Temporal persistence and spatial distribution of an American inoculant strain of the ectomycorrhizal basidiomycete *Laccaria bicolor* in a French forest plantation

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Abstract

Selected strains of ectomycorrhizal fungi, such as the basidiomycete *Laccaria bicolor*, are currently being used as inoculants in nurseries to improve growth of forest trees after out-planting. Information is needed on the survival of these introduced strains in forests and their impact on indigenous biodiversity. Dissemination and persistence of an American strain, *L. bicolor* S238N, were studied 10 years after outplanting in a Douglas fir plantation located at Saint-Brisson (Morvan, France). About 430 *Laccaria* spp. sporophores were collected over 3 years. Inheritance of nuclear ribosomal DNA, as well as RAPD markers, was characterized in *L. bicolor* S238N, using a haploid progeny set of 91 monokaryons. More than 50 markers were identified (19 heterozygous and 33 homozygous or cytoplasmic markers), which unambiguously confirmed that the introduced strain was still present in the inoculated plots. Neither selfing (*P* < 0.0008) nor introgression with indigenous strains was detected although *in vitro* interfertility between the American strain and indigenous *L. bicolor* was identified. No ingress of the introduced genet into adjacent uninoculated plots colonized by various local *Laccaria* genets was detected. It is proposed that the spatial distributions identified have developed through mycelial propagation of the introduced strain and intraspecific competition with native genets. Although longer-term data is still lacking, the stability of the inoculant strain and the limited disturbance to indigenous populations described support large-scale nursery production of this host-fungal combination.

Keywords: basidiomycetes, ectomycorrhizal fungi, inoculation, introgression, *Laccaria* spp., population structure, strain survival

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Introduction

Intercontinental flow of species and genes has progressively increased as a result of improved travel opportunities and commercial exchanges. However, few reports deal with fungal introductions. Efforts have concentrated on introduction of agronomically important rusts and pathogenic ascomycetes, and their invasion routes (Nagarajan & Singh 1990; McDermott & McDonald 1993). Similar studies of nonparasitic fungi are limited to unintentional introductions of these fungi (Singer 1977; Priou 1985; Parent & Thoen 1986). Successful acclimatization of plants often requires the deliberate introduction of their soil symbionts. *Bradyrhizobium japonicum*, a nitrogen-fixing bacteria nodulating soybean (*Glycine max*), naturally absent from European and American soil, has been introduced in the cultivation of soybean, whilst *Rhizobium etli* has been imported with its host, *Phaseolus vulgaris*, to Europe (Martinez-Romero & Caballero-Mellado 1996).

Ectomycorrhizal fungi that form mutualistic associations with tree roots (Smith & Read 1997) have frequently been introduced into tree seedling nurseries, either as indigenous or introduced inoculant strains (Le Tacon et al. 1992). Attempts to grow *Pinus* spp. in tropical areas
Over the last two decades, an American strain of the ectomycorrhizal fungus *L. bicolor* (strain S238N, see Di Battista et al. 1996), has been used as an inoculant for improved nursery cultivation of Douglas fir (*Pseudotsuga menziesii*) in France (Villeneuve et al. 1991; Le Tacon et al. 1992; Henrion et al. 1994). This represents an ideal model for investigating the effects of introduction of exotic fungal strains as native *L. bicolor* is found associated with forest trees, in young and old stands (Last et al. 1987). Sporophores of this fungus are abundantly produced, allowing population analysis through sporophore sampling (Baar et al. 1994; De la Bastide et al. 1994). *L. bicolor* is heterothallic with a bifactorial mating-type system and multiallelic mating-type factors. Fusion of haploid monokaryons gives rise to dikaryotic mycelia bearing clamp connections (Fries & Mueller 1984; Casselton & Economou 1985). Dikaryons represent the vegetative stage that is associated with roots in mycorrhiza; they give rise to sporophores that produce meiotic spores whose germination leads to the growth of monokaryons. Both monokaryons and dikaryons can be grown in pure culture (Fries 1983), allowing *in vitro* assessment of genetic interactions between genotypes (Gardes et al. 1990; De la Bastide et al. 1995c) and segregation analysis (Doudrick et al. 1995; Selosse et al. 1996). Seeding inoculation in nurseries has been performed with *in vitro* cultivated dikaryotic mycelium of the *L. bicolor* S238N strain (Villeneuve et al. 1991). Persistent colonization of Douglas fir roots by an inoculant *L. bicolor* strain after 1.5 years in nursery soil was confirmed following polymorphism analysis of PCR-amplified ribosomal DNA (Henrion et al. 1994). Seedlings inoculated with a dikaryotic *L. bicolor* strain and receiving abundant basidiospore inoculum still harboured the inoculant dikaryon after 20 weeks (De la Bastide et al. 1995b).

