Molecular markers detecting an ectomycorrhizal *Suillus collinitus* strain on *Pinus halepensis* roots suggest successful inoculation and persistence in Mediterranean nursery and plantation

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**Abstract**
Survival of the ectomycorrhizal fungal strain *Suillus collinitus* Sc-32 on *Pinus halepensis* after inoculation and outplanting was monitored in a Mediterranean plantation. Three molecular fingerprints were developed: RFLP of the internal transcribed spacer ribosomal DNA, intersimple sequence repeat, and a specific sequence-characterized amplified region marker. The inoculant was demonstrated to survive on inoculated seedlings 4 years after outplanting (56 months after inoculation), although *S. collinitus* was not fruiting. The designed markers set allows reliable and inexpensive monitoring of inoculated seedlings and suggests that *S. collinitus* is suitable for inoculation of Mediterranean *Pinus*. These data are discussed in the framework of suillloid population ecology.

**Introduction**

*Suillus* are basidiomycetes from temperate and Mediterranean forests that associate with tree roots, forming ectomycorrhize (ECM), mainly on *Pinaceae* but also on deciduous species (Courtecuisse & Duhem, 1994; Kretzer et al., 1996; Wu et al., 2000). Within this group, several *Suillus* spp. were thoroughly investigated as models of ECM fungal population, because they form abundant fruitbodies (or ‘sporophores’, the fleshy structures that bear the meiotic spores) that allow easy genotyping (see below). Genets were shown to reach metric size and to last over many years (Dahlberg & Stenlid, 1990, 1994; Jacobson et al., 1993; Bonello et al., 1998; Zhou et al., 1999, 2000), often increasing in size in ageing forests (Dahlberg & Stenlid, 1990, 1994, but see Zhou et al., 2000 for an exception). They behave as early colonizers, propagating by spores (Dahlberg & Stenlid, 1994, 1995; Zhou et al., 1999) in young forests or after disturbance (Bruns et al., 2002). Besides this ruderal strategy, their competitive abilities allow their persistence in older forests; late colonization may also occur in old populations, continuously giving rise to small genets (Dahlberg, 1997; Boneillo et al., 1998).

Two ecological traits, abundance and persistence, confer on *Suillus* spp. a potential interest as inoculants for forestry purposes. *Suillus collinitus* (Fr.) O. Kuntze forms ECMs with various *Pinus* hosts (Torres et al., 1991; El Karkouri et al., 1996; Bonfante et al., 1997; Rincon et al., 1999; Manian et al., 2001). In Mediterranean forest environments with drought stress and nutrient deficiency, *S. collinitus* is used as a mycorrhizal inoculant in nurseries and experimental plantations to improve pine growth, mineral nutrition and survival (Mousain et al., 1994; Torres & Honrubia, 1994; Roldán et al., 1996; Argillier et al., 1997; El Karkouri et al., 2002, 2004; Gonzalez-Ochoa et al., 2003). A critical problem in mycorrhizal inoculation is survival of inoculants that can be out-competed by other ECM fungi, either in the nursery or forest during, as well as after, outplanting (Le Tacon et al., 1992, 1997; El Karkouri et al., 2002). In addition, when using selected strains, meiotic sporulation and mating, likely to occur in species producing fruitbodies, can lead to replacement by different, possibly less efficient strains.
Molecular markers detecting an ectomycorrhizal fungal strain

(introgressive replacement, Selosse et al., 1998a, 1999). It was also demonstrated for *S. pungeis* that selfing can occur by formation of dikaryotic basidiospores after meiosis (secondary homothallism, Jacobson & Miller, 1994; Bonello et al., 1998), also modifying the selected genetic background. Last, mycorrhizal persistence should ideally be assessed directly on ECMs, not only by fruitbody surveys (Hönig et al., 2000; Weber et al., 2002). Although good congruence between below-ground genets and fruiting genets exists in some *S. grevillei* populations (Zhou et al., 2001b), methods investigating ECMs are required for *S. collinitus*, as this species rarely produces fruitbodies under young trees, in young Mediterranean plantations.

Various methods are available to distinguish *Suillus* spp. genets. *S. collinitus* shows somatic incompatibilities among genets (El Karkouri et al., 1996), as many other *Suillus* spp. (Dahlberg & Stenlid, 1990, 1995, 1994; Dahlberg, 1997), but this method is time-consuming and necessitates *in vitro* isolation. It may also not distinguish closely related strains (Jacobson et al., 1993). Intraspecific variability of isozymes and intersimple sequence repeat (ISSR) fingerprints were also used for *S. collinitus* strain typing (El Karkouri et al., 1996; Bonfante et al., 1997), but their direct use on ECMs, that consist of tissues of both symbionts (plus contaminants), remains questionable. In a recent study of the related species *S. grevillei* in Japan, ISSR successfully revealed the population structure (Zhou et al., 2000), but analysis of ECMs required development of specific primers amplifying microsatellites specific to this species (Zhou et al., 2001a,b). Thus, there is a need for markers identifying ECMs formed by the *S. collinitus* inoculant, in order to control mycorrhizal persistence on seedlings at the nursery phase and in plantations.

