Genetic structure of a natural population of the ectomycorrhizal fungus _Suillus pungens_

**BY PIERLUIGI BONELLO**, THOMAS D. BRUNS AND MONIQUE GARDES

1 *Dept. of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720–3102, USA*
2 *CESAC/CNRS, Université Paul Sabatier/Toulouse III, 29 rue Jeanne Marvig, 31055 Toulouse Cedex 4, France*

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**SUMMARY**

Sequence-based markers were developed to study the genetic structure and reproductive biology of the ectomycorrhizal fungus _Suillus pungens_ Thiers & Smith in a Bishop pine (_Pinus muricata_ D. Don) forest. Six different basidiome genotypes were found in a 1200 m² area. Five of the six genotypes were represented by single basidiomes. The remaining genotype comprised 13 basidiomes and covered an area of at least 300 m², with maximum measured dimensions of 40 m and 14 m. This is the largest genet of an ectomycorrhizal fungus described to date, and is likely the result of vegetative growth, because analysis of single spore isolates eliminates the possibility of genetic identity resulting from either apomixis or fortuitously indistinguishable recombinant genotypes. Genetic analysis also shows that although out-crossing appears to predominate in the population, at least a low percentage (1±4%) of spores are secondarily homothallic.

The combination of extensive vegetative growth and abundant fruiting suggests _S. pungens_ utilizes more carbon than might be expected for a species which accounts for <3% of the total ectomycorrhizal abundance at the site. Additional carbon might come from either more efficient host–fungus transfer, pooling of carbon from the roots of different host plants, or saprophytism.

Key words: Ectomycorrhizas, population structure, SSCP, _Suillus pungens_ Thiers & Smith.

**INTRODUCTION**

Prior work in a Bishop pine (_Pinus muricata_) forest in coastal California revealed that the ectomycorrhizal fungus (EMF) _Suillus pungens_ was the most frequent producer of basidiomes, yet it accounted for <3% of the ectomycorrhizal roots (Gardes & Bruns, 1996). On the other hand, species of _Russula_ and _Tomentella_, which accounted for much higher percentages of the colonized roots in this forest, were much less frequent in the fruiting record (Gardes & Bruns, 1996). This may be a common pattern for _Suillus_ species: studies in other ecosystems have also found a low percentage of _Suillus_ spp. mycorrhizas along with a high production of basidiomes (Danielson, 1984; Natarajan, Mohan & Ingleby, 1992).

Gardes & Bruns (1996) proposed two hypotheses to explain why _S. pungens_ is an abundant fruiter even though it colonizes few roots: (i) it invests less in vegetative growth and persistence and more in fruiting than other species within this given community, or (ii) it has access to more carbon, either owing to more efficient transfer from its host relative to species that appear to dominate the root community, or to a mix of biotrophic and saprophytic capabilities. Under the first hypothesis _S. pungens_ would behave as a ruderal (R) species, its genets would be small and short-lived, and frequent establishment by spores would be the main mode of persistence (Deacon & Fleming, 1992). In contrast, the higher carbon budget in the second hypothesis is compatible with either a combative or stress (C or S) strategy; it would predict large, persistent individuals capable of out-competing other organisms for resources (Cooke & Rayner, 1984).

To test these hypotheses the genotypes of _S. pungens_ that fruited within a 0–12 ha part of the forest...
studied by Gardes & Bruns (1996) were identified and mapped. Unique fungal genets have commonly been identified by testing somatic incompatibility of *in vitro* cultured samples of the population (Dahlberg, 1995). However, the outcome of somatic incompatibility testing can be ambiguous, sometimes even in self pairings (Jacobson, Miller & Turner, 1993; our own results with *S. pungens*). Furthermore, whereas incompatibility is a sufficient condition to define two isolates as different, compatibility does not necessarily imply that two isolates are genetically identical (Sen, 1990; Jacobson *et al.*, 1993; Rizzo, Blanchette & May, 1995; Rodriguez, Petrini & Leuchtmann, 1995). Another major limitation of somatic incompatibility studies is that the technique can be used only with species that can be easily cultured *in vitro*, and many EMF cannot. Thus, to date, the population structures of species in only two EMF genera, *Suillus* and *Laccaria*, have been studied in some detail using this technique (Fries, 1987b; Sen, 1990; Baar, Ozinga & Kuyper, 1994; Dahlberg & Stenlid, 1994; Dahlberg, 1997), or mating types and isozyme analysis (de la Bastide, Kropp & Piché, 1994), for which *in vitro* culturability is also an essential prerequisite. A recent study of *Hebeloma cylindrosporum* populations was conducted using molecular methods, but even in that case most of the techniques were dependent on the availability of relatively large amounts of DNA (Gryta *et al.*, 1997), which is most easily achieved by isolating the fungi in pure culture.

