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High genetic diversity in a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* in a 150-year-old beech forest

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Abstract

The genetic structure of a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* (Bolt. ex Hooker) Murr. was assessed in a closed 150-year-old beech (*Fagus sylvatica* L.) forest in the Vosges Mountains in northeastern France. During the autumn of 1994 and 1997, sporophores were collected from three 100-m² sampling plots located along a 120-m transect crossing the beech stand. The genetic variation of 676 sporophores was initially estimated using heteroduplex analysis of the ribosomal DNA intergenic spacer (IGS1). Ten unique IGS1 heteroduplex/homoduplex patterns were identified, although three types represented most of the sporophores analysed. Each group of IGS1 type was then analysed using random amplified microsatellite analysis (RAMS). RAMS resolved 388 different genotypes amongst the 634 sporophores analysed from the three plots during the autumn of 1994 and 1997. Density as high as 130 genets per 100 m² was observed during the autumn of 1994. The largest clone covered ≈ 1 m², but most genets covered a few cm² and produced only one to three sporophores. Only eight genotypes identified in 1994 were found in 1997. Although *L. amethystina* has the capacity for vegetative persistence, the present study indicates that its populations maintain a genetic structure more consistent with a high frequency of sexual reproduction. This suggests that beech trees could be recolonized by new genotypes each year. Alternatively, this spatial distribution may also arise from erratic fruiting of underground persistent genets. These features (i.e. numerous genets of small size), typical of ruderal species, contrast with studies carried out on other ectomycorrhizal basidiomycetes occurring in mature closed forests.

Keywords: ectomycorrhiza, genets, *Laccaria amethystina*, microsatellites, population structure, rDNA

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Introduction

Ectomycorrhizal symbiosis, a mutualistic plant–fungus association, plays a fundamental role in the biology and ecology of forest trees, affecting growth, water and nutrient absorption, and providing protection from root diseases (Smith & Read 1997). Tree roots are exposed to several hundred different species of ectomycorrhizal fungi (Dahlberg *et al*. 1997). Each species exists as a population of many genetic individuals, so-called genets, between which there is almost invariably some phenotypic variation (Debaud *et al*. 1999). The fact that trees are exposed to genetically diverse mycobionts is an important consideration in forest ecology. Genets vary in their ability to colonize different genotypes of host plant, their ability to promote plant growth and adaptation to abiotic factors, such as organic/inorganic nitrogen concentration and soil pH. Each of these factors might affect the course of a beneficial symbiosis and the dissemination of a fungal genet in a forest ecosystem. Investigation of the temporal and spatial distributions of populations of ectomycorrhizal fungi, together with study of the origin and maintenance of their genetic variation, are therefore critical for understanding how populations of ectomycorrhizal fungi evolve

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and disappear at different stages of development of forest ecosystems (Dahlberg & Stenlid 1995; Egger 1995). By implication, such an understanding can help in the formulation of programmes where economically important tree species (e.g. eucalyptus, oaks, firs, pines, spruces) are inoculated using selected beneficial ectomycorrhizal fungi (Grove & Le Tacon 1993; Dell & Malajczuk 1997; Le Tacon *et al*. 1997; Selosse *et al*. 1998, 1999).

