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Benoit Marçais, F. Martin, C. Delatour. Structure of *Collybia fusipes* populations in two infected oak stands. *Mycological Research*, 1998, 102 (3), pp.361-367. 10.1017/S0953756297005017. hal-02058967

HAL Id: hal-02058967

<https://hal.science/hal-02058967>

Submitted on 6 Mar 2019

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Structure of *Collybia fusipes* population in two infected oak stands

B. Marçais, F. Martin and C. Delatour

Unité des Ecosystèmes Forestiers, Laboratoires de Pathologie Forestière et de Microbiologie. Institut National de la Recherche Agronomique, 54280 Champenoux, France

Collybia fusipes is the cause of a root rot of oak trees (*Quercus petraea*, *Q. robur*, *Q. rubra*). We studied the structure of *C. fusipes* populations in two infected oak stands by using somatic incompatibility and DNA amplification. Isolates were obtained from different oak root systems or from within the same root system and somatic incompatibility groups (SIG) were identified. Many small SIGs that seldom encompassed more than one root system were present in both stands. More than one SIG was usually present on an individual root system: there were 3.1 ± 1.3 SIGs on the pedunculate oaks and 2.2 ± 0.6 on the red oaks. The largest SIG contained more than 70 % of the isolates obtained from the root system of fourteen of the twenty studied trees. Isolates that belonged to the same SIG usually had the same ribosomal intergenic spacer. It is concluded that *C. fusipes* spreads poorly from tree to tree by vegetative means.

INTRODUCTION

Collybia fusipes (Bull. Ex Fr.) Quél. is a very common fungus in France. It occurs mainly at the base of the trunk on living oaks (*Quercus petraea* (Matt.) Liebl., *Q. robur* L., *Q. rubra* L.) and on stumps (Département de Santé des Forêts, 1994). Although it has been well known by mycologists for a long time, *C. fusipes* was only recently reported to be a root pathogen of mature oaks in forest conditions (Delatour & Guillaumin, 1984; Guillaumin *et al.*, 1985). Thus, very little information is available on the epidemiology and virulence of this root rot fungus. In particular, the way in which *C. fusipes* spreads from tree to tree, a very important epidemiological feature, is unknown.

C. fusipes produces cordlike structures called pseudorhiza (Buller, 1958). These structures remain attached to host roots and are found growing free in the soil only when they grow from infected tissues to the soil surface and support fruit bodies. They are quite common on the surface of infected roots and could be involved in the spread of *C. fusipes* within and between the root systems of host trees (Marçais & Delatour, 1996). *C. fusipes* is known to sporulate abundantly from mid-June to the end of September (Département de Santé des Forêts, 1994) and, therefore, basidiospores also could be important in the spread of this fungus. The population structure of *C. fusipes* within a stand should indicate which method of dispersal, vegetative growth or basidiospores, is prevalent. If spread from tree to tree occurs through root contacts, genets of *C. fusipes* should be infrequent and occupy large

areas, encompassing many trees. By contrast, if basidiospores are an important means of dispersal, genets should be frequent in number and occupy small areas, including only one or a small number of trees.

Population structure has been successfully used to indicate the way other root infecting fungi spread within infected stands. It was concluded that vegetative growth was prevalent for *Phellinus weirii* and *Armillaria luteobubalina* because they formed large foci in which only one or a few large genets were present (Childs, 1963; Kile, 1983; Bae *et al.*, 1994). Other *Armillaria* species use both basidiospores and vegetative growth to spread and their population structure in infected stands can either be formed by a high number of genets, few of them attaining a very large size, or a few large genets (Kile, 1986; Guillaumin & Berthelay, 1990; Smith, Bruhn & Anderson, 1992; Worral, 1994). Basidiospores were deemed the main means of dispersal for *Phaeolus schweinizii* and *Inonotus tomentosus* because their genets were small and encompassed either one or a small number of trees (Barrett & Uscuplic, 1971; Lewis & Hansen, 1991). In all those studies, somatic incompatibility, i.e., the interaction that develops between two different heterokaryotic isolates when paired together in culture, was found to be a very useful tool to characterize the population structure of root infecting fungi.