To circumvent these problems and to employ methods that are generally applicable to non-culturable species, a two-step PCR-based approach was used. In the first step randomly amplified polymorphic DNAs (RAPD), arbitrary primer PCR (AP-PCR), and PCR of the ITS region were used to select monomorphic fragments, i.e. those that are reproducibly present in all of the samples. In the second step the selected fragments were re-amplified and then sequence differences identified among them by using single-strand conformational polymorphism (SSCP) analysis. This latter technique is based on the principle that sequence differences result in different single-strand DNA conformations that can be resolved in native polyacrylamide gels (Lessa & Applebaum, 1993). The technique is sensitive enough to detect point mutations (Orita *et al.*, 1989) in DNA fragments smaller than 1,500 bp (Hayashi, 1991). Thus, most different alleles can be differentiated (e.g. Burt *et al.*, 1996). Furthermore, because single strands of individual loci can be recognized, the markers are co-dominant; thus, homozygous and heterozygous individuals can be distinguished. These characteristics make this technique very powerful for resolving genotypes and assessing the reproductive genetics of natural EMF populations. Elucidating the reproductive strategies of *S. pungens* is germane to this study because the determination of individual genets is dependent on (i) discrimination between selfing and vegetative propagation and (ii) ensuring that basidiospores are not the product of apomixis (i.e. production of spores without fertilization and meiosis – Ainsworth, 1971).

**Materials and Methods**

**Fungal material**

A total of 21 basidiomes of *S. pungens*, collected over 2 yr at the Point Reyes National Seashore, were studied. The physical, climatic and ecological characteristics of the study area have been described by Gardes & Bruns (1996); (our site corresponds to site C in that paper). The locations of the basidiomes were mapped to the nearest 0.5 m within an area of 1200 m² (Fig. 1). Basidiomes 1–11 and 13–16 were collected during the ’93–’94 season, 17–22 in ’94–’95. Of these 21 basidiomes, 18 were collected from within the intensively surveyed sampling grid, whereas the other three (1, 7 and 9) were collected from immediately surrounding areas. One basidiome, 12, was collected at the Berkeley Marina (50 km in aerial distance from the Point Reyes site) in ’93–’94, and used to test the resolving power of the molecular methods used. The basidiomes were freeze-dried and stored at −20 °C as described by Gardes & Bruns (1996).

A total of 19 single spore isolates were obtained from basidiome 6 (filled symbol in Fig. 1) using abietic acid (Fries, 1987a) and charcoal. Mycelia were cultured in liquid modified Hagem’s medium (Heinonen-Tanski & Holopainen, 1994), filter-collected, freeze-dried, stored and processed in the same manner as the basidiomes.

**DNA extraction**

Approximately 50 mg of dry fungal biomass (pulverized with a Cyclone Sample Mill–Udy Corporation, USA) were extracted in 600 µl of TES buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 2% (w/v) SDS) at 65 °C for 45 min. The extracts were centrifuged and the supernatant transferred to fresh tubes. Chloroform (600 µl) was added, the solution emulsified, and then separated by centrifugation for 5 min at maximum speed in a micro-centrifuge. A fraction of supernatant (300 µl) was transferred to fresh tubes and processed using the Gene Clean*® II Kit (Bio 101, Inc., USA) (20 µl of glass milk per tube) (O’Donnell, pers. comm.). The DNA was finally eluted into 50 µl of filter-sterilized, double distilled H₂O (ddH₂O). This DNA stock was stored at −20 °C. The stocks were diluted 1:50–1:400 and the dilutions used as templates for the PCR reactions described below.
Genetic structure of a Suillus pungens population