Ectomycorrhizal basidiomycetes constitute an important component of forest fungal communities (Villeneuve *et al*. 1991; Gardes & Bruns 1996; Dahlberg *et al*. 1997), but little is known about their population structure, genetic variability and reproductive strategies. These fungi rely upon basidiospores, mycelial fragmentation, mitotic sporulation and, occasionally, sclerotia as their major means of dispersal and reproduction (Dahlberg & Stenlid 1995). Each genet arises in a unique mating event and then vegetatively expands as a host root-connected mycelium throughout the humus layer of forest soils. Little is known about the longevity of this symbiotic web, but by mapping fruiting body genotypes onto study sites, it appears that local populations of *Suillus* spp. and *Laccaria* spp. in forest soils comprise a number of spatially discrete genets of varying size (Dahlberg & Stenlid 1990, 1994; Doudrick *et al*. 1995; Jacobson *et al*. 1993; Baar *et al*. 1994; de la Bastide *et al*. 1994; Dahlberg 1997; Gryta *et al*. 1997; Bonello *et al*. 1998; Selosse *et al*. 1998, 1999). Somatic incompatibility groupings in *S. bovinus* (Dahlberg & Stenlid 1990, 1994), and molecular analysis of *S. pungens* populations (Bonello *et al*. 1998), revealed that genets of ectomycorrhizal basidiomycetes may be both large (> 300 m²) and old (> 150 years old). Genets of *L. bicolor* and *Pisolithus tinctorius* are also able to spread vegetatively through soil in excess of 30 m (Baar *et al*. 1994; Anderson *et al*. 1998). In contrast, the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, found in nutrient-poor and unstable sandy soils of coastal sand dunes, produces a high density of short lifespan (< 1 year) genotypes with a limited spatial extension (Gryta *et al*. 1997). Dahlberg & Stenlid (1990) have argued that the occurrence of numerous small genetic individuals may suggest a recent colonization by basidiospores, whereas fewer, larger genets are indicative of old mycelial structures that have grown from a point source over decades; such old individuals are often found in mature closed forest ecosystems (Dahlberg & Stenlid 1995). However, studies of population structure have examined a limited number of ectomycorrhizal species, mostly limited to conifer ecosystems. It is therefore difficult to draw conclusions on the changes occurring in the populations of ectomycorrhizal fungi during the evolution of hardwood forest ecosystems, such as beech and oak forests.

To explore further the population genetics of ectomycorrhizal basidiomycetes, the present study describes the spatial genetic structure of the ectomycorrhizal basidiomycete *L. amethystina* (Bolt. ex Hooker) Murr. in a closed 150-year-old beech (*Fagus sylvatica* L.) forest in the Vosges Mountains (France). *L. amethystina* is a common species in boreal and temperate forests and its genetic variability has been described (Gardes *et al*. 1990, 1991). In the present study, the genetic diversity and spatial distribution of this species was evaluated using heteroduplex analysis of the nuclear ribosomal DNA intergenic spacer (IGS1) and random amplified microsatellites (RAMS).

Materials and methods

Study site

The study was conducted in a monospecific beech (*Fagus sylvatica* L.) stand at Aubure in the northeast of France (Haut-Rhin) (48°12′ N, 7°11′ E) (Fig. 1). The forest is located in the Strengbach catchment at an altitude of 1050–1080 m above sea level in a site exposed to the southeast with an inclination of ≈ 5°. The mean annual temperature is ≈ 6 °C. The average annual precipitation is 1300 mm and is evenly distributed throughout the year. Within the stand, the soil is sandy and rich in gravel. The dominant soil type is a podzosol covered by a thin O-layer (3–5 cm) and the humus was described as a moder type (C : N 20.6; pH 3.7). Atmospheric input, mineral fluxes in the forest stand and surface water chemistry have been monitored in the catchment since 1985 (Probst *et al*. 1990). The beech stand is 150 years old, with closed canopies and sparse understorey vegetation (*Luzulo-Fagetum* with *Polystichum spinulosum* DC. and *Athyrium filix-femina* (L.) Roth.). It originates from natural regeneration and, at the time of the study, the tree density was 429 trees per hectare (ha) with a mean tree height of 22.4 m.

Sampling plots

Sporophores of *Laccaria amethystina* were collected from three different 100-m² sampling plots that were located along a 120-m transect crossing the beech stand and situated between two roadways. The upper plot (III) is located 50 m downward from the upper road, whereas plots I and II are situated 15 m and 30 m upward of the lower roadway, respectively. Each sampling site corresponds to a square of 10 m × 10 m. Beech was the only tree species occurring within 20 m around the plots.

