Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex*

F. Richard¹, S. Millot¹, M. Gardes¹* and M-A. Selosse²*

¹UMR 5174 Evolution et Diversité Biologique, Université Toulouse III Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse Cédex 4, France; ²UMR 5175 Centre d’Ecologie Fonctionnelle et Evolutive, Equipe Co-évolution, 1919 Route de Mende, 34293 Montpellier cédex 5, France; *These two authors contributed equally to the supervision of this work.

Summary

- We analysed the ectomycorrhizal (ECM) fungal diversity in a Mediterranean old-growth *Quercus ilex* forest stand from Corsica (France), where *Arbutus unedo* was the only other ECM host.
- On a 6400 m² stand, we investigated whether oak age and host species shaped below-ground ECM diversity. Ectomycorrhizas were collected under *Q. ilex* individuals of various ages (1 yr seedlings; 3–10 yr saplings; old trees) and *A. unedo*. They were typed by ITS–RFLP analysis and identified by match to RFLP patterns of fruitbodies, or by sequencing.
- A diversity of 140 taxa was found among 558 ectomycorrhizas, with many rare taxa. *Cenococcum geophilum* dominated (35% of ECMs), as well as Russulaceae, Cortinariaceae and Thelephoraceae. Fungal species richness was comparable above and below ground, but the two levels exhibited < 20% overlap in fungal species composition.
- *Quercus ilex* age did not strongly shape ECM diversity. The two ECM hosts, *A. unedo* and *Q. ilex*, tended to share few ECM species (< 15% of the ECM diversity). Implications for oak forest dynamics are discussed.

Key words: *Arbutus unedo*, ectomycorrhizas, ITS–RFLP, Mediterranean forests, molecular typing, old trees, *Quercus ilex*, seedlings.


Introduction

*Quercus ilex* L. (Holm oak) is a characteristic evergreen oak species in the Mediterranean basin (Quézel, 1985; Scarascia-Mugnozza et al., 2000). Despite heavy anthropic pressure, old-growth forests still exist in the island of Corsica (Quézel & Médail, 2003). In such stands, overstorey oaks coexist with understorey chaparral shrubs such as *Arbutus unedo* L. (strawberry tree) and *Erica arborea* L. (tree heath). In general, oak trees do not exceed 200 yr of age (Panaïotis et al., 1997). In old-growth forests, mortality leads to tree falls that create numerous small-scale canopy gaps.

A wide variety of ectomycorrhizal (ECM) fungi are symbionts of many tree species in temperate climatic zones. More than 5000 species from the Ascomycetes and Basidiomycetes form ectomycorrhizas on secondary tree roots (Trappe, 1962; Smith & Read, 1997). The majority of ECM species have large host spectra (Molina et al., 1992). This allows a diffuse interaction, i.e. the sharing of common fungal associates by plant individuals of identical or different species. Ectomycorrhizas are critical for nutrition of both partners, and plant protection against soil parasites and toxic compounds. The mycorrhizal network can also reduce carbon costs of ectomycorrhiza formation for some plants, as the extraradical mycelium is already established and sustained by other plants (Högberg et al., 1999).

The fungi that form ectomycorrhizas with trees also form arbutoid mycorrhizas on the roots of ericaceous plants from the *Arbutoidea* suborder (e.g. *Arctostaphylos* and *Arbutus* spp.; Molina & Trappe, 1982). In addition to the fungal sheath and
hyphal intercellular growth (Hartig net) that are typical of ECM, hyphae penetrate the cell wall and produce intracellular coils in living cells (Smith & Read, 1997). These fungi may mediate interactions between arbutoid plants and ECM trees. For instance, in Californian chaparral Arctostaphylos glandulosa Eastw. may allow the establishment of Pseudotsuga menziesii (Mirb.) Franco seedlings (Horton et al., 1999) by acting as a symbiont reservoir that may contribute to successional transition to forest stages. In the Mediterranean basin Q. ilex naturally establishes in A. unedo-dominated chaparral (Gamisans, 1999). However, sharing of fungal symbionts between Q. ilex and A. unedo has hitherto not been explored. To our knowledge, studies of A. unedo symbionts have mainly focused on mycorrhizal ultrastructure (Fusconi & Bonfante-Fasolo, 1984; Giovannetti & Lioi, 1990; Münzenberger et al., 1992).

Studies of ECM communities are based either on identification of mycorrhizas (the so-called below-ground view), or on monitoring of fruitbody production (above-ground view). Identification of mycorrhizas can be conducted according to root-tip morphotype or using molecular tools, such as restriction fragment length polymorphism (RFLP) or sequencing of the internal transcribed spacer (ITS) region, an efficient way to dissect ECM communities (Garde & Bruns, 1996; Horton & Bruns, 2001; Tedersoo et al., 2003). Fruitbody surveys reveal the presence of ECM taxa in a fast and inexpensive way (Vogt et al., 1992; Richard et al., 2004). However these studies, mainly carried out on fleshy macromycetes, often underestimate the presence of numerous resupinate taxa (e.g. Thelephoraceae or Sebacinaceae), hypogeous fungi, and taxa lacking an apparent sexual stage (e.g. Cenococcum geophilum Fr.) (Horton & Bruns, 2001).

Little is known about the below-ground community of ECM fungi in broadleaved forests. For instance, most descriptions of ECM communities in Q. ilex forests have been based on fruitbody surveys (Signorello, 1996; Laganà et al., 1999; Richard et al., 2004). The problems with the use of fruitbody sampling are obvious to anyone who has collected fungi for many years. First, fruiting may vary tremendously from year to year. Second, sampling must be intensive because fruit bodies of many species are short-lived. Thus, in addition to the analysis of fruitbody patterns, there is a need to explore the ECM community in the soil from either ectomycorrhizas or mycelia. A study conducted recently by De Román & De Miguel (2002) has revealed the presence of numerous species of Thelephoraceae in managed Q. ilex stands. However, further research using molecular tools is necessary to document the below-ground diversity in Q. ilex forests.