Molecular methods based on PCR are potentially specific tools for monitoring mycorrhizal inoculants, at the species and strain levels. For instance, specific primers designed from sequences of the internal transcribed spacer (ITS) of nuclear ribosomal RNA genes or from the random amplified polymorphic DNA (RAPD), led to sequence-characterized amplified region (SCAR) markers, which successfully identified *Tuber* species at the vegetative and reproductive phases (Amicucci et al., 1998; Bertini et al., 1998; Mello et al., 1999). Persistence of inoculated strains was recently investigated on ECM for two species: in *Paullinia involutus*, RFLP patterns of an intergenic spacer of the ribosomal DNA (IGS1) allowed detection of a subgroup of strains, including the inoculant one, among nursery-collected ECMs (Hönig et al., 2000); for *Laccaria bicolor*, SCAR markers generated from RAPD (Selosse et al., 1998a,b) allowed the typing of an inoculant strain on ECMs both in the nursery and plantation (Weber et al., 2002). Besides these studies, few genetic data are available on *in situ* survival of introduced ECM strains. From an ecological point of view, this raises the question of whether natural microbial populations can be ameliorated by inoculation: does inoculation (a way of escaping from the usual recruitment) confer some advantage on the inoculant (1) on the inoculated niche and (2) for reaching new surrounding niches?

In the present study, molecular markers were developed for specific and rapid detection of *S. collinitus* strain Sc-32, either *in vitro* or on *Pinus halepensis* ECMs. Both partners are tentatively used for desertification control in Mediterranean environments (El Karkouri et al., 2004). A combination of three molecular markers, RFLP analysis of the amplified ITS, ISSR fingerprints and a SCAR marker was used to investigate the persistence of the inoculant strain Sc-32 on nursery seedlings and *in silva* outplanted plants, as well as its colonization of surrounding uninoculated seedlings.

**Materials and methods**

**Fungal strains and axenic roots**

For this study we used 24 dikaryotic strains of *Suillus collinitus*, including strain Sc-32 (= strain J3.15.32), and 18 strains of other ECM species commonly occurring in Mediterranean nurseries or forests (El Karkouri et al., 2002, 2004), including phylogenetically related *Suillus* and *Rhizopogon* spp. (Table 1). This sampling encompasses strains from the Rieucoulon site that is used below for *Pinus halepensis* plantation. Vegetative mycelia were obtained from the collection of the Rhizosphere & Symbiose Lab (INRA-Montpellier), or isolated from fruitbodies for this study, as in (El Karkouri et al., 1996). In the latter case, fungal identification was carried out on fruitbody morphology and ensured by RFLP analysis of the ITS (not shown).

Six axenic *P. halepensis* roots were obtained from six uninoculated seedlings (Table 2). All seedlings used in this study share the same origin (seed provenance: ’02-Provence, Vilmorin, France’ El Karkouri et al., 2004). Seeds were immersed in distilled water at 4 °C for 48 h, sterilized for 15 min with *H₂O₂* (30%) and rinsed three times with sterilized and distilled water. They were then germinated on 5% agar with a day/night period of 16 h light at 22 °C and 8 h night at 18 °C. Roots were harvested after 3 weeks.