Survey of sporophores

In the area studied, the fruiting period of *L. amethystina* takes place from early August until the end of October, depending on the climatic conditions. In 1994, 1148 sporophores were collected in early September (9/5/94; sampling 1) and at the end of September (9/26/94;

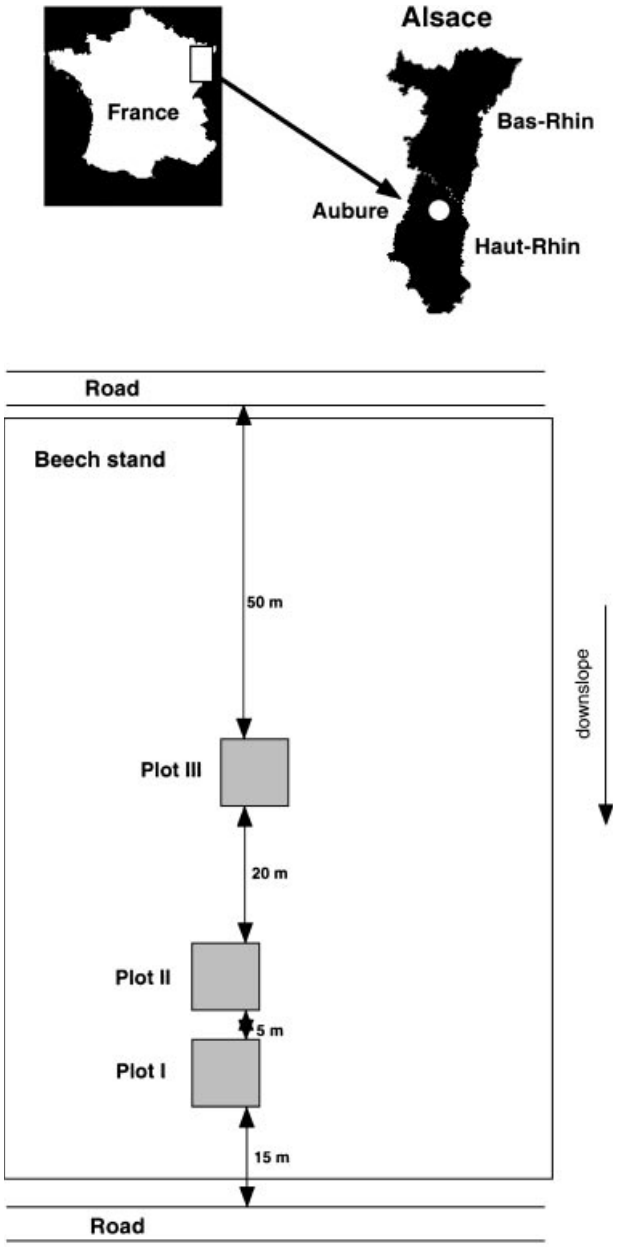

Fig. 1 Maps of the Alsace region and of the study area in the 150-year-old beech forest in the Strengbach catchment (Aubure, Haut-Rhin, France). Each dark grey squares identifies the 10 m × 10 m plots investigated, which were located along a 120-m transect crossing the beech stand.

sampling 2), at the height of the fruiting period, so it can be assumed that only a low proportion of the sporophores was missed. The 1995–97 seasons were noteworthy in that the summers were dry and accompanied by poor fruitings of this species during autumn. In 1995 and 1996, only 12 sporophores were observed in the plots studied. In 1997, only 87 sporophores were found and were collected mid-October (10/20/97; sampling 3) from the three sampling plots. On each occasion, all sporophores were sampled and their coordinates (x, y) were recorded on a sampling grid (10 × 10 m), with a precision of 5 cm, to prepare distribution maps (Fig. 5). Mapped sporophores were numbered, bagged individually, transported to the laboratory within 6 h of sampling and stored at −20 °C pending analysis.

DNA extraction

Total DNA was extracted directly from sporophore tissues using the hexadecyltrimethylammonium bromide (CTAB)/proteinase K protocol, as described by Henrion *et al*. (1994a). Two independent DNA extractions were carried out on 589 sporophores collected in 1994 (321 for the early September and 268 for the end of September samplings) and on the 87 sporophores collected in 1997.