In a previous study (Richard et al., 2004), we analysed the temporal and spatial patterns of fruitbody production in an old Holm oak forest in Corsica during three consecutive fruiting seasons. Fleshy epigamic macromycetes were surveyed in a permanent plot (160 × 40 m) from September 1999 to March 2002. Here we sampled ectomycorrhizas from Q. ilex and arbutoid mycorrhizas from A. unedo shrubs at the same research site in March 2001. On Q. ilex we collected ectomycorrhizas from seedlings, young saplings and old trees. Our objectives were to: (i) document the below-ground ECM richness in an old-growth Mediterranean forest; (ii) investigate two factors potentially shaping this richness, i.e. host age and host species; and (iii) relate the structure of the ECM community, as determined by mycorrhizas, to that obtained from fruitbody surveys. To identify the fungal symbionts on roots, we compared RFLP types from mycorrhizas to those from fruitbodies of known species collected from the same site. Dominant fungal associates of A. unedo were also sequenced to investigate in more detail the composition of the below-ground community. We relied on this typically Mediterranean plant species to ascertain the relative importance of fungal groups not sampled during our fruitbody survey, such as resupinate or hypogeous fungi.

Materials and Methods

Study site

The research transect was the same as that studied in our previous paper (Richard et al., 2004). It was located in the Fango valley (42°20′ N; 8°49′ E) in Corsica, on slightly acidic soils. The vegetation at the research site is an old Q. ilex forest that consists mainly of old Q. ilex trees (= 460 stems ha⁻¹), 2- to 10-yr-old saplings, and 1-yr-old seedlings (Panaíotis et al., 1997). A dense 7 m high oligospecific chaparral develops under oak canopies, made up of Phyllyrea latifolia L., Erica arborea and A. unedo (Panaíotis et al., 1997). Two small individuals of Cistus monspelliensis L. were also present at this site. A detailed description of the research site is provided by Richard et al. (2004).

Sampling of mycorrhizas

To investigate the structure of ECM diversity, we sampled four plant categories: old A. unedo shrubs (A.un.) and three Q. ilex categories representing the age sequence: 1-yr-old seedlings (Q.i.l1); 2- to 10-yr-old saplings (Q.i.l2); and 170-yr-old senescent trees (Q.i.l3) (Table 1). In each category, 30 individuals scattered over the whole study site (from 30 different 100 m² plots) were sampled (Fig. 1). Plant age was determined either by counting shoot ring number (for Q.i.l1 and Q.i.l2) or using data from Panaíotis et al. (1997) (for Q.i.l3). Host species was ascertained by tracking roots to the shoot (for Q.i.l1 and Q.i.l2) or using cambium colour and root architecture which differ between the two host species (for A.un. and Q.i.l3).

Seedlings (Q.i.l1) and saplings (Q.i.l2) were carefully removed from the soil in order to keep the root system intact and avoid fine root disruption. ECM fungal diversity was evaluated in these two plant categories by exhaustive hand-picking of ECM after pulling up. Soil cores were collected for old A. unedo shrubs (A.un.) and Q. ilex senescent trees (Q.i.l3): for each individual, four 10 × 10 cm soil samples
were taken at the four cardinal points, from the humus organic horizon to a depth of 20 cm. Roots were sieved from the soil cores, carefully washed, and samples from a given plant were pooled. For each individual plant, five root samples were randomly selected, and 30 ECMs from 30 distinct aggregates. They were then hand-picked and stored at −20°C in 700 µl CTAB lysis buffer (2% cetyltrimethylammoniumbromide, 100 mM Tris–HCl, 20 mM EDTA, 1.4 mM NaCl). Picking was performed to maximize the number of morphotypes recovered on each plant, in order to obtain the most complete description of the community.

*Cenococcum geophilum* mycorrhizas were considered characteristic enough to be identified by morphology (Agerer, 1987–93). However, in order to ascertain this identification, two randomly selected *C. geophilum* mycorrhizas from five different individuals per plant category were screened by RFLP analysis, and three were sequenced. All other ECM morphotypes were submitted to molecular analysis according to the following sampling design: (i) exhaustive typing for *Q. ilex* and *Q. il* because of the low number of non-*C. geophilum* ECM recovered; (ii) five mycorrhizas per plant for *A. un*.; (iii) seven ECM per plant for *Q. ilex*.

**Polymerase chain reaction**

DNA was extracted as described by Gardes & Bruns (1993). Extracted DNA was resuspended in 30 µl TE buffer (1 mM Tris–HCl, 0.1 mM EDTA pH 8.0) and diluted for PCR with sterile distilled water at a ratio of 1 : 300 (v/v). The internal transcribed spacer (ITS) region of the rDNA was amplified by PCR using the primer pair ITS1–F/ITS4 (White et al., 1990; Gardes & Bruns, 1993) in a PTC 200 DNA thermocycler (MJ Research, Inc., Waltham, MA, USA). A fraction of 12.5 µl of the diluted DNA extract was added to 12.5 µl of the PCR mix (final concentrations: 2 mM MgCl₂, 0.2 mM dNTP, 1 mM of each primer, 1× buffer for Taq DNA polymerase, 1 U Taq DNA polymerase) and amplified using the same temperature profile as Gardes & Bruns (1996). Negative controls without DNA were used to detect DNA contaminations of the reagents in every PCR.