**Nursery-inoculated Pinus halepensis cultivation and ECM sampling in nursery**

*Pinus halepensis* seedlings were cultivated at the Pépinière Forestière de l’Etat (Les Mûles, Aix-en-Provence, Bouches-du-Rhône, France) and inoculated with *S. collinitus* strain Sc-32, as described by El Karkouri et al. (2004). Another set of seedlings (‘control’ seedlings) was grown without inoculation. After 9 months of growing in the nursery, these
Table 1. ECM species and strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Tissue used</th>
<th>Host species</th>
<th>Geographical origins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Suillus collinitus</em> (Fr.) O. Kuntze</td>
<td>Sc-32</td>
<td>Mycelium</td>
<td>P. halepensis M.</td>
<td>Nîmes (Gard)*</td>
</tr>
<tr>
<td></td>
<td>Sc-2</td>
<td>Mycelium</td>
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<td>Rieucoulon (Hérault)*</td>
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<td>Rieucoulon (Hérault)*</td>
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<td>Rieucoulon (Hérault)*</td>
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<td>Rieucoulon (Hérault)*</td>
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<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td></td>
<td>J 3.15.20</td>
<td>Mycelium</td>
<td>P. sylvestris L.</td>
<td>La Joue du Loup (Hautes-Alpes)*</td>
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<tr>
<td></td>
<td>J 3.15.30</td>
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<td>P. sylvestris L.</td>
<td>Coll de Montmirat (Lozère)*</td>
</tr>
<tr>
<td></td>
<td>J 3.15.31</td>
<td>Mycelium</td>
<td>P. sylvestris L.</td>
<td>Les Jaussaux (Hautes-Alpes)*</td>
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<tr>
<td></td>
<td>J 3.15.35</td>
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<td>P. halepensis M.</td>
<td>Levent (Hérault)*</td>
</tr>
<tr>
<td></td>
<td>J 3.15.24</td>
<td>Mycelium</td>
<td>P. pinea L.</td>
<td>La Grande-Motte (Hérault)*</td>
</tr>
<tr>
<td></td>
<td>J B.1</td>
<td>Mycelium</td>
<td>P. halepensis M.</td>
<td>Lunel, Bois de Nabrigas (Hérault)*</td>
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<tr>
<td><em>Suillus mediterraneensis</em> (Jacquetant &amp; Blum)</td>
<td>Sm1</td>
<td>Fruitbody</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td>Redeuil</td>
<td>Sm11</td>
<td>Mycelium</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
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<td></td>
<td>Sm22</td>
<td>Mycelium</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td></td>
<td>Sm4</td>
<td>Mycelium</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lauret (Hérault)*</td>
</tr>
<tr>
<td><em>Suillus granulatus</em> (L.: Fr.) Roussel</td>
<td>S.g. 10</td>
<td>Mycelium</td>
<td>P. contorta Dougl.</td>
<td>ND</td>
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<tr>
<td><em>Suillus variegatus</em> (Sw.: Fr.) O. Kuntze</td>
<td>ECM31</td>
<td>ECM</td>
<td>P. nigra Arnold¹</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<td><em>Suillus bovinus</em> (L.: Fr.) O. Kuntze</td>
<td>ECM57</td>
<td>ECM</td>
<td>P. nigra Arnold¹</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<tr>
<td><em>Xerocomus subtomentosus</em> (L.: Fr.) Quélet</td>
<td>Sst1</td>
<td>Fruitbody</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
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<td>Sst2</td>
<td>Fruitbody</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td></td>
<td>Sst4</td>
<td>Fruitbody</td>
<td>P. halepensis M.</td>
<td>Rieucoulon (Hérault)*</td>
</tr>
<tr>
<td><em>Rhizopogon roseolus</em> (Corda) Th.M.Fr. (= <em>R. rubescens</em> Tul. &amp; C. Tul.)</td>
<td>R 19.1</td>
<td>Mycelium</td>
<td>P. nigra Arnold⁴</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
</tr>
<tr>
<td></td>
<td>B.S.1</td>
<td>Fruitbody</td>
<td>P. nigra Arnold⁴</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<tr>
<td></td>
<td>B.S.2</td>
<td>Fruitbody</td>
<td>P. nigra Arnold⁴</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<tr>
<td></td>
<td>ECM14</td>
<td>ECM</td>
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<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<td></td>
<td>ECM26</td>
<td>ECM</td>
<td>P. nigra Arnold⁴</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
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<tr>
<td><em>Rhizopogon obtexus</em> (Spreng.) S. Rauschert (= <em>R. luteolus</em> auct.)</td>
<td>ECM72</td>
<td>ECM</td>
<td>P. nigra Arnold⁴</td>
<td>Aix-en-Provence (Bouches du Rhône)¹</td>
</tr>
<tr>
<td><em>Thelephora terrestris</em> Fr.: Fr.</td>
<td>T 20.1</td>
<td>Mycelium</td>
<td>ND</td>
<td>Nancy (Meurthe et Moselle)*</td>
</tr>
<tr>
<td><em>Cenococcum geophilum</em> Fr.</td>
<td>Cg SV</td>
<td>Mycelium</td>
<td>Picea sp.</td>
<td></td>
</tr>
</tbody>
</table>

*Forest site.
¹Pinus nigra var. nigra.
²Nursery.
³Pinus nigra var. lancio.
⁴Pinus nigra var. saltzmannii.
ND, not determined.

seedlings were outplanted to the Rieucoulon experimental plantation (see below).

A subset of five inoculated seedlings was used for assessment of *S. collinitus* persistence 4 months after inoculation. Root systems were carefully harvested and washed. Single ECM morphotypes similar to *S. collinitus/P. halepensis* ECMs (Torres et al., 1991) were excised under a binocular microscope, washed once with 10–20 μL of H2O2 (30%) for 30 s and three times with autoclaved H2O in sterilized Eppendorf tubes. In all, 17 ECMs were collected (Table 2).
ECMs from the Rieucoulon experimental plantation

The Rieucoulon site (located at Prades-le-Lez, Hérault, in the south of France) and experimental plantation were described in El Karkouri et al. (2004). After destruction of the previous 20- to 30-year-old forest of Pinus halepensis by fire in 1991 (El Karkouri et al., 2004), two kinds of seedlings were introduced (uninoculated or inoculated with S. collinitus) on a 72 x 45 m plot in December 1996. They were planted out 2.5 m from each other in lines situated 4.5 m apart (see ‘plot II’ in El Karkouri et al., 2004). Neither mature trees nor young naturally-regenerated P. halepensis, Quercus ilex and Q. coccifera were found in 1995. Naturally-regenerated P. halepensis appeared in 1997 only. At Rieucoulon, survival of the inoculant was suggested by significantly greater height increases over 10 and 25 months after out-planting for inoculated seedlings as compared to control ones (+118% and 49%, respectively; P < 0.05 according to the Scheffe test; Mousain et al., unpublished results).