Polymerase chain reaction amplification of the rDNA IGS1

Polymerase chain reaction (PCR) amplifications of the 25S/5S rDNA IGS1 spacer were performed using the primers LB25S1 (5′-GCTACGATCCGCTGAGGTTAA-3′) and 5SA (5′-CAGAGTCCTATGGCCGTGGAT-3′), located, respectively, at the 3′ end of the 25S and the 5′ end of the 5S rDNA subunits. Reactions were carried out in a total volume of 50 µL, including 20 nM of each primer, 20 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton-X-100, 0.2 mM of each dNTP, 20 ng of template DNA and 1 U of *Taq* DNA polymerase (Appligene-Oncor). The thermocycling profile of the GeneAmp PCR System 9600 (Perkin Elmer) was: 3 min denaturation at 94 °C; 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min; followed by a final elongation at 72 °C for 10 min. Amplification products were routinely separated by electrophoresis using 2% (w/v) FMC-MetaPhor agarose/0.25% BRL-agarose gel in 1× Tris–borate–EDTA (TBE) buffer (Sambrook *et al*. 1989). To improve the separation of the homo- and heteroduplex DNA structures generated during the PCR of dikaryotic mycelium carrying different IGS1 alleles, amplification products were resolved in 8% acrylamide gels in 1× TBE (Selosse *et al*. 1996). A negative control reaction, in which DNA was omitted, was included with every run to verify the absence of contamination.

In vitro *formation of IGS1 heteroduplexes*

IGS1 types, which we define as unique fragment patterns, were named RB to RO. For each pattern, we used heteroduplex analysis on acrylamide gels to identify homo- and heterozygotes and to assign genotypes to the locus, as described in Selosse *et al*. (1996).

rDNA IGS1 sequencing

The RR- or RB-IGS1 types were cloned in pBluescript-SK+, and sequenced for both strands as described in Selosse *et al*. (1996). Sequences were deposited in the National Center for Biotechnology Information (NCBI) databases under the following GenBank accession nos: AF124340 and AF124341. Multiple sequence alignments were performed using the MULTALIN program on the WWW ProDom server (INRA).

RAMS

RAMS, or inter-repeat PCR, was carried out as described in Martin *et al*. (1998). Briefly $(GTG)_5$- and $(CCA)_5$-primed PCR amplifications were performed in a total volume of 30 µL, including 300 nM of primer, 20 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton-X-100, 0.3 mM of each dNTP, 1–20 ng of template DNA and 1 U of *Taq* DNA polymerase (Appligene-Oncor). Reactions were performed in a GeneAmp PCR System 9600 thermocycler programmed for 'touchdown' PCR. The thermal cycling started with an initial denaturation step at 94 °C for 3 min followed by eight three-step cycles: 94 °C for 30 s; 'touchdown' annealing temperature (65 °C to 58 °C; temperature was reduced by 1 °C at each subsequent cycle to 58 °C) for 30 s; and 72 °C for 1 min. This was followed by 25 additional cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. A final extension step at 72 °C for 10 min was performed after these 25 cycles. Amplification products were separated by electrophoresis using 2% (w/v) FMC-MetaPhor agarose/0.25% BRL-agarose gel electrophoresis in 1× TBE.

Data analysis

RAMS amplifications were performed twice and only the amplicons that reproduced consistently were scored for presence (1) or absence (0). Only data from unambiguously clear bands were used for genotype comparison and statistical analysis (i.e. 80% of the amplicons). Sporophores with identical banding patterns were regarded as belonging to the same genet and were mapped according to the distribution of collected sporophores. Similarity of RAMS profiles were calculated from binary matrix using Jaccard's similarity index:

$$S = \frac{a_{xy}}{a_{xy} + b_{xy} + c_{xy}}$$

where a_{xy} refers to the number of bands shared by both genets x and y, b_{xy} refers to the number of bands present in genet x and absent in genet y, and c_{xy} refers to the number of bands absent in genet x and present in genet y. A matrix was calculated for each plot. Principal component analysis and unweighted pairgroup method with arithmetic mean (UPGMA) was performed to investigate genetic relationships, on a reduced data matrix (identical patterns were introduced once) using the multivariate analysis ADE-4 program (Thioulouse *et al*. 1997) through the WWW online multivariate analysis system NetMul (http://pbil.univ-lyon1.fr/ADE-4/NetMul.html).