**RFLP analysis**

Aliquots of 8 µl of each amplified DNA were digested using the endonucleases *CfoI*, *Hinfl*, *MboI* and *HaeIII*. PCR products were size-fractionated on 3% agarose gels (38% agarose + 62% NU-sieve agarose (FMC BioProducts, Philadelphia, PA, USA), stained with ethidium bromide, and photographed under UV light. *PhX174* digested by *Hinfl* 1 was used to estimate fragment sizes. Gels were scanned using BioCapt 97.03 (Vilber Lourmat, France) and fragment sizes calculated using Bio1D+97.06 (Vilber Lourmat).

**Molecular identification of mycorrhizas**

To allow identification of mycorrhizas, small pieces of fresh tissue were taken from at least one fruitbody for each of the 166 ECM taxa fruting on the study site between September 1999 and January 2002 (Richard et al., 2004). For 66% of the RFLP-typed species only one fruitbody was used; for 48 species (29%) two fruitbodies were used. For the eight remaining taxa, which belong to abundantly fruiting taxa poorly investigated from a taxonomic point of view, more than two fruitbodies were used as follows (*n* = number of fruitbodies tested): *Cortinarius elatior* Fr. (*n* = 4) and *Cortinarius pseudosalor* J. E. Lange (*n* = 3), *Inocybe tigrina* R. Heim (*n* = 4) and *Inocybe flocculosa* (Berk.) Sacc. (*n* = 3), *Leccinum lepidum* (Bouchet ex Essette) Quad. (*n* = 4), *Russula decipiens* (Singer) Svrček (*n* = 4), *Russula fragilis* (Pers. Fr.) Fr. (*n* = 3) and *Russula persicina* var. *rubrata* Romagn. (*n* = 4). DNA extraction, PCR amplification and RFLP digestion were carried out as for mycorrhizas. RFLP patterns from mycorrhizas and from fruitbodies were matched, and ECMs whose patterns did not correspond to any fruitbody were considered as unidentified taxa.

The most abundant taxa were sequenced to specify their taxonomic position: sequencing was restricted to *A. un*, a

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**Table 1** Sampling design and colonization by the dominant *Cenococcum geophilum* for the four host categories investigated

<table>
<thead>
<tr>
<th>Sampling features</th>
<th>Seedlings</th>
<th>Saplings</th>
<th>Old trees</th>
<th><em>Arbutus unedo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Code name</td>
<td><em>Q. ilex</em></td>
<td><em>Q. ilex</em></td>
<td><em>Q. ilex</em></td>
<td><em>A. un</em></td>
</tr>
<tr>
<td>Number of plants</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Number of mycorrhizas</td>
<td>190</td>
<td>304</td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td>Number of mycorrhizas with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. geophilum</em> morphotype</td>
<td>107 (56.3%)</td>
<td>229 (75.3%)</td>
<td>221 (24.5%)</td>
<td>261 (29.0%)</td>
</tr>
<tr>
<td>Other morphotypes</td>
<td>83</td>
<td>75</td>
<td>679</td>
<td>639</td>
</tr>
<tr>
<td>Colonization by <em>C. geophilum</em>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative abundance per plant (%)*</td>
<td>64.3 ± 31.0 a</td>
<td>73.5 ± 30.4 a</td>
<td>29.2 ± 24.6 b</td>
<td>29.1 ± 14.3 b</td>
</tr>
<tr>
<td>Relative frequency among plants (%)</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mean number ± SD, values followed by different letters differ significantly according to ANOVA (P < 0.05).
typically Mediterranean species. Based on RFLP results, A. unedo symbionts that were represented by at least two mycorrhizas in our sampling were sequenced as described by Selosse et al. (2002), and identified according to BLAST analysis at the NCBI page http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, using default settings. Sequences were deposited in GenBank (Table 2).

**Data processing and statistical analyses**

For each plant category, and for the two plant species, the richness of ECM communities was estimated using various species diversity estimators based on abundance and frequency of taxa. Abundance was defined as the cumulative number of mycorrhizas of a given taxon divided by the total number of mycorrhizas, for a given plant category. Frequency was the number of plant individuals on which a given taxon was found divided by the total number of plant individuals, for a given plant category. Species diversity was estimated using: (i) richness, i.e. the total number of taxa, \( S \); (ii) Simpson's diversity index, \( D \); (iii) the Shannon–Wiener information index, \( H' \); and (iv) Fisher's alpha (Fisher et al., 1943). The rarefaction method (Krebs, 1999) was used to compare one-to-one taxonomic richness of samples of various sizes. This method corrects for differences in sampling size by virtually reducing the size of all samples to that of the smallest one. Calculations were performed with the software BIODIVERSITY PRO 2 (http://www.sams.ac.uk/activities/downloads/software/bdpro.zip) and rarefaction curves obtained were compared graphically for the minimal sample size of the various data sets. Rarefaction analysis was used for comparison between the various plant categories (with minimal sample size \( n = 121 \) ECM tips) and for comparison between above- and below-ground diversity (with minimal sample size \( n = 521 \) ECM tips or fruitbodies). A visual comparison of the distributions of relative species abundance and frequency between above- and below-ground views of the ECM community was performed using rank–abundance curves.

Differences in abundance of C. geophilum (the only species that was abundant and frequent enough to perform parametric tests), according to plant category, were tested by one-way ANOVA with plant category as single factor using MINITAB 12.2 software (MINITAB Inc., Paris, France).

