,e) and electron-dense hyphal cell walls (Fig. 2a,b,d,e). Sebacinales-like basidiomycetes were found on E. cinerea (in n = 1 ultrathin section) and G. poeppigii (n = 2). Both asco- and basidiomycetes formed intracellular coils in living cortical cells of hair roots. The vitality of the host cells was indicated by the mitochondria-rich cytoplasm (Fig. 2a,b,f). Basidiomycetous colonization of cortical cells was found to be exclusive (Fig. 2a) or dual, that is, with both Sebacinales-like basidiomycetes and ascomycetes forming coils within a single host cell (in G. poeppigii, Fig. 2b,e).

**Sebacoids from EEM samples**

The seven EEM root tips of A. unedo from the Fango forest showing a sebacoid morphotype harbour a unique sebacoid sequence (EF030913, Table 2). It differed from the four sequences already obtained by Richard et al. (2005) at this site, suggesting that sebacoids are diverse in this forest. Two Pyroleae (O. secunda and P. chlorantha) from the Chauriat forest produced the same sequence (EF030895 = EF030896, Table 2), so that the related sebacoid was probably not host-specific. A second sebacoid occurred in O. secunda at the Chauriat. A single EEM O. secunda root from Abrahams Lake harboured a sebacoid sequence that differed from the previous ones (Table 2). No sequences were obtained from A. uva-ursi at the two sites investigated (Table 2). Because of the low frequency of sebacoid colonization on other hosts, this does not imply that A. uva-ursi is not a host (pairwise chi-squared tests not significant (P > 0.05), not shown).

**Ultrastructure of fungal colonization in Arbutus unedo EEMs**

Transmission electron microscopy performed on three randomly selected EEM tips of Arbutus unedo (n = 3) from the Fango forest revealed typical arbutoid ectendomycorrhizas with hyphal sheath, Hartig net and intracellular colonization (Fig. 3a,b). Although vitality of the plant cell is not visible, these structures indicate an ectomycorrhizal association. Peripheral hyphae showed thick cell walls (Fig. 3d), as described for ectomycorrhizal sebacoids (Selosse et al., 2002b). The associated fungi have dolipores and imperforate parenthesomes, as is typical for Sebacinales (Fig. 3b,c).

**Phylogeny of sebacoids from Ericaceae**

The results of our molecular phylogenetic analysis involving the nuc LSU sequences from the present study and a comprehensive set of reference sequences published in GenBank are shown in...
Fig. 2 Transmission electron micrographs of ericoid mycorrhiza (ERM) colonized by sebacinoïds. (a) Cortical cell of an *Erica cinerea* hair root colonized by sebacinoïd hyphae (s). Black arrowhead, a dolipore of an intracellular hypha; white arrowheads, mitochondria of the plant cell. Bar, 2 µm. (b) Cortical cell of an ERM of *Gaultheria poeppigii* with dual colonization of ascomycetes (a) and Sebacinales (s). The plant cell cytoplasm is alive, indicated by the nucleus (NU) and mitochondria (white arrowheads); the black arrowhead points to an ascomycetous septum with Woronin bodies. Bar, 2 µm. (c) Detail of the ascomycetous hyphae with septum and Woronin body (arrowhead) of panel (b); M, mitochondria; *, the electron-light hyphal wall. Bar, 1 µm. (d) Dolipore of a sebacinoïd hypha forming ERM with *G. poeppigii*. Arrowheads point to imperforate parenthesomes; *, the electron-dense hyphal wall. Bar, 0.1 µm. (e) Sebacinoïd hyphae (s) with dolipore (arrowhead) and imperforate parenthesomes forming dual colonization with ascomycetes (a) in a cortical cell of *G. poeppigii*. White and black asterisks mark the electron-light cell walls of the ascomycetes and the electron-dense cell walls of the sebacinoïds, respectively. Bar, 2 µm. (f) Detail of mitochondria (M) in the cytoplasm of the plant cell dually colonized by Sebacinales and ascomycetes. Bar, 0.1 µm.
Fig. 4. The outcome of the heuristic ML analysis and the two independent MCMC runs was widely consistent, with few exceptions, the most prominent of which is the different placement of a sebacinoid detected in *Vaccinium vitis-idaea* (EF030882). In the ML tree, this sequence clustered, although without significant support, together with a similar sequence from *Vaccinium vitis-idaea* (EF030883) at the base of Sebacinales group A (Fig. 4). In MCMC analysis, however, the sequence EF030882 was placed within Sebacinales clade B with a posterior probability of 100%, while EF030883 was still placed at the base of clade A with a posterior probability of 97% (not shown). Apart from these exceptions, both ML and MCMC analyses separated the Sebacinales into the known clades A and B.