We studied the structure of *C. fusipes* populations in two infected stands of oaks, using somatic incompatibility and amplification of the ribosomal intergenic spacer. We looked at the population structure at the stand level and also at the individual root system level because we wanted to gain some insight on the way *C. fusipes* infects its host.

MATERIALS AND METHODS

Sample collection and isolation procedures

Two stands were selected for the study. The first was a coppice with standard stand stocked mainly with pedunculate oaks (*Q. robur*) and hornbeams and located at Mersuay (parcelle 5, Forêt communale de Mersuay, Haute Saône, France). The study plot was 2 ha. The second was a 1.1 ha red oak stand (*Q. rubra*), originating from a natural regeneration in 1924-28 and located at Les Barres (Glandée d'Amérique, Arboretum des Barres, Loiret, France). A 0.8 ha portion of this stand was studied.

At Les Barres, the occurrence of *C. fusipes* fruit bodies on 74 trees had been observed for three years (D. Piou, personal communication). Infected root tissues were sampled on all the trees on which *C. fusipes* fruit bodies had occurred in at least one of the three years. At Mersuay, infected root tissues were sampled on all the trees on which *C. fusipes* fruit bodies were present by the end of June 1993. The structure of *C. fusipes* population on individual root systems was also studied on nine trees from Les Barres and eight trees from Mersuay. Root systems were partially excavated (distance of 80 cm from collar and depth of 30 cm), occurrences of *C. fusipes* induced lesions were recorded, and portions of diseased root tissues were sampled from 8-12 scattered locations. Two more pedunculate oaks located in Aillevans and Longeville, close to Mersuay (Haute Saône) were sampled with the same technique. One red oak in Les Barres was completely uprooted and 60 samples were collected. Isolations from infected root samples were made on MAT

selective medium (15 g of Agar, 10 g of Malt, 250 mg of Thiabendazol, 100 mg of penicillin, 100 mg of streptomycin, 1 l of distilled water).

Somatic incompatibility tests

C. fusipes isolates were paired by placing 4 mm² agar blocks containing actively growing mycelium 5 mm apart on cristomalt medium (15 g of Agar, 20 g of cristomalt, 1 l of distilled water). One replicate was done for each pairing. The morphology of the interaction was observed after incubation at room temperature for 15, 30 and 45 days. Pairings were rated as: 0, compatible or no reaction; 1, incompatible, light pigmentation appearing at 30 days after incubation; 2, incompatible, heavy pigmentation present 15 days after incubation.

Isolates originating from the same tree were treated as follows. All isolates were paired against one isolate from the tree. This enabled us to define a first somatic incompatibility group (SIG). The remaining isolates were paired against an isolate that did not belong to the first SIG. This was repeated until the SIG of only 3-4 isolates remained unknown. Those 3-4 isolates were then paired together in all combination. For each SIG, the results were checked in a second experiment by pairing the isolates of each SIG in all combinations.

In each plot, isolates from all trees were paired in all combinations. For the trees on which several isolates had been collected, just one isolate of each SIG present on the root system was included in the study. All pairings that were rated 0 or 1 were repeated in a second experiment.

DNA extraction and PCR amplification

The ribosomal intergenic spacer (IGS) was studied on a total of 68 isolates belonging to 26 of the SIGs (34 from Les Barres and 34 from Mersuay; 5-6 isolates per SIG; see Table 2). All the procedure, DNA extraction, amplification and electrophoresis was replicated 2 times.