However, outplanting of inoculated tree seedlings into forest sites raises several problems. First, persistence of the inoculant strain in competition with indigenous fungi contributes to overall quality of nursery seedling production. Assessment of sporophores or mycorrhiza morphology (Villeneuve et al. 1991; Le Tacon et al. 1992) and characterization of mating type (Buschena et al. 1992) suggest a 1 or 2 year persistence of the *L. bicolor* inoculant after outplanting. Information on (i) genetic identity with the inoculant strain; and (ii) persistence over longer time scales is still lacking. Second, introgression of the introduced genes into indigenous interfertile populations will probably occur if sporophores, and thus meiotic spores, are produced. This would lead to a modification of indigenous populations by gene flow and potential dilution of the introduced genome. Characterization of species and intersterility groups in European *Laccaria* spp. (Fries & Mueller 1984) is not as comprehensive as for the American species (Gardes et al. 1991; Mueller & Gardes 1991). However, as Mueller & Gardes (1991) clearly demonstrated that North American *L. bicolor* range from at least partially to fully compatible with European *L. bicolor*, introgression can be investigated in this model species. The third problem relates to the possibility of loss of local strains through outcompitition by the inoculant strain. The spread of hybrids may lead to extinction of European genotypes by hybridization, a process observed in many species (Rhymer & Simberloff 1996). European ectomycorrhizal fungi are believed to be undergoing drastic community changes (Jaenike 1991; Arnold 1995), as a result of air pollution, increased nitrogen availability in forest soils and changes in forest management practices.

Several molecular tools are available to study populations of ectomycorrhizal fungi (Lubeck & Lubeck 1996), some of which are rapid and require low quantities of DNA template. A useful tool is the analysis of PCR-amplified polymorphic ribosomal DNA loci (Henrion et al. 1994; Selosse et al. 1996). Randomly amplified polymorphic DNA (RAPD) provides reproducible markers for studying ectomycorrhizal populations (Jacobson et al. 1993). RAPD shows sufficient polymorphism to type *L. bicolor* strains (Di Battista et al. 1996) and has enabled construction of genetic linkage map in this species (Doudrick et al. 1995). Here we report on the design of such markers and their use to assess the persistence of an American *L. bicolor* strain 10 years after its introduction into a French site populated by indigenous *Laccaria* spp.

**Materials and methods**

**Strains**

The American strain S238N of *Laccaria bicolor* (Maire) P.D. Orton used for inoculation of Douglas fir was isolated in 1976 from a sporophore under *Tsuga mertensiana* (Bong.) Carr. at Crater Lake, Oregon, USA. This dikaryotic strain has been maintained in Nancy (France) through subculture on synthetic Pachlewski agar medium since 1980 (for
a detailed history, see Di Battista et al. 1996). A haploid progeny set of 91 monokaryons was obtained as previously described (Selosse et al. 1996) and used to test the segregation of identified RAPD markers. Five dikaryons reconstituted from these monokaryons were used to further check inheritance of heterozygous RAPD markers during mating. To discriminate RAPD markers shared by S238N and European Laccaria spp., eight European strains or sporophores were used: L. laccata S1 (sporophore of Bellême, France), L1 (sporophore of the Peyrat-le-Château nursery), L. laccata 83222 (pure cultures isolated from a sporophore of Chamet, Morvan, France), L. bicolor 81306, 83216 and CHAM3 (pure cultures isolated from sporophores of Morvan), L. bicolor S7 (sporophore of Coye-la-Forêt, France), and L. laccata var. moleri (= L. proxima) S2 (sporophore of Bellême). All the described strains are deposited in the Collection of Ectomycorrhizal Fungi (I.N.R.A., Nancy).

**Seedling inoculation and site description**

Seedlings of Douglas fir (Pseudotsuga menziesii (Mirr.) Franco, seed origin: Ashford, Oregon, USA) inoculated on fumigated nursery soil in 1985 as described in Villeneuve et al. (1991) were outplanted 2 years later in Saint-Brisson. This stand is located in the Nièvre (centre of France), at an elevation of 630 m and was previously a mixed forest of beech, oak and birch on a brown podzolic soil over granite. The stand was divided into plots of 7.2 m each containing 49 seedlings and separated by a 3 m nonplanted buffer zone. Of the various seedling treatments, only three were considered in this study (see Fig. 5 later): seedlings grown on (i) sterilized nursery soil artificially inoculated with L. bicolor S238N; (ii) sterilized soil; and (iii) nonsterilized soil. In both the latter treatments seedlings were naturally mycorrhizal with indigenous fungi at the time of outplanting. Soil sterilization in the nursery was achieved through methyl bromide fumigation. The 'local' genets of Laccaria spp. detected were either present in the plots prior to seedling plantation or could have been brought on seedling stock from the nursery (about 200 km from Saint-Brisson). Each plantation plot was thinned twice (February 1992 and February 1995), with half of the trees being cut on each occasion and 16 trees remain on each plot at the time of this study. Total height of inoculated trees has always been significantly higher than the uninoculated controls (about 14% in 1994) and total wood volume was increased by about 50% on L. bicolor inoculated plots (F. Le Tacon and M.-A. Selosse, unpublished).