PCR, RAPDs and AP–PCR

RAPDs and AP–PCRs were carried out at least twice on two separate extractions for each fungal sample. PCR reactions were conducted using the single primers (RAPDs) and primer pairs (PCR, AP–PCR) listed in Table 1. The reactions (12.5 µl) contained 6.25 µl of template DNA, 2.5 µl of filter-sterilized ddH₂O, 1.25 µl of 10× PCR buffer (0.5 M KCl, 0.1 M Tris-Cl (pH 8.3), 25 mM MgCl₂, 0.1% (w/v) gelatin), 1.25 µl of 10× dNTPs (2 mM of each), 0.2 µl of single 50 µM primer (or 0.12 µl of each 50 µM primer in the case of AP–PCR), and 0.25 units of Taq DNA polymerase. Thermal cycling for RAPDs and AP–PCR was carried out as follows: three cycles at 94/35/72°C (5 min each step), followed by 32 cycles at 94/50/72°C (1 min each step), followed by a final 10-min extension step at 72°C. PCR cycling for the ITS region and for re-amplification of RAPD and AP–PCR bands (see below) was carried out as described by Gardes & Bruns (1993) (T_m = 53°C). PCR products were separated in 1% NuSieve® (FMC BioProducts, USA)+0.5% agarose (Life Technologies, Inc., USA) in 0.5× TAE buffer (Sambrook, Fritsch & Maniatis, 1989). Gel images were digitally captured by an EagleVision® image analyser (Stratagene, USA).

PCR–SSCP

Monomorphic bands (i.e. consistently present in all samples) < 500 bp, resulting from RAPD or AP–PCR amplification, were excised using a sterile, aerosol-resistant P1000 pipette tip. The plugs were extruded into the bottom of sterile 500 µl microfuge tubes, 100–400 µl of filter-sterilized ddH₂O was added, and the plugs were melted and diluted at 95°C for 5 min. This diluted DNA was used as a template for re-amplification of specific bands using the relevant primer or primer pairs and the PCR conditions described in Gardes & Bruns (1993) (T_m = 53°C). Once amplification was confirmed (Fig. 2a), the new amplicons were used as templates in a new

**Figure 1.** Schematic depiction of relative positions and arbitrary sizes of *Suillus pungens* genotypes plotted on the reference mapping grid described in the main text. Each mushroom icon identifies an individual basidiome. Enclosures represent separate genotypes (size of single basidiome genets are arbitrary). Basidiome 12 is an off site sample from the Berkeley Marina, c. 50 km in aerial distance from the study site. Open circles represent standing Bishop pine trees, diameters to scale. The area of the shaded surface has been calculated on the basis of triangulations on the map. Basidiome 6, indicated by a filled symbol, is the parent used for the basidiospore studies.
PCR reaction involving radiolabelling of PCR products (hot PCR) (Burt et al., 1996). In the case of the ITS region, the unique products of the first amplification were used directly as templates for hot PCR (gel not shown). The 12-43-µl reactions consisted of 0.5 µl of template, 9.1 µl of filter-sterilized ddH2O, 1.25 µl of 10× PCR buffer, 1.25 µl of a 300-fold dilution of the 10× dNTP stock, 0.2 µl of single 50 µM primer (or 0.12 µl of each 50 µM primer in the case of AP–PCR), 0.25 units of Taq DNA polymerase, and 0.08 µl of 32S dATP and 32P dCTP (Amersham Life Science, Inc., USA) (specific activity >1000, 370 MBq ml–1). Ten PCR cycles were run at 95/50/72 °C (1 min each step), followed by a final 7-min extension step at 72 °C. Three µl of 32S-labelled PCR product were combined with 3 µl of denaturing (formamide-containing) stop solution (USB Corp., USA), heated at 95 °C for 5 min, and snap-cooled on ice. The mixture (6 µl) was loaded on a non-denaturing 0.5 × MDE polyacrylamide gel (FMC BioProducts, USA). The samples were run overnight (16–17 h) at room temperature and at a constant power of 2–10 W. X-ray film was exposed to the dried gels for 2–4 d.