Total DNA was extracted by the hexadecyltrimethylammonium (CTAB) / proteinase K procedure, essentially as described by Henrion, Chevalier & Martin (1994). Amplification of the 25S/5S spacer (IGS₁) was carried out using the primers LB25S1 (5'-GCTACGATCCGCTGAGGTAA-3') and 5SA (5'-CAGAGTCCTATGGCCGTGGAT-3') with priming sites at the 3' end of the 25S gene and the 5' end of the 5S gene, respectively (Henrion, Le Tacon & Martin, 1992). The sequence containing the 5S rDNA plus 5S/17S spacer (IGS₂) was amplified with primers rev5SA (5'-ATCCACGGCCATAGGACTCTG-3'), the reverse complement of primer 5SA, and revNS1 (5'-GAGACAAGCATATGACTAC-3'), the reverse complement of primer NS1 (White *et al.*, 1990), with priming sites at the 5' end of the 5S gene and the 5' end of the 17S gene respectively. Primers were supplied by Bioprobe Systems (Montreuil-sous-bois, France). PCR amplifications were carried out in reactions lots of 25 µl containing 0.3 - 0.6 ng DNA, 100 mM TrisHCl (pH 8.8 at 25°C), 1.5 mM MgCl₂, 50 mM KCl, 0.1 % Triton X-100, 200 µM dNTP mix, 10 pmol primers and 0.6 U of Taq Polymerase (Bioprobe Systems). For IGS₂ amplification, the dNTP concentration was increased to 400 µM. Reactions were performed in a Eurojantec ThermoJet cycler under the following regime: initial denaturation at 93°C for 3 min, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min.

For amplification of IGS₂, extension periods were increased to 5 min during cycles and 15 min for final extension. Negative controls (no DNA template) were included for each set of experiments to test for the presence of DNA contamination. The amplified products were subjected to electrophoresis on 0.8 % agarose gels, stained with ethidium bromide and visualized under UV light.

IGS₂ was cut with a restriction enzyme. About 1 - 2 µg of amplified DNA was digested overnight with 5 U of *Hae III* (Sigma chimie) according to the manufacturer's instructions. Restriction fragments were subjected to electrophoresis on NuSieve gels (1.5 % agarose 'Wide Range', Sigma chimie, 0.5 % agarose, Bioprobe systems).

Table 1. Somatic incompatibility groups (SIG) of *Collybia fusipes* present in the root system of twenty oak trees.

Plot Tree	species	Number of isolates	Number of SIGs	Number of isolates in the largest SIG ^a
Mersuay	<i>Q. robur</i>			
M 14		11	1	11 (100 %)
M 45		8	1	8 (100 %)
M 60		11	2	10 (91 %)
M 65		10	5	3 (30 %)
M 66		12	2	11 (92 %)
M 67		10	5	6 (60 %)
M 69		9	2	5 (56 %)
M 70		8	6	2 (25 %)
Aillevans	<i>Q. robur</i>	17	6	6 (35 %)
Longevelle	<i>Q. robur</i>	10	1	10 (100 %)
Barres	<i>Q. rubra</i>			
B 0 ^b		60	1	60 (100 %)
B 39		10	1	10 (100 %)
B 41		10	2	8 (80 %)
B 55		12	3	10 (83 %)
B 91		11	2	10 (91 %)
B 92		10	2	6 (60 %)
B 99		10	1	10 (100 %)
B 100		7	3	5 (71 %)
B 102		11	3	9 (82 %)
B 103		10	4	7 (70 %)

^a Number in parentheses indicates the proportion of the total number of isolates sampled on the tree represented by the SIG