Relatedness in the composition of ECM taxa among plant species and categories was compared using the Jaccard similarity index (Mueller-Dombois & Ellenberg, 1974), 

\[
J = \frac{c}{a + b - c} \times 100,
\]

where \( a \) is the number of taxa found on the first plant category, \( b \) is the number of taxa found on the other, and \( c \) is the number of taxa shared by the two plant categories. A percentage similarity (PS) based on RFLP-type abundance was calculated in order to take distribution of taxa into account (Pielou, 1984): 

\[
PS = \frac{c'}{(a' + b')} \times 100,
\]

where \( a' \) is the number of mycorrhizas formed by taxa found on the first plant category, \( b' \) is the number of mycorrhizas formed by taxa found on the other, and \( c' \) is the number of mycorrhizas formed by taxa colonizing the two plant categories.

**Results**

**General description of the below-ground ECM community**

In all, 2294 mycorrhizas were sampled from 120 plants (Table 1), among which a Cenococcum morphotype largely dominated. DNA extraction and RFLP typing were carried out on 558 ECMs, including 40 ascribed to this Cenococcum...
The 40 Cenococcum ECMs showed the same RFLP pattern, and three of them showed a unique C. geophilum ITS sequence (Table 2). The Cenococcum morphotype was therefore considered as homogeneous, accounting for 35.3% of the total ECM number and present on 97.5% of all investigated plant individuals (Table 1).

In all, including C. geophilum, ITS–RFLP analysis was conducted on 393 ectomycorrhizas (data not shown). The typing produced 140 different RFLP patterns (Table S1, available online as supplementary material), and resulted in high values of species richness estimators (Table 3). Identification of the mycorrhizal symbionts to species or species group was performed using ITS–RFLP matches with fruitbodies and direct sequencing of the ITS for the most common symbionts of A. unedo (Tables 2 and S1). No intraspecific polymorphism was observed whenever more than one fruitbody per species

<table>
<thead>
<tr>
<th>Abundance rank on A. unedo roots*</th>
<th>Tentative identification†</th>
<th>GenBank accession number</th>
<th>Closest GenBank species</th>
<th>BLAST expected value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>C. geophilum</td>
<td>AY825508</td>
<td>AY394919 Cenococcum geophilum</td>
<td>0.0</td>
</tr>
<tr>
<td>2 (4)</td>
<td>Thelephoraceae #5</td>
<td>AY825526</td>
<td>AJ534912 Tomentella sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>3 (6)</td>
<td>Inocybe tigrina†</td>
<td>AY825515</td>
<td>AY310829 Uncultured ECM</td>
<td>1 e-124</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AY751556 Inocybe sp.</td>
<td>1 e-123</td>
</tr>
<tr>
<td>4 (14)</td>
<td>Thelephoraceae #3</td>
<td>AY825524</td>
<td>AF272915 Tomentella cinerascens</td>
<td>0.0</td>
</tr>
<tr>
<td>5 (17)</td>
<td>Sebacinaeae #1</td>
<td>AY825518</td>
<td>AJ534907 Sebacinaeae sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>6 (18)</td>
<td>Sebacinaeae #3</td>
<td>AY825520</td>
<td>AF440646 Sebacinomyxorrhiza</td>
<td>0.0</td>
</tr>
<tr>
<td>7 (5)</td>
<td>Sebacinaeae #4</td>
<td>AY825521</td>
<td>AY243531 Uncultured mycorrhiza (sebacinoid)</td>
<td>0.0</td>
</tr>
<tr>
<td>8 (20)</td>
<td>Laccaria laccata†</td>
<td>AY825516</td>
<td>AY634142 Uncultured ECM (Inocybe)</td>
<td>1 e-100</td>
</tr>
<tr>
<td>9 (30)</td>
<td>Inocybe #1</td>
<td>AY825514</td>
<td>U83467 Thelephoraceae sp.</td>
<td>1 e-174</td>
</tr>
<tr>
<td>10 (41)</td>
<td>Thelephoraceae #1</td>
<td>AY825522</td>
<td>U92537 Tomentella sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>11 (68)</td>
<td>Thelephoraceae #4</td>
<td>AY825525</td>
<td>AF440647 Sebacinomyxorrhiza</td>
<td>0.0</td>
</tr>
<tr>
<td>12 (40)</td>
<td>Sebacinaeae #2</td>
<td>AY825519</td>
<td>AF534906 Russula sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>13 (15)</td>
<td>Russula nuragica†</td>
<td>AY825517</td>
<td>AJ534906 Russula sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>14 (27)</td>
<td>Clavulinaceae #1</td>
<td>AY825509</td>
<td>AF534200 Uncultured ECM (Clavulinaceae)</td>
<td>0.0</td>
</tr>
<tr>
<td>15 (47)</td>
<td>Thelephoraceae #7</td>
<td>AY825528</td>
<td>AF272904 Tomentella atramentaria</td>
<td>0.0</td>
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<tr>
<td>16 (69)</td>
<td>Thelephoraceae #2</td>
<td>AY825523</td>
<td>AF430289 Tomentella sp.</td>
<td>ECM 0.0</td>
</tr>
<tr>
<td>17 (66)</td>
<td>Thelephoraceae #6</td>
<td>AY825527</td>
<td>AF465184 Uncultured ECM (Thelephoraceae)</td>
<td>0.0</td>
</tr>
<tr>
<td>18 (16)</td>
<td>Cortinarius #1</td>
<td>AY825511</td>
<td>A8096872 ECM of Salix reinii</td>
<td>6 e-90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A534923 Inocybe sp.</td>
<td>3 e-88</td>
</tr>
</tbody>
</table>

The 18 RFLP types occurring more than once in the sampling were sequenced. Closest sequences from identified species, as found by BLAST analysis, are indicated.