Segregation analysis
Segregation of four loci was studied by analysing the basidiospore isolates using SSCP. These four loci were selected because they were easily scored and were heterozygous in the parent. If nuclear assortment follows a random distribution, i.e. if apomixis or other non-random assortment mechanisms (Summerbell et al., 1989) are not occurring, the expected proportion of spores simultaneously heterozygous at all four independent loci (the parental types), is 0.54, or 6.25%.

Expected and observed frequencies of parental types were compared using a χ2 test with Yates correction for continuity (Zar, 1984).

Table 1. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′−3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNL12</td>
<td>CTGAACGCCTCTAAGTCAG</td>
<td>R. Vilgalys (unpublished)</td>
</tr>
<tr>
<td>ITS1</td>
<td>TCGTAGGTGAACTCTCGGG</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>ITS1F</td>
<td>CTTGGTCATTTAGGAGAATTTAAGAA</td>
<td>Gardes &amp; Bruns (1993)</td>
</tr>
<tr>
<td>ITS2</td>
<td>GCTGGCTTCTCT CCTAGATGC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>ITS3</td>
<td>GATCGATGAAGAACGCACTG</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>ITS4</td>
<td>TTCCTCCGCTTATTTGATATGC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>NL13</td>
<td>CAAGCGAATCTTTACATGACAGC</td>
<td>R. Vilgalys (unpublished)</td>
</tr>
<tr>
<td>NS2</td>
<td>GGCCTGCTGACCGACGACGTC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>NS3</td>
<td>GCAAGTCGTTGTGCAACGACGCC</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>NS5</td>
<td>AACCTAAAGAAGATTGACGGAAG</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>NS6</td>
<td>GATCGACGGCCTTATTTGATGCT</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>TW15</td>
<td>CTTGGAGACCTGCTGCGG</td>
<td>T. J. White (unpublished)</td>
</tr>
</tbody>
</table>

Primer pairs ITS1F–ITS2 and ITS3–ITS4 were used to amplify ITS1 and ITS2, respectively, i.e. the two components of the ITS region. All primers are derived from the nuclear small and large rDNA but, because they are used as either unpaired single primers or as mis-paired sets, the specific rDNA regions are not the targets.

Population genetic parameters
Observed and expected average heterozygosities (H0 and Ho, respectively) were calculated using all unique genotypes present at the site, including the ones collected outside the sampling grid (Fig. 1). The same four diallelic loci used for the spore analysis were used to calculate Ho and He. Ho, i.e. the average proportion of heterozygous individuals at each locus, is given by:

\[
\bar{H}_o = \frac{1}{n} \sum _{i=1} ^{n} \frac{x_i}{N},
\]

where \(x_i\) is the number of heterozygous individuals at locus \(i\), \(n\) is the number of loci, and \(N\) is the size of the population (total number of individuals). He, i.e. the average proportion of heterozygous individuals at each locus under Hardy–Weinberg expectations (Berg & Hamrick, 1993), is given by:

\[
\bar{H}_e = \frac{1}{n} \sum _{i=1} ^{n} 2p_i q_i,
\]

where \(p\) and \(q\) are the proportions of individual alleles at locus \(i\), and \(n\) is the number of loci. If \(H_0 \geq H_o\) there is no evidence for an inbreeding effect in the population. Significance of the difference was tested by summing the \(\chi^2\) values and degrees of freedom over the four polymorphic loci via calculation of the fixation index \(F = 1 - H_o/H_e\) for each locus, as described in Berg & Hamrick (1993).

Basidiospore staining and germination
A spore print was obtained aseptically from a basidiome collected at the Berkeley Marina in January 1996. Spores were picked up in sterile water and deposited on microscope slides coated with a thin film of filtered poly-d-lysine (1 mg ml–1 in...
Figure 2. (a) Re-amplification of RAPD and AP–PCR fragments of Suillus pungens basidiome. Amplicons are named after the primers used to generate them. Re-amplification products are adjacent to the original more complex patterns from which they were obtained, and their lanes are marked with asterisks. In lane 15, the band corresponding to that in lane 16 is very faint (white arrow). Lanes 1 and 17: 100 bp ladder (Pharmacia Biotech); lane 10: λ DNA cut with EcoRI and HindIII. (b) Example of SSCP run, showing both basidiome and single basidiospore isolate patterns for the same NL13 locus. Lane 1, parental basidiome 6; lanes 2–20, basidiospore isolates 1–19. Interestingly, only one of the two alternate alleles is present among the homozygous spores.