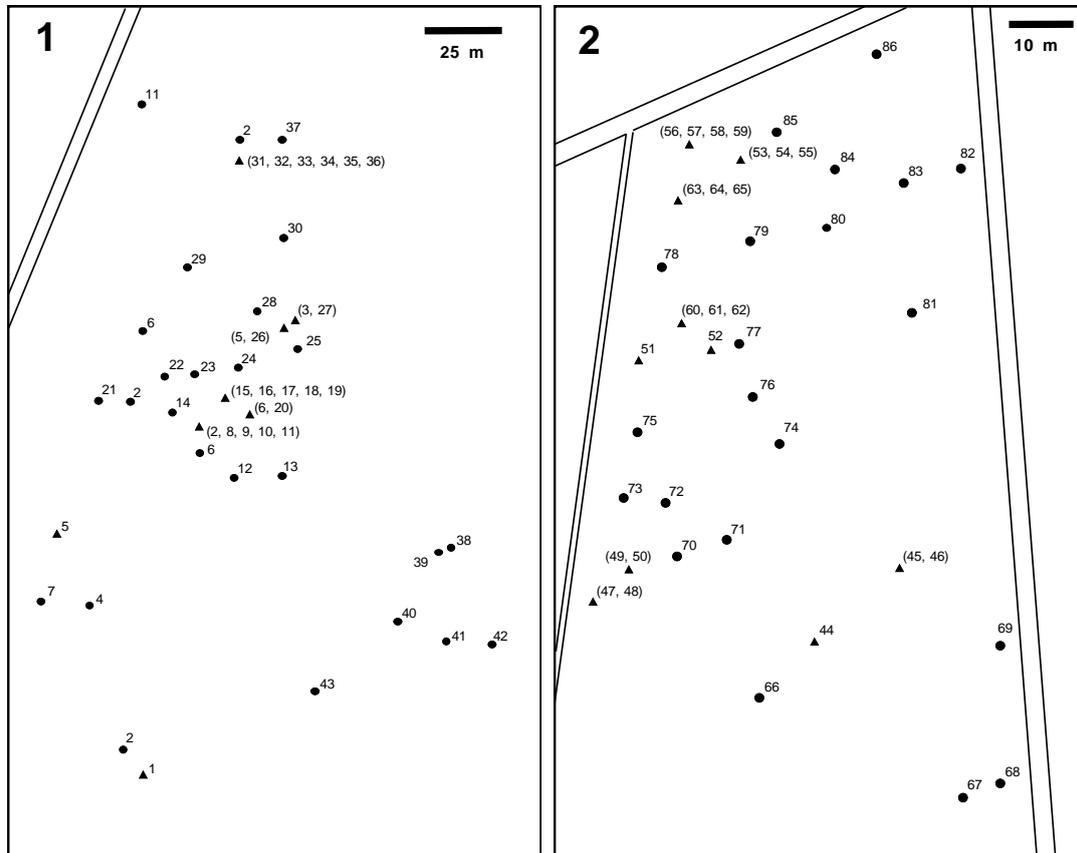
^b Tree completely uprooted

RESULTS

Somatic incompatibility tests

Isolates of *C. fusipes* were obtained from 34 different pedunculate oaks at Mersuay and from 31 different red oaks at Les Barres (Figs 1 & 2). Pairings between isolates from the same tree gave a compatible interaction (rating of 0) in 65 % of the cases. When the compatible interactions were repeated in a second experiment, all were rated 0 again. Among the incompatible interactions, 14 % for Mersuay and 18 % for Les Barres were rated as 1 (lightly pigmented interaction). The mean number of

SIGs per root system was of 3.1 ± 1.3 for the pedunculate oaks (Aillevans, Longeville and Mersuay) and of 2.2 ± 0.6 for the red oaks (Les Barres). However, for the majority of the root systems, most of the isolates belonged to one large SIG (Table 1). When several SIGs of comparable size were present on a root system, isolates which belonged to the same SIG were spatially close (Fig. 3).



Figures 1 - 2. Distribution of *Collybia fusipes* somatic incompatibility groups (SIG). Fig.1. Mersuay. Fig. 2. Les Barres. ●, trees for which one *C. fusipes* isolate is available; ▲, trees for which several *C. fusipes* isolates are available. Numbers indicate the SIGs present on the tree.