*Values within parentheses indicate the rank of RFLP types based on their relative abundance in the whole ECM community.
†Inocybe tigrina, Laccaria laccata and Russula nuragica were identified from perfect matches between RFLP patterns of mycorrhizas and fruitbodies.
‡BLAST expected value represents the number of sequence matches expected by random chance (the smaller the value, the better the match to the reported NCBI database sequence).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Below-ground</th>
<th>Above-ground</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q. il.1</td>
<td>Q. il.2</td>
</tr>
<tr>
<td>Number of taxa*</td>
<td>37 (13)</td>
<td>25 (11)</td>
</tr>
<tr>
<td>Estimated number of taxa based on rarefaction analysis†</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Simpson's diversity index</td>
<td>0.579</td>
<td>0.244</td>
</tr>
<tr>
<td>Shannon–Wiener information index</td>
<td>3.68</td>
<td>1.70</td>
</tr>
<tr>
<td>Fisher’s alpha</td>
<td>14.8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Above-ground diversity (based on fruitbody surveys) from Richard et al. (2004).

*Taxa are RFLP types for below-ground diversity and species based on a morphological concept for above-ground diversity. Values in parentheses indicate number of taxa for which RFLP patterns from mycorrhizas and fruitbodies from the same study site successfully matched.
†Rarefaction analysis conducted on Q. il.1, Q. il.2, Q. il.3 and A. un. (minimal sample size, n = 121 root tips).

morphotype (Table 1). The 40 Cenococcum ECMs showed the same RFLP pattern, and three of them showed a unique C. geophilum ITS sequence (Table 2). The Cenococcum morphotype was therefore considered as homogeneous, accounting for 35.3% of the total ECM number and present on 97.5% of all investigated plant individuals (Table 1).

In all, including C. geophilum, ITS–RFLP analysis was conducted on 393 ectomycorrhizas (data not shown). The typing produced 140 different RFLP patterns (Table S1, available online as supplementary material), and resulted in high values of species richness estimators (Table 3). Identification of the mycorrhizal symbionts to species or species group was performed using ITS–RFLP matches with fruitbodies and direct sequencing of the ITS for the most common symbionts of A. unedo (Tables 2 and S1). No intraspecific polymorphism was observed whenever more than one fruitbody per species.
was examined, except for *R. fragilis* and *R. persicina* var. *rubrata* which produced two different patterns (data not shown). Out of 140 RFLP types, 60 (42.9%) were identified at the genus level (Table S1).

Of the 140 RFLP types, 70 were represented by a single mycorrhiza (Fig. 2a), so that the community harbored a large number of rare taxa. *Cenococcum geophilum* and *R. decipiens* were

Table 4 Abundance of main fungal groups (excluding *Cenococcum geophilum*) on three *Quercus ilex* plant categories based on matching with fruitbody RFLP patterns

<table>
<thead>
<tr>
<th>Species/family</th>
<th>Q. il I.1</th>
<th>Q. il I.2</th>
<th>Q. il I.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Russula</em></td>
<td>22 (13)</td>
<td>24.4 (10)</td>
<td>27.1 (45)</td>
</tr>
<tr>
<td><em>Cortinarius</em></td>
<td>5.1 (3)</td>
<td>0</td>
<td>7.2 (12)</td>
</tr>
<tr>
<td><em>Inocybe</em></td>
<td>1.7 (1)</td>
<td>2.4 (1)</td>
<td>4.8 (8)</td>
</tr>
<tr>
<td><em>Amanita</em></td>
<td>0</td>
<td>9.8 (4)</td>
<td>1.8 (3)</td>
</tr>
<tr>
<td>Others*</td>
<td>3.4 (2)</td>
<td>12.2 (5)</td>
<td>11.4 (19)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>67.8 (40)</td>
<td>51.2 (21)</td>
<td>47.6 (79)</td>
</tr>
</tbody>
</table>

Values are either percentage of total number of typed mycorrhizas (number of mycorrhizas in parentheses), or percentage of total number of RFLP types (number of RFLP types in parentheses).

*Tricholoma, Laccaria, Hygrophorus, Lactarius, Inocybe, Hebeloma and Clavulinaceae.*

Fig. 2 Dominance–diversity curves for above-ground (fruitbodies, open circles) and below-ground (ectomycorrhizas, filled circles) ECM communities based on either (a) relative abundance or (b) relative frequency of taxa. Left to right, most frequent to less frequent species. Abundance data are numbers of fruitbodies (above-ground) or mycorrhizas (below-ground) per species. Frequency data are numbers of 100 m² plots in which a species fruited (above ground) and number of plant individuals on which an RFLP type was found (below ground). Above-ground data (based on fruitbody surveys) are from Richard et al. (2004).
Distribution of the ECM community among the two host species

In all, 46 RFLP types were found on *A. unedo* and 112 on *Q. ilex* (Table 3). In a rarefaction analysis performed to account for differences in sample size (Table 3), the diversity for *A. unedo* was intermediate between that of senescent oaks and those of seedlings and saplings. Similarly, Shannon entropy, Simpson diversity index and Fisher’s alpha were higher for *A. unedo* than for young *Q. ilex* (*Q.i1* and *Q.i2*), but lower than for old *Q. ilex* trees (*Q.i3*, Table 3), suggesting that *Q.i3* harbored more rare species than the three others. Only 18 of the 140 RFLP types occurred both on *A. unedo* and *Q. ilex* roots (Fig. 3). These two-host taxa represented 12.9% of the taxonomic diversity, but colonized 69.4% of all mycorrhizas sampled (Fig. 3) because of the abundance of *C. geophilum* on both hosts. Relative taxa frequencies had similar distributions on the two hosts, with 50.9 and 60.9% of the RFLP types found only once on *Q. ilex* and *A. unedo* roots, respectively (data not shown). At the other extreme, only three RFLP types were found on at least four plant individuals of the same species (data not shown). Because of the low number of mycorrhizas representing each RFLP type (probably caused by the size of our sample), differences in abundance between hosts could not be tested statistically.