deionized water (DW)). After drying at 60 °C on a hot plate the spores were processed following protocols for DAPI (4',6-diamidino-2-phenylindole) and haematoxylin staining. DAPI is a fluorochrome that selectively binds to DNA and haematoxylin is a stain specific for chromatin (O’Brien & McCully, 1981). Half of the slides were fixed in modified Newcomer’s fluid (Newcomer, 1953), i.e. isopropanol: propionic acid: acetone (6:3:1) for 2 h. Fixed spores were rinsed in running DW for 10 min. Fixed and unfixed slides were stained with DAPI (0.1 mg ml⁻¹ in DW) by depositing a drop of solution on the slide and by heating it to 60 °C on a hot plate for a few minutes. Slides were rinsed in running DW for 10 min, mounted in DW, and observed under epifluorescence using a Zeiss® Axiophot compound microscope. A second batch of fixed and unfixed slides was rinsed, hydrolysed and stained with haematoxylin as described by Treu & Miller (1993), except that the slides were left in the stain over a weekend.

Basidiospores from the same basidiome were seeded on a plate of MMN medium (Marx, 1969) (containing glucose in place of sucrose) supplemented with 100 µg ml⁻¹ abietic acid (Fries, 1987a). Spores were incubated in the dark at room temperature for 2 months, but began germinating after 3 wk.

RESULTS

Determination of genotype frequency, position and size

The ITS 1 and ITS 2 spacers of the ITS region were amplified and analysed separately (with primer pairs ITS1F-ITS2 and ITS3-ITS4, respectively). Both ITS fragments produced simple patterns of two to
with this interpretation (e.g. Fig. 2) within single spore isolates in a manner consistent with putative alleles at these and other loci also segregated respectively (Lessa & Applebaum, 1993). Individual strands from a homozygous or heterozygous locus, to the mobility differences of the two or four single strands from a homozygous or heterozygous locus, to the mobility differences of the two or four single SSCP fragments, corresponding to the single spore isolates. Those that did were then treated as loci. For instance, primers producing multiple fragment sets were NS3 (two simple, apparently linked loci), and TW15 (four unlinked loci, TW15/1, TW15/2, TW15/3 and TW15/4). Multiple loci were considered physically linked when, with each primer or primer pairs, different sets of closely spaced SSCP bands remained associated across the different basidiomes. Even when SSCP patterns were too complex to establish which fragments represented alternative alleles, they were sufficiently clear and reproducible for pairwise visual comparisons of identity between samples. Samples were deemed identical when they shared all strong reproducible bands in an SSCP profile. Information about complexity of SSCP patterns for each primer or primer pair, and individuality of basidiome genotypes, is summarized in Table 2.

The genotypic discrimination thus obtained with each DNA fragment was outlined on the basidiome map (Fig. 1) and this resulted in the definition of six different genotypes in the study plot (1200 m²). Among these, a large genotype could be recognized, spanning the distance between basidiomes 6 and 16, c. 40 m, whereas the distance between 11 and 14 is c. 14 m (Fig. 1). By joining the outermost vertices (basidiomes) with straight lines to form a polygon (Worrall, 1994), the surface area of the genotype was estimated to be c. 300 m² (based on triangulations on the map).

**Genetic parameters**

SSCP analysis showed that all 19 single basidiospore isolates analysed were heterozygous at one or more of the four loci scored, indicating that they were all