**Sampling and isolation**

The present study focused on one of the plots where Douglas fir had been inoculated with L. bicolor S238N in the Peyrat-le-Château nursery. Neighbouring plots were planted with either seedlings inoculated with L. bicolor S238N or uninoculated seedlings (i.e. grown on disinfected soil or on nondisinfected soil, but spontaneously mycorrhizal with nursery fungi) (see Fig. 5, later). All sporophores produced in the plot and in the surrounding nonplanted buffer zone were collected during the autumn of 1995, i.e. 269 samples from an area of 175 m². They were morphologically identified as L. bicolor or L. laccata and their ground location was mapped with a precision of 5 cm. Isolation of vegetative mycelium was attempted from all collected sporophores on a modified HAGEM medium (NH₄Cl 0.5 g/L, KH₂PO₄ 0.5 g/L, (NH₄)₂HPO₄ 0.5 g/L, MgSO₄ (H₂O)₇ 0.5 g/L, glucose 5 g/L, malt extract 5 g/L, thiamine HCl 50 µg/L, FeCl₃ 5 mg/L, agarose 10 g/L) with chloramphenicol and rifampicin (100 mg/L) added to prevent bacterial contamination. Emergent mycelium was screened for the presence of septa and clamp connections and subcultured twice to obtain noncontaminated cultures. Further sporophores were randomly sampled in the autumn of 1994 and 1996 on the same plot (i.e. 28 sporophores in 1994 and 32 sporophores in 1996), but analysed directly, to study yearly stability in the sporophore population. Sporophores from the surrounding plots were randomly sampled over 3 years (1994, 1995 and 1996) to check the ability of the inoculant strain to invade trees of neighbouring plots. A total of 100 sporophores (41 in 1994, 16 in 1995 and 43 in 1996) were collected from nine plots (three inoculated with L. bicolor S238N and six uninoculated plots; see Fig. 5).

**Spore germination and mating of monokaryons**

Sporophores were collected from two L. bicolor sporophores present in the inoculated plot, N321 (genet B, identical to S238N, see below) and N203 (genet D, see below), and were germinated according to the protocol of Fries (1983). Haploid progeny were confirmed by checking for the absence of clamp connections. For each sporophore, 15 monokaryons were paired in all possible combinations, as described by Selosse et al. (1996), to isolate progeny of the four mating types (Table 1). To test for fertility between the two sporophore genets, four monokaryons representing the four mating types were selected in each of the progeny and paired in all combinations. They were also paired to four monokaryons representing the four mating types of the S238N progeny used for analysis of marker segregation. Dikaryon formation was verified by identification of clamp connections.

**DNA extraction and PCR amplification**

Total DNA was extracted from cultured vegetative mycelium, monokaryotic mycelium (91 progeny of S238N),
or directly from sporophore tissues using the hexade-
cyltrimethylammonium bromide (CTAB)/proteinase K
protocol essentially as described by Henrion et al
(1994).

All PCR were run in 0.2 mL tubes in a GeneAmp PCR
System 9600 (Perkin Elmer) and contained 1 reaction
buffer (20 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl,
0.1% Triton X-100), 0.2 mM each dNTP (0.4 mM for IGS2),
0.02–2 ng/µL template DNA and 0.06 units/µL
Taq DNA
polymerase (Appligène, France). The thermocycling pro-
file for all amplifications (except IGS2 amplification, see
Selosse et al. 1996) was: 3 min denaturation at 94 °C, 30
cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min,
followed by a final elongation at 72 °C for 10 min. The 25S/5S
spacer (IGS1) and the 5S/17S spacer (IGS2) were amplified
with previously described primers (Selosse et al. 1996).

RAPD amplification was carried out in conditions spe-
cially designed to enhance reproducibility: stringent
annealing temperature (50 °C) and high primer concen-
tration: 3.5 µM, except for 007 (7 µM) and 014 (30 µM) (see
primer sequence in Table 2). All RAPD reactions were per-
formed at least twice and, for sporophores whose
mycelium was not obtained in pure culture, two indepen-
dent DNA extractions were used. All primers were sup-
plied by Bioprobe Systems (Montreuil-sous-Bois, France).

Amplification products were separated by electrophore-
sis using 8% acrylamide gels in 1× Tris–borate–EDTA
buffer, except for the amplified IGS2 products which were
separated on a 1% agarose gel because of their larger size.

Analysis of RAPD markers

In the segregation study, only major reproducible bands
were scored (see caption of Table 2 for marker coding).
Goodness of fit to the expected Mendelian segregation
ratio (1:1) was tested by Chi-square analysis. The linkage
relationships of the Mendelian markers were analysed
with MAPMAKER 2.0 for Macintosh. We used the HAP-
LOID model that assumes that all markers are in the cou-
pling phase and consequently does not recognize linkages
for markers in repulsion. Repulsion phase linkages can be
detected by analysing recoded data (i.e. presence recoded
to absence, and vice versa) together with the original data
set. Analysis of the combined data yielded twice the
expected number of linkage groups corresponding to the
two homologues for each chromosome. Markers were
assigned to linkage groups using a LOD score (log₁₀ of the
odds ratio) at least equal to 2.5 and recombination fraction
of q < 0.40. Recombination fractions were converted into
genetic distances using the Kosambi mapping function,
i.e. taking multiple crossing-over into account.