On *Q. ilex* roots, 48 RFLP types (42.9%) were identified to genus, species or family level, based on RFLP matches with fruitbodies (Table 4) or sequence analysis of taxa that were shared with *A. unedo* (Table 2). The genus *Russula* was the most represented (Table 4), accounting for 22–27.1% of the identified ECM and between 12 and 16.7% of the corresponding taxonomic diversity, depending on plant age. At the species level, apart from *C. geophilum*, *R. decipiens* (a species linked to the genus *Quercus*) was the most abundant (10.5% of the total number of *Q. ilex* mycorrhizas) and most frequent species (present on 28.3% of sampled *Q. ilex*, data not shown).
On *A. unedo* roots the molecular analysis allowed identification of 28 taxa, accounting for 81.4% of the typed mycorrhizas and 60.9% of the species richness (data not shown). Thelephoraceae was the most represented, accounting for 25% of taxonomic diversity and 35.7% of the identified ECMs (Fig. 4). To a lesser extent, the genus *Inocybe* (21.4% of the ECMs) and the Sebacinaeae family (18.6% of the ECMs) were well represented (Fig. 4). The genera *Russula, Cortinarius* and *Laccaria*, as well as the Clavulinaceae family, were also present (Table 2). At the species level, the ECM community on *A. unedo* was strongly dominated by *C. geophilum*, present on all 30 sampled plants (Table 1). In addition to *C. geophilum*, only three species, two Thelephoraceae species and *I. tigrina*, were present on at least three plant individuals (data not shown).

**ECM community and Quercus ilex age**

The three *Q. ilex* categories were dominated by *C. geophilum* ECM (Table 1). In addition to *C. geophilum*, variable numbers of RFLP types were found (Table 3), and rarefaction analysis suggested that the ECM richness was higher under senescent trees than for *Q. il.1* and *Q. il.2* (Table 3). For each age, *R. decipiens* was among the most frequently recorded, and the only species found on more than three plant individuals (data not shown). Most RFLP types (90 out of 112) were found on one *Q. ilex* age only, while 19.6% were shared by at least two categories (Fig. 5). Only *C. geophilum, R. decipiens*, and two unidentified species were found on the three plant categories (Fig. 5). *Cenococcum geophilum* was significantly more abundant on *Q. il.1* and *Q. il.2* than on *Q. il.3* (Table 1). Based on taxonomic diversity, *Q. ilex* of various ages shared few fungal partners (between 6.9 and 14.8%; Table 5). However, based on RFLP type abundances, similarities ranged from 21.4% (24 RFLP types) to 80% (9 RFLP types) between each category, while similarities were 70% (46 RFLP types) between *Q. il.1* and *Q. il.2* (Table 5). Values are percentage of total number of RFLP types (*n* = number of RFLP types).

**Comparison between above- and below-ground views**

In all, 260 ECM taxa were revealed on the studied transect by combining the present ECM typing and the 166 species from fruitbody identifications by Richard *et al.* (2004) (Table 3). Rarefaction analysis (data not shown) suggested that, for the minimal samples size *n* = 521, below-ground richness was higher (127.3 taxa) than above-ground diversity (92.4 taxa). Only 17.7% (46 species) of these taxa were found only below-ground (Table 3). In addition, nonfruiting species do exist on this site, as exemplified by *C. geophilum*. Reciprocally, 46.1% of the total taxonomic diversity (120 species) that fruited was not found on roots (Table 3).

**Dominance–diversity curves**

Similarity estimators showed very similar distributions of above- and below-ground diversities. The ECM community was strongly dominated by rare species (Fig. 2a): more than 60% of the taxa produced less than one fruitbody per 1000 m², while 50% of the taxa were represented by only one mycorrhiza. Similarly, both distributions showed a few very abundant species (Fig. 2a): three dominant species (*Lactarius laccata, I. tigrina* and *Lactarius chrysorrheus*) produced 32.9% of all fruitbodies (Richard *et al.*, 2004), while *C. geophilum* accounted for more than a third of all mycorrhizas (Table 1). An ordination based on either relative abundance (Fig. 6a) or frequency (Fig. 6b) of RFLP types was used to visualize correspondences between above- and below-ground views. Of the 22 most abundant ECM RFLP types (including *C. geophilum*), only eight produced epigeous conspicuous fruitbodies, including the three most productive ones, *L. laccata, I. tigrina* and *L. chrysorrheus* (Fig. 6a). Using mycorrhizas and fruitbody frequencies, a similar relative discrepancy was observed,

**Table 5** Comparison of ectomycorrhizal fungal communities between plant species and plant categories based on presence/absence of taxa, using a Jaccard similarity index (*J*) or quantitative comparisons (abundance of taxa, considering the dominant *Cenococcum geophilum* or not), using a percentage of similarity (PS)