<table>
<thead>
<tr>
<th>DNA fragments</th>
<th>SSCP pattern complexity*</th>
<th>Individuality of fruit body genotypes, including 12 (outlier)</th>
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<tbody>
<tr>
<td>ITS region</td>
<td>Simple</td>
<td>1 = 19; 13 = 20 = 21; all others indistinguishable from each other</td>
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<tr>
<td>ITS1-NS5</td>
<td>Complex</td>
<td>18 = 19 = 20; all others indistinguishable from each other</td>
</tr>
<tr>
<td>NL13</td>
<td>Simple</td>
<td>all indistinguishable from each other</td>
</tr>
<tr>
<td>NS2-CNL12</td>
<td>Complex</td>
<td>1; 7; 12; 13; 19 = 20 = 21; all others indistinguishable from each other</td>
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<tr>
<td>NS3</td>
<td>Simple</td>
<td>1 = 7 = 12; 13; 19; 20 = 21; all others indistinguishable from each other</td>
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<tr>
<td>NS6-CNL12</td>
<td>Complex</td>
<td>1; 9; 13; 18 = 19 = 20; all others indistinguishable from each other</td>
</tr>
<tr>
<td>TW15-CNL12</td>
<td>Complex</td>
<td>12; 19 = 20 = 21; all others indistinguishable from each other</td>
</tr>
<tr>
<td>TW15/1†</td>
<td>Simple</td>
<td>7; 13; all others indistinguishable from each other</td>
</tr>
<tr>
<td>TW15/2†</td>
<td>Complex</td>
<td>1 = 7; 9; 12; 13; 19; 20 = 21; all others indistinguishable from each other</td>
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<tr>
<td>TW15/3†</td>
<td>Complex</td>
<td>1 = 7; 9; 12 = 13; all others indistinguishable from each other</td>
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<tr>
<td>TW15/4†</td>
<td>Complex</td>
<td>1; 7; 9; 12; 13 = 20 = 21; 19; all others indistinguishable from each other</td>
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</table>

Except for the ITS region, the fragments are named after the single primers or primer pairs used to generate them. * Simple: 2–4 bands; complex: multiple bands (more than 4). † Part of a single, complex SSCP pattern (see text). N.B.: ITS region, NL13, NS3 and TW15/1 were used in the basidiospore segregation studies (see Table 3).
Table 3. Single basidiospore isolates displaying the parental type (X) heterozygous loci ITS, NS3, NL13, and TW15/1

<table>
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<tr>
<th>Loci</th>
<th>1</th>
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<tr>
<td>NL13</td>
<td>X</td>
<td>X</td>
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<tr>
<td>TW15/1</td>
<td>X</td>
<td>X</td>
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</tr>
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</table>

Parental types at all four loci

| Parental types | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Observed frequency of parental types at all four heterozygous loci is 0 ± 0 (0 in 19 spores), expected is 0 ± 0.5% (0 ± 0.625% in 19 spores) in the case of random assortment. $\chi^2$ corr. = 0 ± 4.25, 1 d.f., 0.50 < $P$ < 0.75, n.s. (see text for details).

Figure 3. Haematoxylin-stained basidiospores of Suillus pungens. Arrow shows a binucleate spore. Spore length: 10 µm.

Discussion

All single basidiospore cultures of S. pungens were heterozygous at one or more loci and had non-parental genotypes. These results indicate a life cycle with a secondarily homothallic (i.e. selfing) component, rather than a typical basidiomycetous heterothallic life cycle with a haploid phase of uninucleate basidiospores. In addition, the percentage of binucleate spores (1.3–1.4%) was similar to the percentage that germinated under laboratory conditions (< 1%). Interestingly, a similar result was obtained in S. granulatus from North America (Jacobson & Miller, 1994). By contrast, Fries (1987a) found higher germination rates and obtained homokaryotic isolates from European S. granulatus and other Suillus spp. However, it is also known that varying percentages of two-spored basidia are produced in several Suillus spp. (Treu & Miller, 1993), and that these basidia are more likely to generate binucleate spores (Kühn, 1980; Tommerup, Bougher & Malajczuk, 1991). Taken together, these observations suggest that only binucleate spores of S. pungens can germinate in vitro. This hypothesis has not yet been tested owing to the difficulty of sorting uninucleate and binucleate spores effectively while maintaining their germination potential.