Comparison of RAPD patterns of the different genets
collected in Saint-Brisson was performed using Jaccard
indices to build a phenetic tree with the UPGMA method
(unweighted pair-group method using arithmetic means)
implemented in the PHYLIP package (Felsenstein 1989).

Results

Characterization of markers of Laccaria bicolor S238N

In order to identify the introduced strain L. bicolor S238N
in the field, we characterized molecular markers of the
strain and their behaviour within haploid progeny. The
ribosomal DNA (rDNA) is a head-to-tail repeat of coding
and noncoding sequences (Fig. 1A) mapping to a single
locus in this strain. The allelic variations in length and
sequence within the two nontranscribed intergenic spac-
ers, IGS1 and IGS2, have been studied elsewhere (Selosse et al. 1996). Ribosomal DNA may be considered as codom-
inant markers (Fig. 1B,C, left lane of both gels): (i) the two

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Table 2 RAPD markers used for characterisation of *Laccaria bicolor* S238N. Markers are referred to by the primer generating them followed by the size (bp) of both alleles in S238N (– indicates an absent band allele). The segregation within a S238N progeny of 91 monokaryons is given. Heterozygous markers are shown in bold; markers also found in European strains of the INRA collection are in italics (see Materials and methods). Genetic distances between linked markers have been established using a Kosambi map function. Unless indicated, linked dominant markers are linked in coupling.

<table>
<thead>
<tr>
<th>Primer Marker</th>
<th>Segregation</th>
<th>Linkage</th>
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<tr>
<td>Ribosomal DNA</td>
<td>α/β haplotypes</td>
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</tr>
<tr>
<td>007 1200 / –</td>
<td>51:40* dominant</td>
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</tr>
<tr>
<td>5’-GTCCGACGA-3’</td>
<td>91:0 homozygous</td>
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</tr>
<tr>
<td>0250 / 0250</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>010</td>
<td>42:49* codominant</td>
<td>15.9 cM‡</td>
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<tr>
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</tr>
<tr>
<td>014 2000 / –</td>
<td>49:42* dominant</td>
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<tr>
<td>5’-TCCGTGTCTTG-3’</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>155</td>
<td>47:44* codominant</td>
<td></td>
</tr>
<tr>
<td>5’-CGCACCACAC-3’</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>0700 / –</td>
<td>42:49* dominant</td>
<td></td>
</tr>
<tr>
<td>012</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>0200 / –</td>
<td>50:41* dominant</td>
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<td>152GC</td>
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</tr>
<tr>
<td>155</td>
<td>1020 / 1020 91:0 homozygous</td>
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</tr>
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<td></td>
</tr>
<tr>
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<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>0820 / 0820</td>
<td>91:0 homozygous</td>
<td></td>
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<tr>
<td>155</td>
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</tr>
<tr>
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<tr>
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</tr>
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<td>0900 / 0900</td>
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<td></td>
</tr>
<tr>
<td>0820 / –</td>
<td>44:47* dominant</td>
<td></td>
</tr>
<tr>
<td>0470 / –</td>
<td>68:23 dominant</td>
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</tr>
<tr>
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<td>91:0 homozygous</td>
<td></td>
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<tr>
<td>157</td>
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<td></td>
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<tr>
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<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>1200 / –</td>
<td>52:39* dominant</td>
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</tr>
<tr>
<td>1020 / 1020</td>
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<td></td>
</tr>
<tr>
<td>0720 / 0720</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>0430 / 0430</td>
<td>91:0 homozygous</td>
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<td>91:0 homozygous</td>
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<tr>
<td>0220 / 0220</td>
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</tr>
<tr>
<td>0190 / –</td>
<td>44:47* dominant</td>
<td></td>
</tr>
<tr>
<td>0170 / 0170</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>174</td>
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<tr>
<td>5’-AACGGGCAGC-3’</td>
<td>91:0 homozygous</td>
<td></td>
</tr>
<tr>
<td>1230 / 2000</td>
<td>47:44* dominant</td>
<td></td>
</tr>
<tr>
<td>1200 / –</td>
<td>60:31 dominant</td>
<td></td>
</tr>
<tr>
<td>0930 / –</td>
<td>42:49* dominant</td>
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</table>
IGS2 fragments are, respectively, the two IGS2 haplotypes; and (ii) in the complex IGS1 patterns, the 2.2 and 2.4 kb fragments are heteroduplexes, indicating heterozygosity. The 0.8 kb fragment contains two allelic homoduplexes of divergent sequence but indistinguishable size (Selosse et al. 1996) that interact to form two heteroduplexes of high apparent molecular weight.