Although suilloid species (S. collinitus and S. mediterraneensis) are present in the forest surrounding the plantation, no Suillus spp. fruitbody was recovered on the plantation between 1996 and 2000 (El Karkouri et al., 2004). However, a molecular survey by ITS typing of 461 ECM on this site in 1998 showed the abundance of suilloids on outplanted P. halepensis roots, such as Suillus collinitus and, at lower frequency, S. mediterraneensis (El Karkouri et al., 2004). In the present study, we further genotype the S. collinitus ECMs already demonstrated to have morphotypes and ITS RFLP patterns identical to those of S. collinitus (El Karkouri et al., 2004). In all, 100 S. collinitus ECMs were available from nine inoculated P. halepensis seedlings (Table 3). In addition, 43 S. collinitus ECMs from three uninoculated and two naturally-regenerated P. halepensis seedlings were also investigated here, in order to look for propagation of the inoculant strain (Table 3).

To further ensure persistence of the inoculant strain Sc-32 on inoculated seedlings in the plantation, 44 suilloid ECMs were collected in the autumn of 2000 from three inoculated seedlings and in vitro isolation of the fungal symbiont was tentatively performed as in (Erland & Soderstrom, 1990). These isolated mycelia were then submitted to molecular analysis at the species and genotype levels.

DNA extraction

All samples were subjected to DNA extraction using the DNAeasy Plant Mini Kit, according to the manufacturer’s recommendations (Qiagen S.A., Courtaboeuf, France). Extracted DNA was stored at −22 °C for molecular typing. DNA concentrations were determined on agarose gel using the low DNA Ladder (GibcoBRL, Life Technologies, Cergy Pontoise, France), and all samples were adjusted to approx. 6 ng μL−1 for mycelia DNA, 30 ng μL−1 for DNA of non-inoculated roots and 2 ng μL−1 solution for ECM DNA.

PCR and RFLP of ITS

The nuclear rDNA internal transcribed spacer (ITS, i.e. ITS1 + 5.8S + ITS2) was amplified by PCR using the universal ITS1 and ITS4 primers set (White et al., 1990) that does not amplify P. halepensis ITS (K. El Karkouri, personal observations). PCR amplifications were carried out in a 50 μL volume obtained by adding 25 μL of diluted sample DNA (two dilution replicates for each PCR: 1/100 and 1/200 for mycelial DNA; 1/5 and 1/10 for ECM DNA) to a 25 μL PCR mix. Final concentrations of the reagents were: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.05% W-1

<table>
<thead>
<tr>
<th>Table 3. Molecular typing of suilloid ECMs of Pinus halepensis collected at the Rieucoulon plantation</th>
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</thead>
<tbody>
<tr>
<td>ECM origin</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Inoculated seedlings</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Uninoculated seedlings</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Regenerated seedlings</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*The ‘Sc‘ type corresponds to an ITS showing the S. collinitus Sc-32 RFLP patterns I for all three restriction enzymes, whereas the ‘Sc+‘ type indicates the presence of faint fragment(s) in addition to a main Sc restriction fingerprint, after PCR or after digestion for at least one enzyme, see Fig. 5a.
†For ISSR types ‘Sc‘, ‘Sc+‘ and ‘non-Sc‘ see Fig. 5b.
‡+ and − denote amplification and non-amplification of the specific SCAR marker, respectively.
§Ratio (to total number of seedlings) of number of seedlings with at least one root tip colonized by a symbiont of fingerprints identical to strain Sc-32.
ISSR-PCR amplification

PCR was carried out in a total volume of 50 μL, with reagent concentrations as above. Dilutions used were the same as above for mycelia and ECMs; 1/100 and 1/500 dilution were used for noninoculated roots. In addition, fungal specificity of ISSR fingerprints was tested on artificial DNA mixtures of *S. collinitus* Sc-32 mycelium and axenic *P. halepensis* root, at different ratios. Fungal DNA was diluted 1/200 (3 × 10^{-2} ng μL^{-1}), and various plant DNA dilutions (1/100, 1/500, 1/1000 or no plant DNA) were added in an equal volume (12.5 + 12.5 μL) as templates. We used the primers (GACA)₄, (GAC)₅ and (GCC)₅ that provided reproducible fingerprints with intraspecific variability among *Suillus* spp., including *S. collinitus* (Bonfante et al., 1997; Zhou et al., 1999). PCR amplifications were carried out on a PTC-100 thermocycler (MJ Research Inc.), using a temperature profile identical to that of Zhou et al. (1999) with only 35 cycles; three different annealing temperatures were tested: 48, 55 and 65 °C. PCR amplifications were performed at least twice with controls (no DNA template), to check for reproducibility and DNA contamination. To summarize ISSR fingerprints, a phenetical analysis was performed using presence (1) or absence (0) of reproducible bands, enabling construction of a squared binary matrix of dissimilarities between *S. collinitus* strains using Jaccard’s distance. A phenogram based on dissimilarity values was constructed by the unweighted pair group method using arithmetic means (UPGMA) cluster analysis (Sneath & Sokal, 1973), with the CLUSTER (http://www2.biology.ualberta.ca/jbrzusto/cluster.php) and TREEVIEW (http://taxonomy.zoology.gla.ac.uk/softwares) (Page, 1996) programs.