Unlike isolates that came from the same tree, those collected on different trees gave incompatible interactions in 98.6 % of the pairings for Mersuay and in all of them for Les Barres. Even trees separated by just few meters were usually infected with isolates belonging to different SIGs (Figs 1 & 2). In the Mersuay plot, the trees infected with isolates from the same SIG were scattered within the stand (SIGs 2, 5, 6 and 11, Fig. 1). About 12 % of the incompatible pairings between isolates from two different trees were rated as 1 in both plots. When repeated in a second experiment, these pairings were all rated 1 again. These weaker interactions showed transitivity (when pairing between A and B and that between B and C were rated as 1, pairing between A and C was always rated as 1). Therefore, isolates showing a weaker incompatibility together could be grouped. Eleven of those groups were

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present in Mersuay and nine in Les Barres. Isolates from those groups were scattered within the stands.

All together, including the single isolates from each tree and the multiple isolates from individual root systems, 43 SIGs were identified in Mersuay and 43 in Les Barres.

Table 2. IGS type of the isolates studied by PCR analysis. Isolates are grouped according to the SIG.

SIG	Isolate	IGS ₁ type	IGS ₂ type	SIG	Isolate	IGS ₁ type	IGS ₂ type
1	M _{45.1} ^a	1	1	44	B _{39.1}	1	12
1	M _{45.2}	1	1	44	B _{39.3}	1	12
1	M _{45.3}	1	1	44	B _{39.5}	1	12
1	M _{45.4}	1	1	44	B _{39.7}	1	12
1	M _{45.8}	1	1	44	B _{39.9}	1	12
2	M ₁₆	1	2	47	B _{91.2}	1	13
2	M ₄₄	1	3	47	B _{91.4}	1	13
2	M _{65.1}	1	4	47	B _{91.6}	1	13
2	M _{65.2}	1	4	47	B _{91.8}	1	13
2	M _{65.10}	1	4	47	B _{91.11}	1	13
3	M _{60.1}	1	5	51	B _{99.1}	1	14
3	M _{60.2}	1	5	51	B _{99.4}	1	14
3	M _{60.5}	1	5	51	B _{99.6}	1	14
3	M _{60.7}	1	5	51	B _{99.8}	1	14
3	M _{60.9}	1	5	51	B _{99.10}	1	14
5	M _{14.1}	1	6	53	B _{55.3}	1	15
5	M _{69.5}	2	4	53	B _{55.5}	1	15
5	M _{69.6}	2	4	53	B _{55.7}	1	15
5	M _{69.7}	2	4	53	B _{55.9}	1	15
5	M _{69.8}	2	4	53	B _{55.11}	1	15
5	M _{69.9}	2	4				
				56	B _{103.4}	1	6
6	M ₆₂	2	ND	56	B _{103.5}	1	6
6	M _{66.3}	3	3	56	B _{103.7}	1	6
6	M _{66.4}	3	3	56	B _{103.9}	1	6
6	M _{66.6}	3	3	56	B _{103.10}	1	6
6	M _{66.8}	3	3				
6	M _{66.10}	3	3	60	B _{100.3}	1	15
				63	B _{102.2}	2	4
7	M ₁₁	2	7	64	B _{102.10}	2	4
9	M _{65.9}	2	4	66	B ₂₃	1	6
15	M _{67.2}	1	8	69	B ₁₈	2	4
16	M _{67.4}	1	9	70	B ₉₃	1	16
22	M ₁₈	2	10	72	B ₉₅	2	15
27	M _{60.4}	2	11	81	B ₈	2	4
28	M ₂₅	2	3	86	B ₂	1	3

^a M_{45.1} is isolate number 1 from the tree M₄₅.

Amplification of the intergenic spacer

The amplification priming sites were selected from highly conserved regions found in the 17S, 5S and 25S regions adjacent to the IGS spacers (White *et al.*, 1990; Henrion *et al.*, 1992). Amplification of the 5S/17S spacer (IGS₂) of *C. fusipes* produced one to three fragments ranging from 2100 to 2700 base pairs (Fig. 4), whereas amplification of the 25S/5S spacer (IGS₁) generated three or four fragments of 1070, 800, 737 and 480 base pairs. Three different banding patterns were evidenced for IGS₁ and sixteen different IGS₂ types were detected (Table 2). Variability in the IGS₂ was mainly linked to different numbers and size of amplified DNA fragments (Fig. 4). However, additional variability was detected among the 64 isolates by cutting the IGS₂ with *Hae* III (Fig. 5). Isolates which belonged to the same SIG generally had the same IGS₁ and IGS₂ type. SIGs 2, 5 and 6 were the only groups in which all isolates did not have both the same IGS₁ and IGS₂. These were also the only SIGs in which isolates from different trees were present (Table 2).