Fifty-five reproducible fragments were generated using eight RAPD primers, and their meiotic segregation was studied in haploid progeny of L. bicolor S238N (see Table 2). Their inheritance during dikaryon formation was also confirmed in five reconstituted dikaryons (data not shown). Thirty-three RAPD fragments did not segregate and therefore represented homozygous nuclear markers (a cytoplasmic origin, although less probable, cannot be ruled out). From 22 other fragments, 14 exhibited a dominant mode of inheritance of alleles, with the present band allele dominant over the absent band allele. The eight other fragments corresponded to four putative codominant markers (i.e. haploids lacking or having both fragments were not identified). Alternatively, the DNA fragments could have been tightly linked in repulsion, but this condition was not considered further. As highlighted in the Chi-square analysis (Table 2), 14 segregations were not significantly different from 1:1 Mendelian segregation at the 0.05 level. Combined linkage analysis of Mendelian and ribosomal markers showed two linkage groups of three markers each. The eight other markers remained genetically isolated. No linkage relationships were found for the four distorted markers following individual map placement. Ambiguous markers were scored through comparison with eight European Laccaria strains or sporophores (see the Material and methods) but only seven markers, all homozygous, were found in European patterns (Table 2). These ambiguous fragments cannot be used to characterize the American strain L. bicolor S238N.

Ribosomal typing of sporophores from the inoculated plot

Of the 269 sporophores of Laccaria spp. (i.e. 69 Laccaria laccata and 200 L. bicolor) collected from the inoculated plot in autumn 1995, in vitro isolation of vegetative cultures from L. bicolor was successful in 154 cases but no cultures of L. laccata were obtained. Although uncontaminated, growth of mycelium of the latter species was arrested shortly after emergence from the sporophore inoculum. Among the amplified IGS1 and IGS2, only nine different ribosomal fragment combinations were identified (A to I, Fig. 1B,C). Identical fragment patterns from pure cultures and corresponding sporophores confirmed that fungal DNA from the sporophores was not contaminated by other organisms. Within the nine ribosomal types, several shared the same IGS1 pattern (namely, C and D or F, G, H and I), but all differed in the IGS2, as

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evidenced by co-migration of amplified products (data not shown). The patterns confirm the three central features relating to IGS polymorphism in *Laccaria* (Selosse et al. 1996): (i) the amplification of one or two IGS2 fragment(s) representing the two respective homozygous or heterozygous IGS2 haplotypes of the dikaryon; (ii) some IGS1 amplifications yield fragments of apparent high molecular weight (bands of type A and B, arrow in Fig. 1B) or a smear (type E in Fig. 1B) that can be interpreted as heteroduplexes (see Selosse et al. 1996 for the experimental procedure to distinguish heteroduplexes); and (iii) aside from these heteroduplexes, IGS1 patterns showed one or two (type A) homoduplexes corresponding to the two IGS1 haplotypes. No other ribosomal types were found within sporophores collected on the same site in 1994 and 1996 (data not shown). Interestingly, the type B fragments matched the heterozygous pattern of *L. bicolor* S238N (also presented in Fig. 1B,C) but the other types did not share either ribosomal haplotype with *L. bicolor* S238N. Further molecular typing was carried out to ascertain the relationship between the B type, accounting for 100 (37.2%) of the collected *Laccaria* sporophores, and the inoculant strain S238N.

**Evidence for persistence of *L. bicolor* S238N**

Primers used to generate the RAPD patterns from the 100 B-type sporophores and the 70 pure cultures isolated from B-type sporophores are presented in Table 2. As with the ribosomal typing patterns, no differences were detected in the RAPD fragment patterns of B-type sporophores and corresponding pure cultures in all the tested samples (Fig. 2A). The identical RAPD patterns corresponded to those of the *L. bicolor* S238N culture conserved in the Collection of Ectomycorrhizal Fungi (I.N.R.A. Nancy) (see comparison on Fig. 2A). The 45 loci scored in this study (unambiguous RAPD loci and rDNA) proved that the inoculant strain S238N had remained and was sporulating in the inoculated plot.

**Absence of detectable introgression**

The eight other *Laccaria* ribosomal types were also analysed by RAPD amplification with the same set of primers. Within each ribosomal type, sporophores shared identical RAPD patterns (Fig. 2B). Pure cultures and sporophores of type A, C, D and E produced the same pattern. Therefore sporophores of type F, G, H and I, for which no pure culture was available, were assumed to be free of any contamination. The RAPD patterns of the nine ribosomal types showed similarities but were all distinguishable (Fig. 3A), i.e. they can therefore be considered as nine genets (Table 3). In agreement with the morphological identification, RAPD patterns of genets A, C, D, and E (assigned to *L. bicolor*) on one hand, and F, G, H and I (assigned to *L. lactea*) on the other hand, were closely related, as underlined in the UPGMA tree (Fig. 3B). Identical clustering patterns were obtained using a neighbour-joining method (data not shown). Careful comparison of the RAPD patterns (including co-migrations, data not shown) revealed that, aside from the B genet, only five markers of *L. bicolor* S238N were present in at least one other genet. These markers, namely 152C (0250/0250) (see Fig. 3A), 152GC (0220/0220), 155 (0370/0370) (see Fig. 2A), 157 (0330/0330) and 157 (0220/0220), were all shown previously to be shared by European *Laccaria* (Table 2), thus not indicating gene flow. These five shared markers were only found in *L. bicolor* genets, so that, in neighbour-joining (data not shown) and UPGMA trees (Fig. 3B), *L. bicolor* S238N was always located at the root of the *L. bicolor* cluster. Therefore, no introgression of the exotic genome within the local genets was detected.