SCAR sequencing and primer design

A high-intensity 880 bp fragment amplified by the (GAC)₅ primer from *S. collinitus* strain Sc-32 only was isolated from agarose gel. It was purified, treated with T4 DNA polymerase and T4 polynucleotide kinase (New England BioLabs Inc., Beverly, MA, USA) (Mello et al., 1999), and cloned into EcoRV-digested and dephosphorylated pBluescript II KS+ (Stratagene, La Jolla, CA, USA) according to the manufacturer’s recommendations. Plasmid DNA was then sequenced by Genome Express (Paris, France) using the universal reverse and forward M13 primers. Two specific primers Sc-32-1 (5′-CCATGAGTGTATGCGTGACG-3′) and Sc-32-II (5′-GAGAACGACACAGGGATGG-3′) were designed in *silico* from the insert sequence. Amplification of the SCAR fragment (expected size: 721 bp) was performed exactly as described above for ISSR-PCR, using 0.4 μM each of the two primers (Eurogentec), with the same DNA dilutions. To further ensure the identity of SCARs amplified from sampled ECM with the Sc-32 reference sequence, we randomly selected a SCAR PCR product from three inoculated seedlings from the nursery and six inoculated seedlings from the plantation (in all, 12 PCR products). They were sequenced by Genome on both strands and compared to the reference sequence using Sequencher™ 3.11 for MacOS9 from Genes Codes (Ann Arbor, Michigan, USA).

Results

**ITS RFLP fingerprints of *Suillus collinitus***

All 24 *Suillus collinitus* strains investigated had a 700 bp ITS, but showed variable RFLP patterns, i.e. two with each of *TaqI* and *Hinfl* (Fig. 1a) and four with *MspI* (Table 2). None of these patterns was identical to those of the other ECM species tested (Table 1). ITS-RFLP analysis distinguished the strain Sc-32 from 14 *S. collinitus* strains, including some growing under *Pinus halepensis* trees, but not from the nine others (Table 2). No ITS was amplified from axenic roots of *P. halepensis* using ITS1 and ITS4 in our PCR conditions (not shown), suggesting that the host ITS will not interact in ECM typing.

**ISSR fingerprints of *Suillus collinitus* in pure culture and in the presence of plant DNA***

Reproducible, multiband fingerprints ranging from 0.25 to 2.5 kb were obtained at annealing temperatures of 48 and 55 °C for the (GACA)₄ and (GAC)₅ primers respectively (Fig. 2), the (GCC)₅ primer produced no exploitable fingerprints at any temperature (not shown). ISSR-PCR fingerprints gave 27 distinct and reproducible fragments, allowing the distinction of 11 and eight ISSR patterns in *S. collinitus* using the (GACA)₄ and (GAC)₅ primers respectively (Fig. 2a, Table 2). In all, 12 different genotypes were characterized, with a unique ISSR fingerprint for strain Sc-32 (Fig. 2).
In order to use ISSR analysis directly on ECMs, we also investigated fingerprints of axenic roots of *P. halepensis* seedlings. On all investigated seedlings (Table 2), the primer (GACA)$_4$ always amplified an identical fingerprint, called R1 (Fig. 3a, lane 6) and the primer (GACA)$_5$ always amplified the fingerprint R2 (Fig. 3b, lane 6), suggesting a lack of polymorphism on this seedling source. We then investigated the influence of large amounts of plant DNA on the Sc-32 fingerprint by artificially mixing plant and fungal DNAs at different ratios. All expected fragments of *S. collinitus* Sc-32 were successfully amplified using the (GACA)$_4$ primer (Fig. 3a). But unexpectedly, only some fragments of the R1 fingerprint were present at the higher fungus:plant ratios, no plant fragment was amplified at the 1:1000 dilution. Using the (GACA)$_5$ primer, all fragments of *S. collinitus* Sc-32 were also successfully amplified, whereas those of *P. halepensis* seedlings were not, at the three mixing ratios tested (Fig. 3b). These low or null amplifications of plant DNA, in spite of the high concentrations used in our assays, suggest that clear ISSR fungal fingerprints can be obtained directly from *P. halepensis* ECMs.

**Specific distinction of *Suillus collinitus* Sc-32 by a SCAR marker**

In order to obtain a more specific marker, an 880-bp fragment specific to strain Sc-32 amplified with (GACA)$_5$ (arrowed on Fig. 3b) was cloned and sequenced (GenBank accession no. AY551007). No significantly similar sequence was retrieved from GenBank by Blastn analysis. A pair of internal primers (see Materials and methods), for which no similar fungal sequence was found in GenBank (not shown), was designed. They allowed specific amplification of a 721-bp fragment from strain Sc-32 (Fig. 4a), but neither from the 23 other *S. collinitus* strains (Fig. 4a, Table 2), nor from the other fungi tested (Fig. 4a, Table 1) and axenic *P. halepensis* seedlings (Fig. 4b, Table 2).