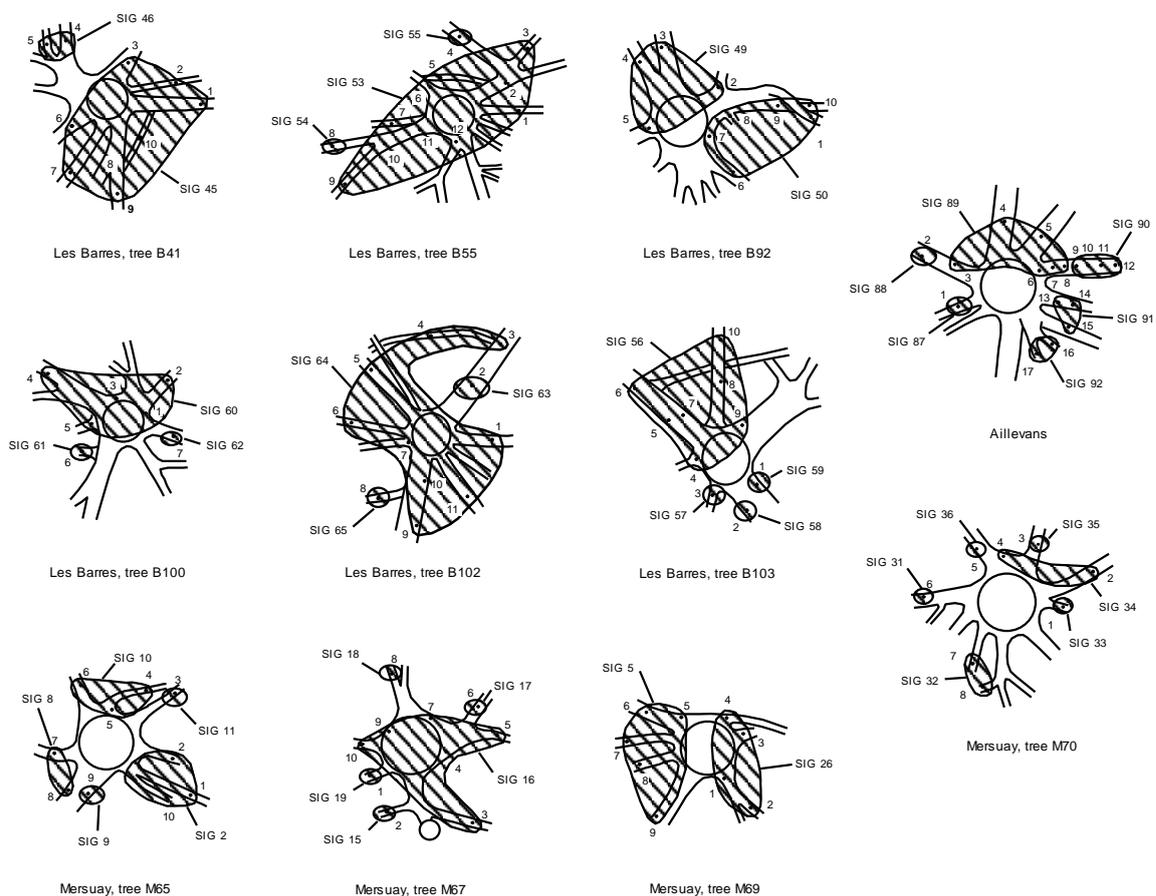
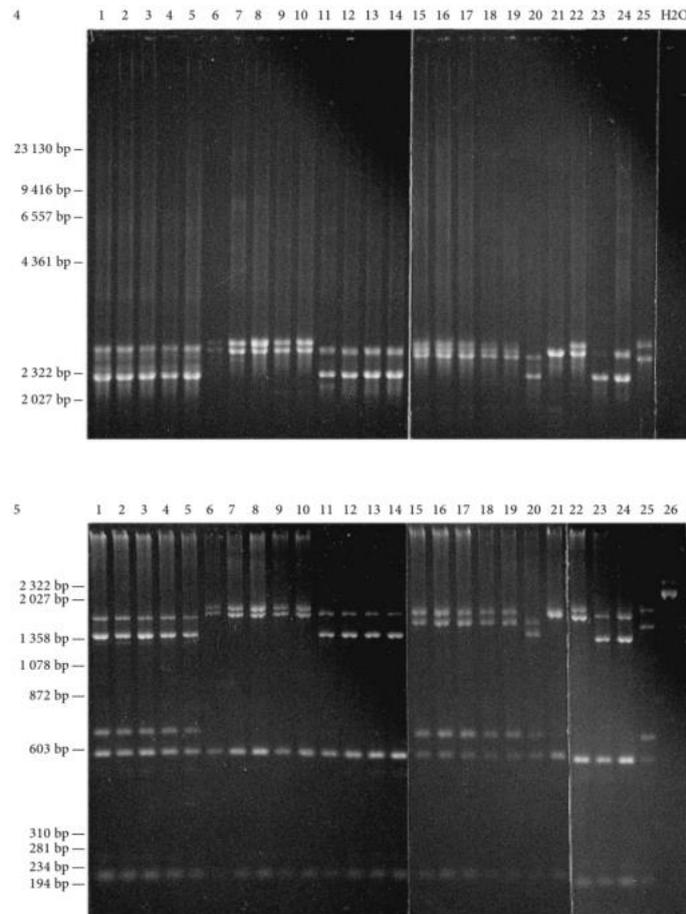