**Spatial distribution of the genets on the inoculated plot**

The spatial domains occupied by the nine genets were delimited on the basis of sporophore type and location.
Sporophores growing in close vicinity (< 5 cm) always belonged to the same type and were therefore mapped collectively to a single point (i.e. only 149 positions on the map). The introduced S238N genet occupied the centre of the plot of inoculated Douglas fir, restricting the indigenous genets to the border of the plot. These genets appeared not to be fragmented. The only exception was genet G, which was fragmented into at least three ramets, one of which (two sporophores at the same position) was located in the middle of the plot. Molecular typing of 28 and 32 sporophores, respectively, collected in 1994 and 1996, identified the presence of all but one of the genets (I) and two of the genets (I and F) in the respective sampling years. The lack of detection of these genets could be attributed to the random sampling procedure, as all sporophores present in the plot were not typed. The inoculant strain (S238N) accounted for 36 of the 60 collected sporophores, and the spatial distribution in 1994 and 1996 (shaded zone of Fig. 5) was identical to that in 1995, indicating a stable sporophore distribution.

**Interfertility of S238N with indigenous genets**

The lack of introgression may have reflected intersterility barriers between the American strain S238N and European *L. bicolor* strains. No spore germination was achieved from *L. laccata* genets. Spore germinations were obtained from two of the *L. bicolor* sporophores, namely N203 (genet D) and the N321 (genet B, identical to the S238N strain). The four mating types of the N203 progeny were successfully paired with the four mating types recovered in the progeny used for analysis of marker segregation (Table 1). In spite of its distant origin, *L. bicolor* S238N is thus sexually compatible with European strains.

**Lack of spread of *L. bicolor* S238N from the inoculated plots**

In order to follow the spread of strain S238N away from the inoculated plots, monitoring of the sporophores appearing in surrounding plots was performed over the 3 years, in a sampled population of 100 sporophores. Sporophores were first typed using rDNA and RAPD with primers 152C and 174. The identity of putatively S238N-related sporophores was further checked with the other RAPD primers described in Table 2. No indications of introgression were detected during the study of S238N

![Fig. 3](image-url) **Fig. 3** Comparison of the eight indigenous genets with the inoculant strain S238N. A. Comparison of RAPD patterns obtained with primer 152-C on 8% acrylamide. Size of the markers (see Table 2) is given on the left with heterozygous markers in italic. Molecular size markers (Phi-X-174 digested by HaeIII) are given on the right. B. Phenetic tree obtained by UPGMA comparing the RAPD patterns of the nine genets present on the inoculated plot (type B was identical to the inoculant American strain). Total number of fragments generated by the primers of Table 2 are indicated in brackets for each genet.
spread (data not shown). The resulting map (Fig. 5) showed that the introduced strain (represented by 32 sporophores) fruited abundantly on inoculated plots, where it dominated or even excluded other genotypes. The strain was also found in the buffer zones around inoculated plots, but was not detected in uninoculated plots. Indigenous sporophores, found on and around uninoculated plots, belonged to a great number of genets (at least 30, data not shown), suggesting a high genetic diversity in these plots.

**Discussion**

Molecular markers were utilized to assess the persistence of an American strain of the ectomycorrhizal fungus *Laccaria bicolor* inoculated on Douglas fir outplanted in a French forest stand. In addition to rDNA, a heterozygous marker in this strain (Selosse *et al.* 1996), 51 RAPD markers were characterized. Segregation analysis in haploid progeny indicated that 18 markers were heterozygous (i.e. a heterozygosity level of 0.35) and four had strong segregation bias. The distorted segregation in *L. bicolor* progeny observed here has already been identified at a much higher frequency by Doudrick *et al.* (1995). Differential viability or reduced germinative ability *in vitro* due to recessive deleterious alleles may explain the low level of spore germination observed in this species (Fries 1983). This could lead to a segregation bias in the identified markers linked to these alleles. Although non-neutral *in vitro*, these markers may be Mendelian under natural conditions, and were therefore not rejected for the field study. Two noteworthy methodological results of this investigation were: (i) the good resolution of IGS2 typing, each RAPD-defined genet having a distinct IGS2 pattern (Fig. 1B); and (ii) the quality of sporophore DNA, which was not contaminated by sporophore-colonizing organisms.
During the 3-year survey, these molecular markers confirmed that, 7–10 years after introduction, *L. bicolor* S238N remained in inoculated plots of the Saint-Brisson forest (France), accounting for 168 of the 429 sampled *Laccaria* sporophores (Figs 4 and 5). The molecular identity, especially for the five co-dominant loci studied, probably excludes any selfing of the strain, although this was shown to be possible in vitro (Table 1). Assuming that for a dikaryotic F1 progeny, the probability of retaining heterozygous markers is 0.75 for dominant markers and 0.5 for codominant markers (i.e. no segregation bias in natural conditions), the probability of a F1 identical for five co-dominant and 14 dominant independent markers is:

\[ P = (0.75)^{14} (0.5)^5 \]

i.e. about 0.0005. In this case, the two linkage groups (Table 2) imply a slightly different formula:

\[ P = XY (0.75)^{10} (0.5)^3 \]

where X and Y are the probabilities of retaining the parental phenotype for each of the linkage groups. 