Consistent with earlier studies, group A also includes all sequences retrieved from fruitbodies, from ectomycorrhizal samples and from heterotrophic orchids (Fig. 4). Sebacinoid sequences from EEMs (from *Arbutus unedo*, *Orthilia secunda*, *Pyrola chlorantha*) also appear in this group. All the sequences obtained from ERMs, on the other hand, clustered within Sebacinales clade B, the clade that also includes all sequences from cavendishoid mycorrhizas and liverwort thalli (Fig. 4). Clade B also contains the sequences of *Sebacina vermifera* isolates obtained mostly from Australian green, autotrophic orchids (Warcup, 1988), as well as of *Piriformospora indica*.

Within clades A and B, the sequences were not strictly grouped according to the mycorrhizal types from which they were obtained. Furthermore, no larger biogeographical subgroup was resolved. For example, a well-supported subgroup (97% in ML bootstrap, 100% MCMC support) contains sequences from Australia, Argentina, Ecuador, Canada, France, Germany, and Estonia.
Discussion

Sebacinales as common mycorrhizal fungi in Ericaceae roots

Out of 600 samples of Ericaceae roots, a total of 89 produced a sebacoid sequence (Tables 1, 2), representing 74 different sequences (because of some identities). Among them, five were obtained after cloning, suggesting that the 48 PCR products from ERM roots that we were not able to sequence directly contain sebacoid sequences (Table S1). Cross-contamination of samples seems unlikely, as most sequences are different. Moreover, we provide evidence that hyphae with sebacoid dolipores and parentheses can be detected by TEM analysis and that they occur in (or around) living cells, forming typical ERM (Fig. 2) or EEM (Fig. 3) on the roots of at least three host species. This underlines the utility of combining molecular and TEM approaches, if possible on the same sample, as already used to demonstrate that sebacoids form ectomycorrhizas (Selosse et al., 2002b) and cavendishiod mycorrhizas (Setaro et al., 2006a). In this study, the use of specific primers probably enhanced molecular detection.

Sebacinales form ERM-like interactions in hair roots of Ericaceae, whereas only ascomycetes were reported to be involved so far (Smith & Read, 1997). Although more species should be investigated at the ultrastructural level, this is in accordance with the molecular diversity of Sebacinales previously found on hair roots of Vaccinioideae and Staphyleoideae species from Canada (Gaultheria shallon, Berch et al., 2002; Allen et al., 2003), Australia (Epacris pulchella, Bougoure & Cairney, 2005), and the Neotropics (Cavendishia nobilis, Setaro et al., 2006a). It extends this diversity to the Ericoideae tribe. With the exception of C. nobilis (Setaro et al., 2006a), and probably Calluna vulgaris (Bonfante-Fasolo, 1980), we are not aware of direct observation of sebacoids in ERM. Some species investigated in the present study failed to produce positive PCRs (Table 1). This could be the result of undersampling, as Sebacinales abundance might vary among species. On G. shallon they represented more than half of cloned ITS sequences (Allen et al., 2003; accordingly 75% of roots did not yield fungal isolates) compared with less than a quarter on E. pulchella (Bougoure & Cairney, 2005; 8% of roots did not yield fungal isolation). This suggested that Sebacinales colonized larger root portions on G. shallon than on E. pulchella. However, these percentages are not directly comparable, since isolation procedures were not identical.