Figure 3. Distribution of *Collybia fusipes* somatic incompatibility groups (SIG) on the root system of *Q. robur* and *Q. rubra*. Just the trees in which the largest SIG represent less than 90 % of the total number of isolate are shown.



Figures 4 - 5. Ribosomal DNA intergenic spacer IGS₂ in *Collybia fusipes* isolates from Mersuay (Haute Saône, France). Fig. 4. DNA fragments amplified with the primer pair NS1' / 5SA'. Fig.5. DNA fragments amplified with the primer pair NS1' / 5SA' and cut by *Hae III*. Lines 1: M_{45.1}; 2: M_{45.2}; 3: M_{45.3}; 4: M_{45.4}; 5: M_{45.8}; 6: M_{60.1}; 7: M_{60.2}; 8: M_{60.5}; 9: M_{60.7}; 10: M_{60.9}; 11: M_{66.3}; 12: M_{66.4}; 13: M_{66.6}; 14: M_{66.8}; 15: M_{69.5}; 16: M_{69.6}; 17: M_{69.7}; 18: M_{69.8}; 19: M_{69.9}; 20: M₁₁; 21: M₁₄; 22: M₁₈; 23: M₂₅; 24: M₄₄; 25: M_{60.4}; 26: M_{66.3}, uncut by *Hae III*.

DISCUSSION

Somatic incompatibility interactions among isolates of *C. fusipes* collected from two oak stands indicate that the population structure of this fungus is quite heterogeneous and composed of many genets within an infected stand.