<table>
<thead>
<tr>
<th>Category</th>
<th>J</th>
<th>PS (+ C.g)*</th>
<th>PS (− C.g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q. il.1 and Q. il.2</td>
<td>6.9</td>
<td>81.7</td>
<td>20.0</td>
</tr>
<tr>
<td>Q. il.1 and Q. il.3</td>
<td>13.0</td>
<td>63.9</td>
<td>31.1</td>
</tr>
<tr>
<td>Q. il.2 and Q. il.3</td>
<td>14.8</td>
<td>71.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Q. il.1 and A.un.</td>
<td>10.7</td>
<td>59.6</td>
<td>21.2</td>
</tr>
<tr>
<td>Q. il.2 and A.un.</td>
<td>4.4</td>
<td>71.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Q. il.3 and A.un.</td>
<td>13.0</td>
<td>53.8</td>
<td>28.9</td>
</tr>
</tbody>
</table>

*Including Cenococcum geophilum.†Without Cenococcum geophilum.*

63.9 to 81.7% (or at least 17.9% excluding *C. geophilum*), with successive age categories being more similar than the distal ones, *Q. il.1* and *Q. il.3* (Table 5).
with only nine taxa found above ground (including the four most frequent) belonging to the 22 most represented ECM RFLP types (Fig. 6b). A single fruiting species, *R. decipiens*, was present among the seven most frequent RFLP types found in this study (Fig. 6b). This taxon was not abundant (ranking 29th) and relatively infrequent (ranking 28th) above ground (data not shown). Symmetrically, some very infrequent species producing few fruitbodies were more represented below ground, such as *Cortinarius* subspg. *Phlegmacium*-3 and *Inocybe* subspg. *Inocybium*-3. They produced, respectively, one and three fruitbodies over 3 yr (Richard et al., 2004), but their ECM occurred on three different plant individuals (Fig. 6b).

**Discussion**

The present study confirms the remarkable species richness of the fungal community measured by fruitbody surveys of epigeous macrofungi at the same site (Richard et al., 2004). High values of species diversity estimators were obtained, as illustrated by Fisher’s alpha (Table 3), an estimator linking the number of taxa to the number of individuals sampled, which is not unduly affected by sample size (Tokeshi, 1993). Assuming that each RFLP type corresponds more or less to one species (see below), a total of 140 species were detected based on analysis of mycorrhizas (one RFLP type per 2.5 tips investigated), excluding *C. geophilum*, compared with 166 species that have been found using fruitbody surveys (Richard et al., 2004). Based on these two approaches together, there were at least 260 ECM fungal species at the site between 1999 and 2002. This is more than in most previously described late successional stands covering similar areas (Jonsson et al., 1999; Bidartondo et al., 2000, 2001), although similar values were found in old temperate coniferous forests, either by ECM typing (Dahlberg et al., 1997; Luoma et al., 1997) or fruitbody surveys (Villeneuve et al., 1989; O’Dell et al., 1999; Smith et al., 2002).

Our results suggest that ITS–RFLP data are robust for characterizing community diversity, for two reasons. First,
from 158 morphologically defined species that were used in the DNA analysis, 144 (91% of total) yielded a single species-specific RFLP type (Table S1 and data not shown). Second, intraspecific variation was a minor problem. Of the 58 species represented by at least two fruitbodies, 56 (96%) yielded a single RFLP type for all fruitbodies with the exceptions of \textit{R. fragilis} and \textit{P. persicina var. rubrata} (Table S1 and data not shown). Together our results highlight the fact that ITS–RFLP data are a valuable tool for grouping ECM species, and for identification of the mycorrhizal symbionts with the fruitbody RFLP-matching approach. These results are similar to those reported by Horton (2002), who also investigated the use of ITS–RFLP patterns to assess diversity of ECM fungi collected across a 7 km coniferous forest. In addition, Kärén \textit{et al.} (1997) already reported that intraspecific variation was not a problem on a local scale. In the two polymorphic species of \textit{Russula}, the RFLP variation found in the ITS is the result of variation in two of the four endonucleases (data not shown). Currently, we do not know if the variation observed is a reflection of cryptic species.

The below-ground method revealed the same distribution pattern as the above-ground survey with respect to the relative proportion of abundant vs rare taxa. The below-ground community was characterized by a few common types and a large number of rare types (Fig. 2). This pattern was also observed using fruitbodies (Fig. 2; Richard \textit{et al.}, 2004). Below ground, 50% of the RFLP types collected were represented by one mycorrhiza. The two dominant species were \textit{C. geophilum} (Table 1) and \textit{R. decipiens} (Fig. 6a). \textit{Cenococcum} alone contributed to 35% of the ectomycorrhizas. Of the 120 total plants, 117 (98%) were colonized by this fungus (Table 1). \textit{Russula decipiens} was found on 17 oaks (data not shown). However, several questions remain concerning the below-ground diversity because of the large number of rare types observed at our site. Which proportion of the local community is really sampled? Would comparable patterns be obtained at another time? Are all abundant species included? Our ability to detect community similarity (e.g. \textit{Arbutus} vs \textit{Quercus}) based on species abundance is also limited by the inherent distribution of the diversity.

Fungal species richness was comparable above and below ground, but the two levels exhibited little overlap (< 20%; Table 3) in fungal species composition. This result confirms and extends earlier observations on the complementarity of the two levels in obtaining a comprehensive view of community composition (Gardes & Bruns, 1996; Jonsson \textit{et al.}, 2000; Peter \textit{et al.}, 2001). For instance, without the below-ground approach we would have missed \textit{C. geophilum}, an ascomycetes species that does not produce fruitbodies at all. It was particularly abundant on oak seedlings and saplings (Table 1). Its high dominance and frequency at our site may arise in part from its ability to sustain xeric conditions by formation of sclerotia (Lilleskov \textit{et al.}, 2004). The role of this fungus in ecosystem functioning is also intriguing – could it provide drought protection to plant roots, as suggested by Jany \textit{et al.} (2003)? Or, alternatively, could \textit{C. geophilum} be purely opportunistic, with little relevance to tree physiology?