Can one say that Sebacinales are ERM mycorrhizal? If mycorrhizal interaction is defined as a morphogenetic process uniting roots and soil fungi, a definition we favour and use here, then we observed ERM mycorrhizas involving sebacoids. If the definition additionally implies a mutualistic relationship, a feature sometimes violated in associations considered as mycorrhizal (Kiers & van der Heijden, 2006) and difficult to establish, then the question requires further investigation. Mutualism has been experimentally shown for relatives of the ERM sebacoids in clade B. Piriformospora indica is beneficial for the growth of several plants (Rai et al., 2001; Varma et al., 2001; Peškan-Berghöfer et al., 2004; Waller et al., 2005) and even inducits systemic resistance to fungal diseases and tolerance to salt stress in barley (Waller et al., 2005). Together with another clade B sebacoid, it enhanced growth of Nicotiana tabacum, but at the expense of herbivore resistance (Barazani et al., 2005). Similar positive effects on plant growth have also been shown for several isolates of the Sebacina vermisfera species complex (Deshmukh et al., 2006). Such beneficial effects might thus be expected in the interaction between group B sebacoids and Ericaceae, but await further studies, for example using in vitro synthetic associations.

Evolution of mycorrhizal structures in the Sebacinales

Mycorrhizal types are not evenly distributed over the phylogeny of Sebacinales (Fig. 4), in agreement with previous investigations (Weiß et al., 2004). Clade B encompasses species that grow intracellularly, in hepatics (Kottke et al., 2003), ERM roots (our data), orchid roots (Warcup, 1988), and many other hosts for P. indica (the model species Arabidopsis thaliana (Peškan-Berghöfer et al., 2004) and Nicotiana tabacum (Barazani et al., 2005), but also species in Fabaceae and Rhamnaceae (Varma et al., 2001), Asteraceae and Solanaceae (Rai et al., 2001) as well as Poaceae (Waller et al., 2005)). However, the interaction is somewhat different at the cellular level for P. indica, which was shown to enhance apoptosis of plant cells, and colonize dead cells instead of living cells as observed here (Deshmukh et al., 2006). Several types of interaction can therefore be expected at the structural level in clade B. Some well-supported terminal clades within group B do not at present contain sequences from different host types (Fig. 4), but this might well be the result of an undersampling of Sebacinales diversity. Within clade B, species involved in cavendishiod mycorrhizas (Setaro et al., 2006a) form abundant extracellular mycelium, in addition to the usual intracellular colonization. This results in an EEM-like association that probably evolved secondarily among ERM plants (Setaro et al., 2006b).