Although the germinated spores were secondarily homothallic, their potential ecological significance is not clear. In theory, dikaryotic spores could produce secondary mycelium directly, and eventually basidiomes in nature, without outcrossing. This mycelium would thus be the result of selfing. For fungi in which post-meiotic nuclear assortment is random, selfing would over time lead to a significant decline (inbreeding) in the observed average heterozygosity level of the population, relative to that expected under Hardy–Weinberg conditions ($H_0$ and $H_e$, respectively) (Hartl & Clark, 1989). If such behaviour were a consistent and quantitatively important component in the reproductive strategy of S. pungens, significantly low heterozygosity should be observed in the population. On the contrary, $H_e$ was significantly larger than $H_0$; this result appears inconsistent with a high frequency of selfing. However, for fungi in which post-meiotic nuclear assortment is not random, maintenance of high population heterozygosity is possible (Summerbell,
et al., 1989). Neither of these situations appears to be the case with *Suillus pungens*, as loss of heterozygosity was evident in the segregation of individual loci (Fig. 2b) and recovery of parental genotypes was not observed (Table 3). From this finding it can be inferred that the ecological role of the monokaryotic (presumably haploid) basidiospores is probably more important than that of dikaryotic spores. This is not surprising given that at least 98% of the observed spores are monokaryotic, and probably haploid. However, a mixed homothallic–heterothallic reproductive strategy, which has been suggested for other *Suillus* spp. (Treu & Miller, 1993), cannot be excluded for *S. pungens*.

Genetic analysis of the germinated spores demonstrates that identity among basidiomes is unlikely to have resulted from either apomictically produced spores or from fortuitously similar recombinant genotypes. Given random assortment, the chance of recovering a parental genotype with four heterozygous loci from the selfed progeny is $0.5^4 = 0.0065$. The chance of recovering an identical recombinant genotype in the basidiome study, however, is actually much lower because a total of 11 polymorphic SSCP patterns, including the four used in the progeny study, were compared. Seven of these were complex and so comparisons were based on identity only, rather than on explicit determination of alleles present. However, even if only one of the seven contained heterozygous loci, the probability of recovering identical recombinant genotypes drops to $0.5^3 = 0.0031$. Thus, the largest genotype is, with a high probability, a single mycelial individual (genet). Although it may be fragmented into ramets, this genet is likely to be the result of mycelial spread from a single point source since no other mechanisms for dispersal and establishment of active vegetative propagules in *Suillus pungens*, or other ectomycorrhizal basidiomycetes, is known. The distance between the outermost basidiomes of this individual is $>40$ m, making this the largest EMF genet reported to date. Although direct correspondence between topographic basidiome distribution and genet size is unlikely, this linear dimension, and the measured area of $c. 300 \text{ m}^2$, are probably underestimates of the genet’s true extension, since these basidiomes lie on the perimeter of the study plot, which is a fraction of the area of the forest stand that contains it.

To obtain a rough estimate of a minimum growth rate, a simplifying assumption can be made that this genet started growing midway between the outermost basidiomes $c. 40$ yr ago at the time the current forest replaced a previous Bishop pine forest following a fire (Gardes & Bruns, 1996). A growth rate of $0.5 \text{ m yr}^{-1}$ is then required to account for the genet size. Uncertainties about the full size of the genet would make this an underestimate, and if different ramets of the genet survived the last fire then the rate is an overestimate. Nevertheless, a rate of $0.5 \text{ m yr}^{-1}$ is similar to those of some other EMF (Read, 1992) and on a par with some important forest saprotrophs and necrotophs like *Armillaria* spp. and *Heterobasidion annosum* (Smith, Bruhn and Anderson, 1992; Garbelotto et al., 1997).

The large genet of *Suillus pungens* reported here is reminiscent of the patterns seen in Europe with *S. bovinus* and *S. variegatus* (Dahlberg & Stenlid, 1994; Dahlberg, 1997). In all three species there is now evidence for temporal persistence and vegetative growth of large genets. These patterns are not what is expected for species exhibiting strictly ruderal (R) characteristics, such as *Hebeloma cylindrosorum* (Gryta et al., 1997), in which rapid reproduction and transient establishment are typical. Instead, these results suggest that *Suillus* spp. have a strategy combining R, C, and S characteristics, i.e. they are competitors that are able to both persist in time and expand in space while maintaining high, if irregular, reproductive potential (Cooke & Rayner, 1984).