The observation that \textit{C. geophilum} often dominates in ECM communities, for example in Spanish \textit{Q. ilex} forests (De Román & De Miguel, 2002); in the Californian chaparral (Borchers & Perry, 1990); or in temperate \textit{Fagus sylvatica} forests (Blaise & Garbey, 1983), leads us to question the existence of ectotypes or cryptic biological species (Shinohara \textit{et al.}, 1999).

Combining the species composition viewed above and below ground, the following patterns were observed. Apart from \textit{Cenococcum}, the community appeared to be dominated by members of the genus \textit{Russula} and, to a lesser extent, by the genus \textit{Inocybe} as well as members of the Thelephoraceae and Sebacinaeae (Fig. 4; Richard \textit{et al.}, 2004). In Spanish managed \textit{Q. ilex} forests, thelephoroid morphotypes accounted for a quarter of the root tips investigated by De Román & De Miguel (2002). Russulaceae and Thelephoraceae also dominated the community in two other Californian Mediterranean ecosystems, the chaparral (Horton \textit{et al.}, 1999) and the bishop pine forest (Gardes & Bruns, 1996), whereas Sebacinaeae were among the most frequently encountered taxa in \textit{Eucalyptus} sclerophyllous forests in Australia (Glen \textit{et al.}, 2002). An intriguing feature is the absence of hypogeous fungi (at least among dominant taxa on \textit{A. unedo}, Table 2 and S1), which is perhaps caused by environmental conditions. For instance, the lack of species of \textit{Tuber} may be explained by acidic soil conditions.

Tree diversity has been suggested to favour ECM diversity on a local scale (Nantel & Neumann, 1992; Kernaghan \textit{et al.}, 2003). We tested the hypothesis that the hosts contribute to ECM fungal diversity. Only 12.9% of the taxa were shared (Fig. 3), less than what was found in mixed forest stands by Horton & Bruns (1998), Cullings \textit{et al.} (2000) and Kennedy \textit{et al.} (2003), where multihost fungi dominated, accounting for 30 to 90% of the ECM fungal community in all three studies. Unfortunately our sampling is insufficient to provide statistically significant data, because of the high species richness of the community. Most species were too infrequent to draw conclusions about their distribution, a problem that is often limiting in studies of ECM communities (Horton & Bruns, 2001; Taylor, 2002). Nevertheless, even if restricted to a limited number of fungal taxa, sharing of symbionts may have ecological consequences as \textit{Q. ilex} seedlings successfully establish and survive in \textit{A. unedo}-dominated chaparral (Gamisans, 1999). This pattern suggests that \textit{A. unedo} shrubs may provide conducive conditions for \textit{Q. ilex} seedlings in early stages of forest succession, perhaps by providing a compatible fungal network.

Despite the important width of the age sequence, the ECM community was quite similar at the various developmental stages of \textit{Q. ilex} investigated. We observed: (i) similar rank–abundance curves (reflecting high taxonomic diversity and a
dominance of rare taxa, Table 3); (ii) among the identified taxa, similar dominance of genera such as Russula, Cortinarius and Amanita (Table 4); and (iii) a high abundance of C. geophilum (Table 1). Our findings support the conclusion that established seedlings recruit ECM symbionts in an opportunistic way among mycobionts colonizing the old surrounding trees. Similar observations were made in multi-aged stands dominated by conifers such as Pinus sylvestris (Jonsson et al., 1999) or Tsuga heterophylla (Kranabetter, 1999; Kranabetter & Friesen, 2002).

An intriguing question is whether or not the sharing of ECM partners between seedlings and old trees is under natural selection. Seedlings may take benefit from established ECM fungi that already have large extraradical soil-exploring mycelia built at the oldest trees’ expense (Högberg et al., 2002). In addition, shared symbionts may even transfer carbon from high-canopy trees to understory seedlings (Simard et al., 1997; Lérat et al., 2002), counterbalancing low light influx. For the related species Quercus rubra, seedling nutrition and mycorrhization (infection level and diversity) are improved in the vicinity of adult conspecifics (Dickie et al., 2002). Symbiont sharing between seedlings and older Q. ilex could thus result in favouring of kin, as most Q. ilex acorns remain around the mother tree due to barochory (Darley-Hill & Carter Johnson, 1981).

Conclusions
This first report on the below-ground ECM diversity in a Mediterranean hardwood old-growth forest revealed a striking diversity of ECM fungi. The ascomycete C. geophilum and members of the Russulaceae, Cortinariaceae (genus Inocybe), Thelephoraceae and Sebacinaeae are the most abundant taxa on roots. Exhaustive inventory of the diversity is a real challenge because of the large number of rare types. Our results suggest that both above- and below-ground levels have to be explored to obtain a comprehensive overview of the composition of the ECM fungal community. The composition and diversity of the ECM community does not depend on host age. Our results also question the ecological importance of symbiont sharing between Q. ilex and A. unedo in old-growth forest dominated by Q. ilex.

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Supplementary material
The following material is available as supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1382/NPH1382sm.htm. These supplementary data include (i) for each ITS-RFLP type, DNA fragment sizes in base pairs and (ii) for the 60 taxonomically identified taxa, genus- or species-level identification obtained by comparing ITS-RFLP types to those from voucher specimens of sporocarps or by BLAST analysis of the ITS sequences.

Table S1 ITS-RFLP types of ectomycorrhizas collected on the 120 sampled plants.

References


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