\[ X = 0.294 \text{ for the group including the rDNA and} \]
\[ Y = 0.386 \text{ for the other group, therefore } P = 0.0008. \]

The probability of a phenotype identical to S238N decreases with further inbreeding, so the hypothesis that type-B sporophores arose through independent matings can be safely rejected.

Persistence of inoculant ectomycorrhizal fungi has been confirmed through morphological identification of mycorrhiza (Villeneuve *et al.* 1991; Thomson *et al.* 1996). The survival, 2 years after outplanting, of *L. bicolor* inoculated on container-grown black spruce (*Picea mariana*) was suggested through the detection of identical mating-type combination in the population (Buschena *et al.* 1992). However, selfing cannot be excluded, as these two loci are necessarily heterozygous in any dikaryotic progeny. Persistence of *L. bicolor* S238N on the Douglas fir root system was also proven using rDNA both in the nursery (Henrion *et al.* 1994) and on a formerly cultivated farmland 2 years after outplanting (Di Battista 1997). To our knowledge, this study is the first to report on the genetically stable persistence of an introduced fungal strain in natural populations over a 10-year period. Control trees were naturally colonized by indigenous ectomycorrhizal fungi, such as *Laccaria* spp. (Fig. 5), either in nursery and/or after outplanting. Colonization of disturbed forest sites and young plantations has already been reported for *Laccaria* spp. (Last *et al.* 1987; Buschena *et al.* 1992). The persistence of the inoculant strain may explain the significantly higher growth of the inoculated trees in the Saint-Brisson stand (F. Le Tacon and M.-A. Selosse, unpublished), as suggested in other studies (e.g. Villeneuve *et al.* 1991; Buschena *et al.* 1992; Le Tacon *et al.* 1992). Indigenous strains that colonize control trees do not induce similar plant growth promotion, emphasizing the benefit of early nursery inoculation with selected strains.

The strongly divergent RAPD profiles of the inoculant strain S238N and local *Laccaria* spp. genets (Fig. 3) allowed us to study its introgression (about 87% of markers in Table 2 are characteristic). These molecular markers revealed no interbreeding in the Saint Brisson plantation. The common fragments that were detected in S238N and local strains had been previously shown not to characterize the American strain. However, the lack of introgression was rather unexpected, as North American *L. bicolor sensu lato* has been reported to be at least somewhat intercompatible with Swedish strains (Mueller & Gardes 1991; De la Bastide *et al.* 1995a). In particular, one of the American intersterility groups (*L. bicolor sensu stricto*) is fully compatible with Swedish strains. The inoculant strain was also fully compatible with at least the *L. bicolor* genet D from Saint-Brisson (Table 1). Intercontinental fertility has already been reported for homobasidiomycetes of the northern hemisphere, e.g. within saprophytic fungi (Brasier 1987) and forest tree pathogens (Korhonen 1987). It has been proposed that these species are undergoing an allopatic speciation (with allelic differentiation) without development of intersterility barriers (Brasier 1987; Otrosina *et al.* 1993).

The absence of both introgressed and self-crossed progeny of S238N was unexpected because of the high sporophore production (e.g. 37.2% of the sporophores on the inoculated plot in autumn 1995). Spore germination, which was possible in vitro, could contribute to gene flow. Other studies have shown that abundant spore inoculum does not prevent the persistence of a stable genet, e.g. over 10 weeks on seedlings inoculated with a dikaryotic *L. bicolor* strain (De la Bastide *et al.* 1995b) or over thousands of years in a forest population of *Armillaria* (Smith *et al.* 1992). Our data suggest that the loss of a ‘window of opportunity’ for spore establishment does not allow development of new genets. In established populations, colonization of new root tips and population development mainly result from vegetative mycelial propagation (Dahlberg & Stenlid 1995). This interpretation does satisfactorily explain the stability of the sporophore population observed over three sampling years. Alternatively, introgression and/or selfing could have lead to lasting genets that remain undetected in our study. Recombinant genets may not be able to form sporophores because of: (i) a reduced fruiting ability (Di Battista *et al.* 1996); (ii) post-fertilization sterility in hybrids; or (iii) a low mycorrhizal ability that would limit their access to photoassimilates required for sporophore development (Lamhamedi *et al.* 1994). Resource allocation to sporophore formation was shown to vary greatly among species (Jansen & de Nie © 1998 Blackwell Science Ltd, *Molecular Ecology*, 7, 561–573
Intraspecific differences are just as likely to operate, and sporophore analysis may underestimate the total number of genets. Recombinant genets may also fruit later in the development of the plots but, in this study, no significant evolution of the sporophore population was noticed over the 3 years, in agreement with the stability of natural Laccaria populations under Norway spruce (De la Bastide et al. 1994).