By contrast, clade A mycorrhizal association usually presents a hyphal sheath (Selosse et al., 2002b; Urban et al., 2003), and sometimes intracellular colonization, as in some ectomycorrhizas (Selosse et al., 2002b) and in some heterotrophic orchids (Selosse et al., 2002a; Taylor et al., 2003). All EEM sebacoids from Ericaceae belong to clade A, as observed in independent studies of Monotropoideae mycorrhizal associates (Pyrola chlorantha and Orthilia secunda, Tedersoo et al., 2007; P. rotundifolia, M.-A. Selosse, unpublished; discussed later). This corroborates the observation that EEM fungi usually belong to clades with ectomycorrhizal abilities, in both Arbutoideae (Richard et al., 2005) and Monotropoideae (Bidartondo, 2005; Tedersoo et al., 2007).
Fig. 4 Phylelogenetic relationships within the Sebacinales, with part of the tree relating to clade A and clade B. Phylogram derived by heuristic maximum-likelihood (ML) analysis from an alignment of nucLSU sequences, using a time-reversible model of nucleotide substitution, additionally assuming a portion of invariable sites and gamma-distributed substitution rates at the remaining sites (GTR + I + G). Branch support values were calculated from 1000 replicates of nonparametric ML bootstrap analysis (first numbers), and from Bayesian Markov chain Monte Carlo analysis, also using the GTR + I + G substitution model (second numbers). Values below 50% are indicated with asterisks or omitted. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The tree was rooted with Auricularia auricula-judae. Mycorrhizal types: ECM, ectomycorrhiza; CVM, cavendishoid mycorrhiza; EEM, ectendomycorrhiza; ERM, ericoid mycorrhiza; JMM, jungermannialean mycorrhiza-like thalli; ORM, orchid mycorrhiza. Sample origin: A, Austria; AUS, Australia; ARG, Argentina; CAN, Canada; CHL, Chile; CHN, P.R. China; ECU, Ecuador; EST, Estonia; FRA, France; GER, Germany; GUY, Guyana; IND, India; NOR, Norway; REU, la Réunion; SPA, Spain.
Fig. 4 continued
Clades A and B therefore differ in mycorrhizal associations. The occurrence of ERM sequences basal to group A in Fig. 4 is not strongly supported and might have two alternative explanations: either future works will support this (in which case features specific to clade A are derived in Sebacinales evolution, such as mycorrhizal associations with sheath), or this is an artifactual grouping. Indeed, these sequences appeared in various positions within group B in preliminary analyses, and there is a discrepancy concerning this pair of sequences between ML and MCMC analysis (as detailed in the Results section). For the moment, we regard the placement of these sequences in the phylogenetic tree as unstable. Addition of more sequences might resolve this problem in future analyses.

Evolution of mycorrhizal structures in the Ericaceae

Available Ericaceae phylogenies (Freudenstein, 1999; Kron et al., 2002a) suggest a single most parsimonious scenario for evolution of mycorrhizal association (Fig. 5). Starting from a plesiomorphic association with arbuscular mycorrhizal fungi, still observed in Enkianthoideae (Abe, 2005), a shift occurred to EEM association involving ectomycorrhizal fungi, as currently retained in Monotropoideae + Arbutioideae. Then, ERM association arose once in the Ericoideae + Styphelioideae + Harrimanelloideae + Cassioioideae + Vaccinioideae clade (Fig. 5; Callings, 1996). Among these, more derived conditions evolved, such as the cavendishoid mycorrhizas in the Andean clade of Vaccinioideae (Setaro et al., 2006a,b), or perhaps the additional presence of arbuscular mycorrhizal fungi in Gaultheria poeppigii (Urcelay, 2002), in Vaccinium spp. and Styphelia tameiameiae from Hawaii (Koske et al., 1990). Significantly, although data on the smaller subfamilies Harrimanelloideae and Cassioioideae are lacking so far, the EEM to ERM shift was associated with a shift from clade A to clade B sebacinoids (Fig. 5).

Interestingly, two sequences of clade B sebacinoids were found in cloning of fungal ITS in the Ericaceae Pyrola chlorantha, in addition to EEM mycorrhizal fungi (Tedersoo et al., in press): cloning allows recovery of various endophytic fungi, and this may mean that clade B sebacinoids even occur in EEM Ericaceae as endophytes, together with the main mycorrhizal fungus. Strikingly, Heliotiales, the ERM ascomycetous associates of Ericaceae, also encompasses root endophytic species (Vrålstad et al., 2002), raising an intriguing hypothesis. At the emergence of ERM association in Ericaceae, some endophytic fungal clades (namely the Heliotiales and the Sebacinales) would have been recruited as mycorrhizal partners and/or excluded the former EEM symbionts in the ancestor of ERM Ericaceae.

We did not detect any significant difference in frequency of colonization, or specialization for any sebacinoid subclade in Ericoideae, Vaccinioideae, or in any frequently sampled plant species (Table 1). Mycobionts from the same ericaceous species are distributed over group B in several cases (Fig. 4). However, Sebacinales obtained from some host species from different localities are very similar, if not identical (e.g. Vaccinium myrtillus mycobionts from France and Germany), and there is a cluster (near the top of the clade B tree) uniting mycobionts from several Vaccinium species. Our sampling design and effort are nevertheless not suitable for reliable conclusions to be drawn regarding specificity.