Spread and persistence of a large *S. pungens* genet fruiting abundantly implies high physiological activity and thus a large C budget, and this appears to be at least partly inconsistent with its low mycorrhizal percentage (3% of total: Gardes & Bruns 1996). Three possibilities, which are not mutually exclusive, are suggested to explain this phenomenon: (1) *S. pungens* is much more efficient at obtaining C from its host, thus many fewer mycorrhizal connections are needed for significant C transfer; (2) a larger size enables individual genets to visit the roots of more trees and to pool more total C than species which are more common, but produce smaller genets; and (3) a fraction of the C is obtained saprophytically.

In support of the first hypothesis, others have previously found evidence for differential C demands among mycorrhizal fungi (Finlay & Söderström, 1992). In fact, *Suillus* can kill seedlings under some conditions (Piola, Rohr & van Aderkas, 1995), which suggests that its biotrophic C demand might be too high in some cases. A more efficient C transfer for Suillusord fungi is also consistent with the specialist hypothesis of Cullings, Szaro & Bruns (1996).

The large genet size and abundant hyphal cords of *S. pungens*, and the fact that some other mycorrhizal and saprophytic fungi have the potential to transport resources through cords over relatively long distances (Finlay & Read, 1986; Wells, Boddy & Evans, 1995), are the main reasons the second hypothesis is proposed. By comparison, *Russula amoenoens* and *Tomentella sublitacina*, two species which are much more abundant on the roots at this site, but which appear to be uncommon fruiters (Gardes & Bruns, 1996), do not form cords.

In support of the third hypothesis, various levels of saprophytic capabilities have been demonstrated for several EMF, including *Suillus* spp. (Haselwandter, Bobleter & Read, 1990; Entry, Donnelly &
Cromack, 1991; Majiela, Fagerstedt & Raudaskoski, 1991; Durall, Todd & Trappe, 1994; Bending & Read, 1995). Laiho (1970) recognized that *Paxillus involutus* can grow saprophytically, but needs connections to a live host to produce basidiomes, and Wang (1995) has observed symbiotic, pathogenic, and saprophytic behaviors in different stages of the life cycle of the EMF *Tricholoma matsutake*, the matsutake mushroom. A saprophytic growth mode could allow individual genets of *S. pungens* to survive short periods of live host absence, for example following wildfires, by persisting on the dead host root systems or other dead organic matter. Since the study plot has recently burnt in a wildfire (autumn 1995), the survivability of the large genet through such a catastrophic disturbance has now become testable.

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**References**


*Note added in proof*

Dr Annette Kretzer has pointed out to us that the probability of maintaining heterozygosity at a locus within a dikaryotic spore is not 0.5 (as we state in the paper), because nuclei are packaged into spores from a pool of either four (in bisporic basidia) or eight (in tetra-sporic basidia) nuclei, without replacement. Thus, if four meiotic nuclei are packaged into two basidiospores, the probability of maintaining heterozygosity is actually 0.67, and if eight nuclei (four meiotic and four mitotic, due to post-meiotic mitosis in the basidium) are packaged into four basidiospores, the probability is 0.57. Although the eight nuclei/four spore model is a better fit to the observations in Table 3, neither model can be rejected: 0.05 < P < 0.1 and 0.25 < P < 0.50, for the four nuclei/two spore, and for the eight nuclei/four spore model, respectively. In any case, the manner in which spores are formed affects the probability of seeing identity due to chance from establishment of secondarily homothallic spores. Assuming either the four nuclei/two spore, or the eight nuclei/four spore model, the probabilities of recovering heterozygous spores would be \( P = 0.67^n \) or \( P = 0.57^n \), respectively, where \( n \) is the number of independent loci being considered. To reach a probability below 0.05, we thus need eight or six heterozygous loci (\( 0.67^8 \approx 0.040, 0.57^6 \approx 0.034 \)), respectively. Therefore, either four or two of the seven complex markers listed in Table 2 would need to be heterozygous to achieve these probabilities, rather than only one as stated in the discussion. On the other hand, a further possible source of fortuitously identical genotypes comes from random mating of non sibling, uninucleate, haploid basidiospores. If we assume panmixia, and use the expected population allele frequencies (data not shown), the four loci given in Table 3 are alone sufficient to reduce the probability of observing identity to 0.033.