Strong sporophore dependence on host photosynthates (Lamhamedi et al. 1994; Smith & Read 1997) and similarities in sporophore spread and mycorrhizal colonization (De la Bastide et al. 1994; Gryta et al. 1997) suggest that the sporophore maps (Figs 4 and 5) do reflect the subterranean mycorrhizal distribution of fruiting genets, although nothing is known about the distribution of nonfruiting genets. The inoculant genet dominated the inoculated plots and the inner part of surrounding buffer zones. Prior establishment on a root system probably increases competitiveness through: (i) maintenance of vegetative mycelium throughout the year; (ii) vicinity of formerly and newly developed roots; and, thus, (iii) greater access to host photosynthates. This was further supported by the lack of sporophores of other mycorrhizal species in inoculated plots (except some Clathriporus piperatus in autumn 1996, data not shown). However, some indigenous genets were present in inoculated plots (Figs 4 and 5); for example, genet G was fragmented and two sporophores formed in the middle of the plot. Such a pattern can be explained by an early colonization event in fumigated nursery soil, followed by fragmentation during outplanting. Uninoculated plots (Fig. 5) and the outer border of the inoculated plot (Fig. 4) were colonized by various genets of L. bicolor and L. laccata. Sporophores collected in the buffer zone between plots may have arisen from mycelium that emanated from mycorrhizal roots of neighboring, uninoculated trees. The spatial domains of the indigenous genets (Fig. 4 except genet G) were small (fruited area covered 0.5–13 m²) and not fragmented, but their fragmentation into several contiguous ramets cannot be excluded.

Colonization by numerous small-sized genets, with high fruiting rates, is reminiscent of young natural populations of ectomycorrhizal fungi, e.g. Suillus bovinus under Scots pine (Dahlberg & Stenlid 1990), L. bicolor under Norway spruce (De la Bastide et al. 1994) and Hebeloma cylindrosporum, a ruderal species adapted to pioneer sites (Gryta et al. 1997). These population structures are postulated to arise from multiple colonization of disturbed stands by basidiospores. Further growth of the genets leads to possible fragmentation and competitive elimination, i.e. an increase in average size and a decrease of genetic diversity (Dahlberg & Stenlid 1995). This phenomenon explains the population structure and genet size of L. bicolor under 17-year-old Scots pine (Baar et al. 1994). This may lead to genetically uniform old populations, such as described for the parasitic tree-root colonizer Armillaria bulbosa (Smith et al. 1992). In the natural oak forest of Saint Brisson, Laccaria spp. are scarcely present and their successful establishment on Douglas fir may have required basidiospore colonization, leading to various, although genetically similar (Fig. 3), indigenous genets of small size. As no new genets were detected in our 3-year survey, further indigenous colonization seemed to have been inhibited. However, the small size of the indigenous genets does not preclude their future ability to outcompete the inoculant strain. Some of the indigenous genets found on the border of inoculated plots (Fig. 4), or in the buffer zone between inoculated plots (Fig. 5), may have colonized inoculated root systems. In contrast, the inoculant genet is more reminiscent of older forest populations, with larger size of genets, mycelial propagation and reduced fruiting density (Fig. 4). Inoculation thus artificially provides the inoculant strain with traits normally exhibited by old genets.

Inoculation-linked gain in competitiveness does not seem to allow the spread of the strain into neighbouring plots (Fig. 5). Other genets may already have colonized these plots and outcompete the inoculant strain, limiting its impact on indigenous Laccaria populations over the 10 years. However, the spores produced by L. bicolor S238N may be dispersed over long distances, a phenomenon that could be of importance for the colonization of new stands (Dahlberg & Stenlid 1995). Assessment of the impact of genet introduction will require further analysis of the below-ground mycorrhizal populations associated with tree roots in the field, as well as in simplified model systems (Timonen et al. 1997), and a longer-term field monitoring. A better knowledge of ectomycorrhizal populations, e.g. the turnover rates for genets and the conditions for successful establishment of new genets, will also be necessary. Establishment of exotic basidiomycetes has been described in Europe (Singer 1977), such as Clathrus archeri (Parent & Thoen 1986) or Amanita singeri (Priou 1985). The strain of L. bicolor used in the present study does not show similar invasive traits, but longer-term potential with respect to distances and time remains to be assessed. However, establishment of the introduced S238N genet is highly favoured in the Saint-Brisson stand which supports a compatible tree species from the same geographical origin as the fungal genet.

The data presented here support the development of large-scale nursery inoculation of Douglas fir with the ectomycorrhizal fungal genet S238N. In future, the genus Laccaria will probably develop as a model for both mycorrhizal inoculation assessment and the study of disturbed fungal populations.

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References


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