**Detection of *Suillus collinitus* Sc-32 on inoculated seedlings from the nursery**

A total of 17 suillloid ECMs from five *P. halepensis* seedlings inoculated with strain Sc-32 and grown in nursery containers (Table 2) were investigated to test (1) our markers on
ECMs and (2) the success of inoculation. All 17 ECMs showed a fungal ITS identical in size and RFLP pattern to those of strain Sc-32 for the three restriction enzymes used (Fig. 1b, Table 2). ITS RFLP typing is therefore possible on P. halepensis ECMs and suggests persistence of strain Sc-32. In ISSR analysis, all ECMs produced the Sc-32 pattern using the (GACA)<sub>4</sub> primer, as well as three major fragments of the P. halepensis R1 fingerprint (Table 2, Fig. 3c). Using the (GACA)<sub>4</sub> primer, all ECMs produced only the fingerprint of strain Sc-32 (Table 2, Fig. 3d). As expected from artificial mixes of fungal and plant DNAs, both ISSR primers successfully allowed the detection of S. collinitus Sc-32 directly on P. halepensis ECMs. Last, we amplified the S. collinitus Sc-32 SCAR marker from all the 17 ECMs (Table 4b). This suggests that our marker sets can be used on ECMs and that the strain Sc-32 successfully formed ECMs on P. halepensis and survived at least in the nursery.

**Detection of Suillus collinitus Sc-32 on inoculated seedlings from the Rieucoulon plantation**

Using this set of markers, we then analyzed 143 suillus ECMs from a plantation with inoculated, noninoculated and naturally regenerated P. halepensis seedlings (Table 3). In a
previous study (El Karkouri et al., 2004), these ECMs were all shown to involve *S. collinitus* based on ITS RFLP and sequence. In more detail, two kinds of ITS RFLP fingerprints, indistinguishable from that of strain Sc-32, were found among the 143 ECMs used in the current study (Fig. 5a and Table 3): one displayed ITS RFLP-type I (pattern ‘Sc’), the other (pattern ‘Sc+’) showed faint fragment(s) in addition to the main Sc fingerprint, for at least one enzyme. The latter fingerprint, with fragments of unequal intensity adding up to more than 700 bp, suggests that at least one other fungus, either soil-borne contaminant or endophytic, was present in low amounts together with *S. collinitus*.

Using the ISSR primers (GACA)_4 and (GAC)_6, three different patterns were retrieved. (1) The so-called ‘ISc’ pattern was identical to the fingerprint from a mix of plant + Sc-32 DNA and from nursery ECMs for both primers (i.e. all fungal fragments with or without some R1 plant fragments, Table 3; Fig. 5b), suggesting that the strain Sc-32 survived on these roots. (2) Some fingerprints lacked at least one of the Sc-32 fragments, and sometimes also contained additional fragments: various fingerprints were of this type (‘non-ISc’, Table 3; Fig. 5b), indicating that these roots were colonized by *S. collinitus* genotypes differing from Sc-32. (3) The ‘ISc+’ pattern (Table 3; Fig. 5b) encompassed fingerprints identical to ‘ISc’, but with at least one additional fragment for one or two primers. This may result either from a contamination, or from a different fungal partner. Interestingly, all ‘ISc+’ ISSR types were found on ECM with an ‘Sc+’ ITS fingerprint already suggesting contamination. Out of the 143 analyzed ECMs, 90 were amplified with the Sc-32 SCAR primers, suggesting that they were colonized by the strain Sc-32 (Fig. 5c; Table 3). To further ensure their identity with strain Sc-32, 12 randomly chosen PCR products were sequenced (11% of all SCAR PCR products, see Materials and methods): all showed full sequence identity to the sequence of strain Sc-32 (not shown). Moreover, all ECMs allowing amplification of the SCAR sequence belonged to the ‘ISc’ or ‘ISc+’ ISSR types that were already suggested to be colonized by strain Sc-32 (Table 3).

All ECMs colonized by the last strain Sc-32 were from inoculated seedlings, and at least one root of each of these seedlings was colonized by Sc-32 (Table 3). Overall, four of these nine seedlings had roots colonized by a non-Sc-32 *S. collinitus* genet. Sc-32 colonized in all 90% of the inoculated seedlings’ roots. In contrast, strain Sc-32 was completely absent from the uninoculated and naturally regenerated seedlings (Table 3).

Last, we tried to reisolate strain Sc-32 from suilloid ECMs of inoculated seedlings. Suilloid isolates were obtained from only five out of 44 ECMs collected in 2000, among which ITS RFLP analysis identified four *S. collinitus* isolates (identical to ITS RFLP type 1) and one *S. mediterraneensis* isolate. Among *S. collinitus* isolates, three were identical to strain Sc-32 for all available markers, the fourth matched...
ISSR type II (Fig. 2) and did not allow amplification of the specific SCAR marker (not shown). Despite the low isolation rate (16%), this demonstrated the strain survival, up to 4 years after outplanting.

**Discussion**

We developed a set of markers allowing specific detection of *Suillus collinitus* strain Sc-32 in pure culture or on *Pinus halepensis* ECMs. Specificity was ensured by comparison with other *S. collinitus* strains, mainly collected at the Rieucoulon outplanting site (Table 1). These markers demonstrated survival of strain Sc-32 in the nursery and after outplanting.