The variability among isolates was very high for the two markers we studied, SIGs and IGS. As many as 43 different SIGs were detected in both stands on 31 and 34 trees, respectively. Among the 34 studied isolates from each stand, 9 and 14 different IGS (IGS₁ + IGS₂) were detected, respectively. The IGS₂ was the most diverse. Even without restriction by *Hae III*, at least six different IGS₂ types can be separated in Mersuay (Fig. 4) while just three IGS₁ types were present in the two stands (Table 2). The differences between the various IGS₂ were mainly differences in the size and number of the amplified DNA fragments (Fig 4), which has already been observed (Bruns, White & Taylor, 1991). The occurrence of these multiple bands may arise from heterogeneous IGS in the studied *C. fusipes* heterokaryons

(i.e., several loci, divergent sequences in the two nuclei or within the same rDNA repeat). The presence of two bands for the IGS₂ in several isolates was likely to have arisen from a difference in the sequence between the two nuclei because monokaryons obtained from some of those isolates had just one of the two bands (results not shown). Slower moving fragments of PCR products may also correspond to concatemers or heteroduplexes formed by cross-hybridization between slightly different amplification products (Jensen & Straus, 1993; Selosse *et al.*, 1996). Sequencing of the fragment obtained using the primer pair revNS1 and rev5SA permitted us to check that we indeed amplified the IGS₂ (result not shown).

A compatible interaction between two heterokaryons of the same species has often been taken as an indication that they belong to a same genet (Childs, 1963; Barrett & Uscuplic, 1971; Kile, 1983; Kile, 1986; Guillaumin & Berthelay, 1990; Holmer, Nitare & Stenlid, 1994). It just indicates that the two isolates have common alleles at a small number of loci controlling the somatic incompatibility (Leslie, 1993). However, that SIGs are indeed genets has been recently verified by DNA analysis for *Armillaria* sp. (Smith *et al.*, 1992; Smith, Bruhn & Anderson, 1994) and for *P. weirii* (Bae, Hansen & Strauss, 1994). This work showed that *C. fusipes* isolates belonging to the same SIG from the same tree always had identical IGS. In comparison, two isolates that belonged to different SIGs had both the same IGS₁ and the same IGS₂ in just 52 out of the 2153 cases, i.e. 2.4 % (Computed from Table 2). Moreover, isolates belonging to the same SIG and from the same tree were clustered on the root systems. Thus, the SIGs present on one tree could well be genets. Conversely, SIGs on a root system could represent groups of closely related isolates. However, synthetic heterokaryons of *C. fusipes* obtained by pairing sibling haploid strains together were shown to be compatible in only 10 % of the cases (Marçais *et al.*, in preparation). Moreover, the lightly pigmented interaction, rated as 1 in this work, represented about 65 % of incompatible interactions between those sib-composed heterokaryons. These weaker interactions accounted for only 14 % of the incompatible interactions between isolates from the same tree in Mersuay and for only 18 % in Les Barres, a rate very close to the 12 % found in both plots for incompatible interactions between isolates from different trees. Thus, our results do not support the second hypothesis and the SIGs are more probably genets. In contrast, in the few cases where *C. fusipes* isolates of the same SIG were present on different trees, they always had a different IGS₁ or a different IGS₂ and are thus likely to be different genets having common alleles at the loci controlling somatic incompatibility.

Isolates from neighbor infected trees were always found to belong to different genets. Thus, we found no evidence that *C. fusipes* is able to spread from tree to tree through root contacts. Infection and colonisation by this fungus occur mainly on the collar root of the tree (Marçais & Delatour, unpublished results). This might partly explain why it spreads poorly by root contact to neighbor trees. The high number of genets occupying small areas within the two plots indicates that *C. fusipes* spreads in infected stands mainly by basidiospores. This is in agreement with the scattered distribution of *C. fusipes* in oak forest (Marçais & Delatour, unpublished results). The tree might become infected by basidiospores germinating at the surface of the bark or alternatively, by mycelium growing free in the soil, as described for *Phaeolus shweinitzii* (Barrett & Greig, 1984).

We want to thank S. Crocco and J. E. Ménard for their technical assistance and P. Wargo for reviewing the manuscript. We also want to thank D. Piou and the C.E.M.A.G.R.E.F. for their help at the Les Barres stands and the D.S.F. for their help at the Mersuay stand.

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