Analysis of the genetic structure of the population

Analysis of the population structure was performed using rDNA IGS1 polymorphism of the genets found in 1994 (sampling 1 and 2), as distinguished by RAMS analysis (i.e. each genet was attributed a ribosomal genotype). Allele distinction was based on: (i) the size of the amplified homoduplex on acrylamide gel; and (ii) its ability to form heteroduplex structures. The intrapopulation heterozygote deficit (F_{IS}) and the interpopulation heterozygote deficit (F_{ST}), among pairwise population samples (Weir & Cockerham 1984), were analysed using GENEPOP, version 3.1b (Raymond & Rousset 1995).

Results

IGS1 typing of sporophores of Laccaria amethystina

Owing to the large number of sporophores collected over the three plots, their genetic variability was initially assessed by investigating the variability of the rDNA IGS1, a region showing a high intraspecific variability within *Laccaria* spp. (Henrion *et al*. 1992; Selosse *et al*. 1998, 1999; Martin *et al*. 1999). In total, 10 IGS1 patterns were identified among the 676 sporophores analysed (Fig. 2). The different IGS1 types differed in the number and size of the amplification products. The total number of IGS1 bands ranged from one (e.g. RB and RR types) to three (e.g. RV type), observed following agarose-gel electrophoresis (Fig. 2A). These multiple PCR products resulted from heteroduplex formation between IGS1 alleles of divergent sequences (Selosse *et al*. 1996), as shown by conformation-sensitive polyacrylamide gel electrophoresis (Fig. 2B). For example, co-denaturation of a mixture of RB- and RR-IGS1 types produced the heteroduplex profile observed for the RV type (data not shown), suggesting that the RV type was heterozygous (genotype *a*/*b*) and contained an RB-like allele (*a*) and an RR-like allele (*b*). Sequencing of the RB- and RR-IGS1 types (GenBank accession nos: AF124340 and AF124341, respectively) revealed a low divergence (0.8%) for most of the sequence, but a 47-bp indel at position 318 (see http://mycor.nancy.inra.fr/Aubure/Aubure.html for the sequence alignment). This indel generated heteroduplex structures containing regions of single-stranded DNA (Selosse *et al*. 1996). *In vitro* assays established the allelic composition of the rDNA locus in each ribosomal type (Table 1). This analysis