**Genotyping of *Suillus collinitus* ECMs**

Although ITS polymorphism does not characterize the strain Sc-32, fungal ITS-RFLP patterns can be obtained from ECMs and provide a preliminary screening by distinguishing six RFLP patterns among *S. collinitus* strains (Fig. 1, Table 2). Intraspecific ITS polymorphism is already reported in some ECM basidiomycetes (Horton, 2002; den Bakker et al., 2004). However, ISSR-PCR produced a characteristic pattern ‘Iscc’ for strain Sc-32 at both the mycelial and ECM stages, unambiguously distinguishable from all other genotypes (or genets), especially at Rieucoulon (Fig. 2b; Table 2). One ISSR fragment was specific to Sc-32 (Fig. 2a), whereas two to five others were present in at least one of the 17 other tested strains. For each ISSR fragment found among these 17 strains, the frequency of individuals similar to strain Sc-32, i.e. also having this fragment or lacking it, can be calculated. Leaving aside the fragment specific to Sc-32, the probability of a strain showing all five other Sc-32 fragments and none of the other fragments occurring at Rieucoulon can then be calculated as $p = 1 - p_0$, assuming linkage equilibrium among all markers. Here, $p = 0.013$ (or $p = 0.009$ when additionally considering the frequency of the Sc-32 ITS-RFLP pattern). The frequency of the Sc-32 specific fragment cannot be taken into account in this calculation, as $p_0 = 0$ at Rieucoulon: this may result from the geographic distance of the site where Sc-32 was collected (40 km), leading to genetic isolation. In all, the ‘Iscc’ pattern characterizes Sc-32, and likely did not occur before outplanting at Rieucoulon.

The molecular polymorphism of *S. collinitus* (especially for ITS Horton, 2002) could reflect morphologically cryptic species, as demonstrated for American *Cantharellus* spp. (Dunham et al., 2003): indeed species circumscription remains unclear in suilloids (Kretzer et al., 1996). At least the Sc-32 ITS RFLP pattern, shared by all Rieucoulon suilloid ECMs, does not relate to a cryptic species specific to *P. halepensis*, as strain J3.15.24 from a *P. pinea* stand shares the same ITS. In addition, the polymorphic ISSR fragments shared by the various strains (see high similarities in Fig. 2b) suggest that independent genetic pools do not occur within *S. collinitus*.

At Rieucoulon, no *S. collinitus* fruitbodies were found, forcing us to carry out direct investigations on ECMs. The (GACA)$_4$ primer only amplified weak, nonpolymorphic fragments from *P. halepensis*, the (GAC)$_3$ primer gave no PCR products (Fig. 3). Annealing sites for these primers may be less abundant, or surround less variable regions in the plant than in the fungal genome. Therefore, amplification of pattern ‘Iscc’ was possible on ECMs (Tables 2 and 3). Indeed, a study published when this paper was under review implicitly suggests that ISSR pattern is amplifiable from ECMs without interference of host DNA for *Suillus pictus* (Hirose et al., 2004). Compared to SCARS, ISSR fingerprinting (1) produces more markers in a single step, (2) requires limited preliminary work before typing, and (3) avoids loss of specificity during SCAR choice, due to the design of internal primers (Weber et al., 2002). Direct ISSR thus has interesting features that counterbalance risks of amplifying plant endophytes or contaminants. It is an inexpensive method for routine nursery control. To further confirm identification, we designed a SCAR marker specific to *S. collinitus* Sc-32 (Fig. 4), so that a combination of seven loci was used here to characterize the strain Sc-32.

**Suillus collinitus Sc-32 survival**

The inoculated strain Sc-32 likely survived on Rieucoulon ECMs (Fig. 5). Additional faint fragments sometimes occurred in ITS-RFLP (‘Sc+’ pattern) and ISSR (‘Iscc’ pattern, Fig. 5 and Table 3), probably due to contamination by other microorganisms. Indeed, *P. halepensis* harbours root endophytic fungi (Girlanda et al., 2002) that can entail weak PCR contaminations, as they colonize roots less densely than ECM fungi. We suggest that all ‘Sc’/‘Iscc’ ECMs are colonized by strain Sc-32, as they all exhibit the specific SCAR marker (Fig. 5 and Table 3). Alternatively, ‘Sc’ may represent recombinant genotypes (although no *S. collinitus* fruitbody was seen, see below): at least, this does not allow us to draw quantitative conclusions on Sc-32 survival and breeding.

Limiting our conclusion to identical ISSR patterns, this demonstrates successful inoculation of *P. halepensis* by strain Sc-32, up to 4 years after outplanting (i.e. 56 months after inoculation). Persistence of inoculated suilloids is commonly reported in microcosms and the nursery (e.g. Timonen et al., 1997) despite competition with spontaneous strains (El Karkouri et al., 2002). Genetic evidence of inoculant on ECM after outplanting is so far restricted to *L. bicolor* (Selosse et al., 1998a, 1999; Weber et al., 2002), based on two SCARS, and *Paxillus involutus* (Hönig et al., 2000), based on rDNA RFLP polymorphism.

But do the ‘Sc’ and ‘Iscc’ patterns truly identify the dikaryotic inoculant Sc-32, or its progeny? Outcrossing is
unlikely, as fingerprints would include additional fragment(s) from another genetic pool (all indigenous patterns at Rieucoulon differ from pattern 'IsC' by at least one additional fragment, see Fig. 5b). However, selfing could preserve an IsC-like pattern, with a probability ranging from 1 (assuming a very unlikely situation where all markers are homozygous in Sc-32) to 0.95 = 3.1 \times 10^{-2} (assuming that each ISSR marker is heterozygous). Selfing can arise by mating after basidiospore germination in soil (Weber et al., 2002) or by direct formation of dikaryotic basidiospores on Sc-32 basidia (as in some suilloids Jacobson & Miller, 1994; Bonello et al., 1998). This seems highly unlikely, as no S. collinitus fruitbodies were seen in our regular investigations, either in the nursery or plantation. This precludes formation of haploid basidiospores and thus selfing of strain Sc-32. Such a situation contrasts with inoculated Laccaria species, which fruit early and abundantly after inoculation (Selosse et al., 1999), but may be common for ECM fungi fruiting poorly, or not, such as Cenococcum geophilum (Iany et al., 2002).