Perspectives on Sebacinales

Our data add to the amazing diversity and ecological abundance of sebacinoids. So far, no biogeographical subgroup is detectable in Sebacinales. One well-supported subgroup (97%, with a
long branch; Fig. 4) contains Sebacinales from Europe, South America and Australia. Some well-supported terminal clades at present do not include sequences from different continents; however, ensuring that they represent regional clades would require much more additional sampling. Finding sebacinoids among ascomycetes in ERM roots (sometimes in the same cell, Fig. 2b) again underlines their frequent coexistence with ascomycetes (Warcup, 1988; Selosse et al., 2002a; Urban et al., 2003; Setaro et al., in press), an intriguing and hitherto unexplained feature.

Interestingly, in >60% of the ERM samples successfully amplified, a single or, at most, two nearly identical sequences (when two bases are present at certain positions) were obtained. In Andean Ericaceae investigated by Setaro et al. (2006), different sebacinoids co-occurred in a given sampled root. The sequence homogeneity observed on our samples encompassing many roots from a given root system, on is of unclear significance, since the meaning of the differences between $28S$ rDNA sequences is unknown. If these differences represent interspecific variations, we observe here patchiness in species distribution that often leads to the colonization of a root system by a single species (including possibly several individuals), and a huge specific diversity exists among Sebacinales. If differences between sequences represent intraspecific polymorphism, then a single individual (or several closely related ones) often covers each root system. Although there is evidence from other basidiomycetes that the variability of $28S$ rDNA sequences is low at the intraspecific level (Fell et al., 2000), no rigorous conclusion can be drawn. This frustrating problem arises for sebacinoids because the absence of meaningful morphological characters makes distinction of morphological species impossible. Similarly, the absence of haploid cultures for most ERM and EEM species makes distinction of morphological species impossible. Similarly, the absence of haploid cultures for most ERM and EEM species does not allow distinction of biological species using inter-fertility tests. If other loci were available, the phylogenetic species concept (Taylor et al., 2000) would be interesting to apply: the fingerprint of recombination in a taxon is that phylogenies of individuals of the same biological species are not congruent for different loci. Conversely, in a comparison between phylogenetic trees of different loci, the level at which congruence between loci arises delineates biological species (Taylor et al., 2000). This potentially applies to taxa known only from environmental DNA, if at least two loci are available. For sebacinoids, specific primers should be designed, since environmental DNA extracts harbour DNA from many fungal species. This is a common problem with mycorrhizal taxa mostly reported from molecular studies, such as tulasnel-loids (Bidartondo et al., 2004) or thelephoroids (Koljalg et al., 2001).

More diversity and biological interactions may still be found in Sebacinales. They may colonize many other hosts, as suggested by their isolation from roots that were first supposed to be arbuscular mycorrhizal (Williams, 1985; Milligan & Williams, 1987). In particular, clade B sebacinoids may grow in roots of many plant taxa: some were recovered from *Piriformospora indica* (Neubert et al., 2006) and hePC (Kottke et al., 2003), and *P indica* showed compatibility with tremendously diverse hosts in glasshouse experiments, as mentioned earlier. Additionally, sebacinoid mycelia may be shared by different root systems. Thus, we might expect a major role in interplant interactions, or even in carbon transfers between plants, as already demonstrated for clade A sebacinoids associated with achlorophyllous orchids (McKendrick et al., 2002; Selosse et al., 2002a,b; Taylor et al., 2003). A role for sebacinoids in shaping plant communities is an intriguing possibility raised by their frequent detection in molecular microbial ecology.

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**References**


### Supplementary Material

The following supplementary material is available for this article online:

Table S1 List of the 239 samples of ericoid mycorrhizal (ERM) roots investigated in this work, in alphabetic order of Ericaceae host name, with geographic origin and GenBank accession numbers of the recovered sequences of the ITS+28S rDNA

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02064.x

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