Also, strains differing from Sc-32 by ISSR ('non-IsC' type) and lacking SCAR fragment (Fig. 5 and Table 3) are also present on roots of 10% of inoculated seedlings; they were the exclusive partners of uninoculated and regenerated seedlings. As already observed for Laccaria and Paxillus spp. inoculants in plantations (Selosse et al., 1998a; Weber et al., 2002), the competitive ability of inoculants is restricted to inoculated root systems, with only rare invasions of new root systems (Selosse et al., 1999). The number of investigated root systems prevents us from detecting any rare event, but such a colonization seems unlikely at least on planted seedlings. Mycelium growth rate is 0.47–1.1 m year⁻¹ for L. bicolor (Selosse et al., 1998a, 1999) and 0.4–0.5 m year⁻¹ for S. pungens (Bonello et al., 1998; Bruns et al., 2002): Sc-32 genets would therefore reach at most 1.6 m, 1.5 years after outplanting, not overcoming the 4.5 m distance between the lines of outplanted seedlings.

As far as the ecological consequences of inoculation are concerned, our data and previously published findings (Selosse et al., 1998a, 1999; Weber et al., 2002) support a general model where inoculation successfully allows early establishment of the inoculant, perhaps thanks to reduced competition on young root systems, but little affects further intraspecific competition out of the inoculated root system. This is congruent with a model of genet development proposed for Leccinum durisculum (Selosse, 2003): genets can grow as long as recruitment is not finished, but stop growing at that time as contacts (and thus competition) with neighbouring genets impede further growth. Nursery inoculation thus favours an earlier settlement of the ECM inoculant and allows a better growth as long as competing genets are not present. After establishment of other genets, ECM inoculants do not have special advantages for colonization of surrounding root systems.

**Suillus collinitus ecology**

In contrast with other suillloid populations studied so far under wetter climates (Zhou et al., 2001b; Hirose et al., 2004), ECM formation at Rieucoulon was not accompanied by fruiting, up to year 2000 (El Karkouri et al., 2004; El Karkouri, pers. obs.). Our data reveal a discrepancy between above- and below-ground views that is unexpected for suilloids, where below- and above-ground distributions were supported to be congruent (Zhou et al., 2001b; Hirose et al., 2004). Indeed, other population analyses are somewhat biased by the fact that ECMs were sought under fruitbodies (Zhou et al., 2001b; Hirose et al., 2004), an approach that cannot reveal nonfruiting mycelia. Here, ECMs were systematically searched for, in the absence of fruitbodies. Alternatively, this discrepancy may relate to the hard, xeric Mediterranean conditions that could disfavor S. collinitus fruiting, especially in young plantations lacking canopy.

Our observations can be explained by vegetative persistence of the inoculant, together with multiple colonizations by indigenous genets. The latter may arise from resident propagules (e.g. spores or rhizomorphs) of the plantation site (El Karkouri et al., 2004). Alternatively, if S. collinitus does not resist fire, as described for S. pungens (Bruns et al., 2002), they could arise from surrounding P. halepensis forest by spore dispersal, or even from the nursery. The observed population is thus congruent with the combination of competitive (C) and ruderal (R) strategies characterizing suilloids (Dahlberg & Stenlid, 1994, 1995; Dahlberg, 1997; Bonello et al., 1998; Zhou et al., 1999), and allowing them to be present at all stages of forest dynamics (multistage species) (Visser, 1995). C attributes explain the growth of the inoculant strain Sc-32, as suilloid genets can survive up to tens of years (Dahlberg & Stenlid, 1990, 1994, 1995; Dahlberg & Stenström, 1991; Dahlberg, 1997; Bonello et al., 1998; Zhou et al., 1999, 2000), whereas R attributes (Zhou et al., 2000; Bruns et al., 2002) explain fast colonization of uninoculated and regenerated P. halepensis by new genets. Indigenous genets even colonize inoculated seedlings at Rieucoulon (10% of root tips, Table 3), exactly as reported for L. bicolor on inoculated Douglas fir (Selosse et al., 1998a, 1999).

**Conclusions**

Our marker set allows strain detection on ECMs and monitoring of mycorrhizal inoculant. The data show that strain *Suillus collinitus* Sc-32 is a suitable, lasting (up to 4 years) inoculant for *Pinus halepensis* which is promising for forestry in harsh Mediterranean environments (water- and nutrient-poor soils), where forest survival is additionally threatened by anthropic pressure and fire. Suilloids currently emerge as inoculants in soils polluted by heavy metals (Colpaert et al., 2000): they may therefore provide ECM
inoculants in adverse soil environments, and also interesting models for comparative studies of ECM populations in stressing conditions.

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