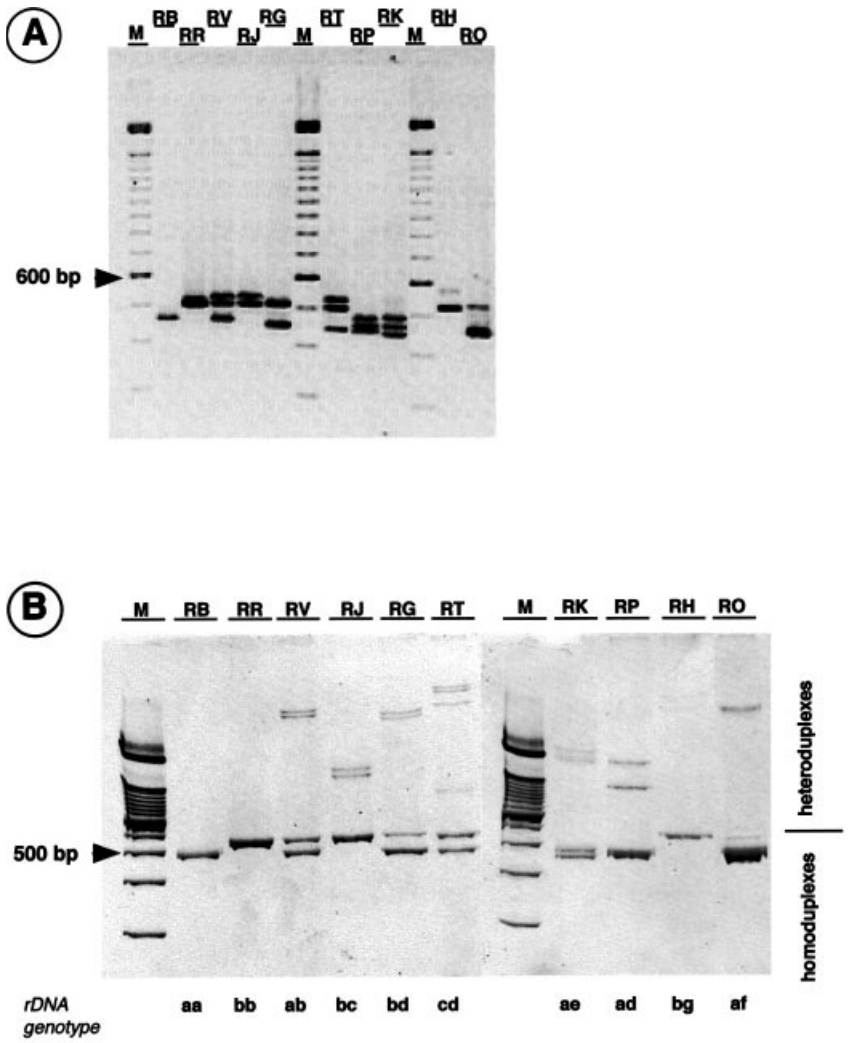

Fig. 2 Gels showing the 10 amplified intergenetic spacer (IGS1) patterns (RB to RO) from *Laccaria amethystina* sporophores found on the three plots studied in the 150-year-old beech forest at Aubure. Multiple bands resulted from heteroduplex formation during polymerase chain reaction (PCR) and revealed sequence differences in IGS1 alleles. Bands with retarded migration in (B) correspond to heteroduplexes. rDNA genotypes are indicated at the foot of (B). The PCR products were separated either on 2% FMC-MetaPhor agarose/0.25% BRL-agarose gel (A) or on acrylamide gels (B). M, Gibco-BRL 100-bp ladder.

Table 1 Total number of sporophores and relative contribution (%) of the different intergenetic spacer (IGS1) types of *Laccaria amethystina* identified in the Aubure beech stand

	Sampling 1 (1994)						Sampling 2 (1994)						Sampling 3 (1997)					
IGS1 type (genotype)	Plot I		Plot II		Plot III		Plot I		Plot II		Plot III		Plot I		Plot II		Plot III	
RV (*ab*)	58	***48.7%***	39	***39%***	37	***36.2%***	57	***43.1%***	28	*32.5%*	15	*30%*	9	*28.1%*	15	***55.6%***	12	***42.9%***
RB (*aa*)	25	*21.0%*	6	*6%*	11	*10.8%*	26	*19.7%*	14	*16.3%*	7	*14%*	10	***31.2%***	7	*25.9%*	6	*21.4%*
RR (*bb*)	16	*13.4%*	25	*25%*	28	*27.5%*	25	*18.9%*	33	***38.4%***	23	***46%***	9	*28.1%*	2	*7.4%*	9	*32.1%*
RJ (*bc*)	3	*2.5%*	5	*5%*	16	*15.7%*	7	*5.3%*	5	*5.8%*	3	*6%*	1	*3.1%*	3	*11.1%*	1	*3.6%*
RG (*bd*)	4	*3.4%*	8	*8%*	3	*2.9%*	0	*0*	0	*0*	1	*2%*	0	*0*	0	*0*	0	*0*
RT (*cd*)	4	*3.4%*	5	*5%*	3	*2.9%*	10	*7.5%*	2	*2.3%*	0	*0*	0	*0*	0	*0*	0	*0*
RO (*af*)	9	*7.6%*	0	*0*	0	*0*	2	*1.5%*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
RH (*bg*)	0	*0*	2	*2%*	0	*0*	0	*0*	1	*1.2%*	0	*0*	1	*3.1%*	0	*0*	0	*0*
RP (*ad*)	0	*0*	7	*7%*	2	*2.0%*	5	*3.8%*	2	*2.3%*	0	*0*	0	*0*	0	*0*	0	*0*
RK (*ae*)	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	1	*3.1%*	0	*0*	0	*0*
Unknown	0	*0*	3	*2%*	2	*2.0%*	0	*0*	1	*1.2%*	1	*2%*	1	*3.1%*	0	*0*	0	*0*
Total per Plot	119		100		102		132		86		50		32		27		28	
Total per sampling			**321**						**268**						**87**			

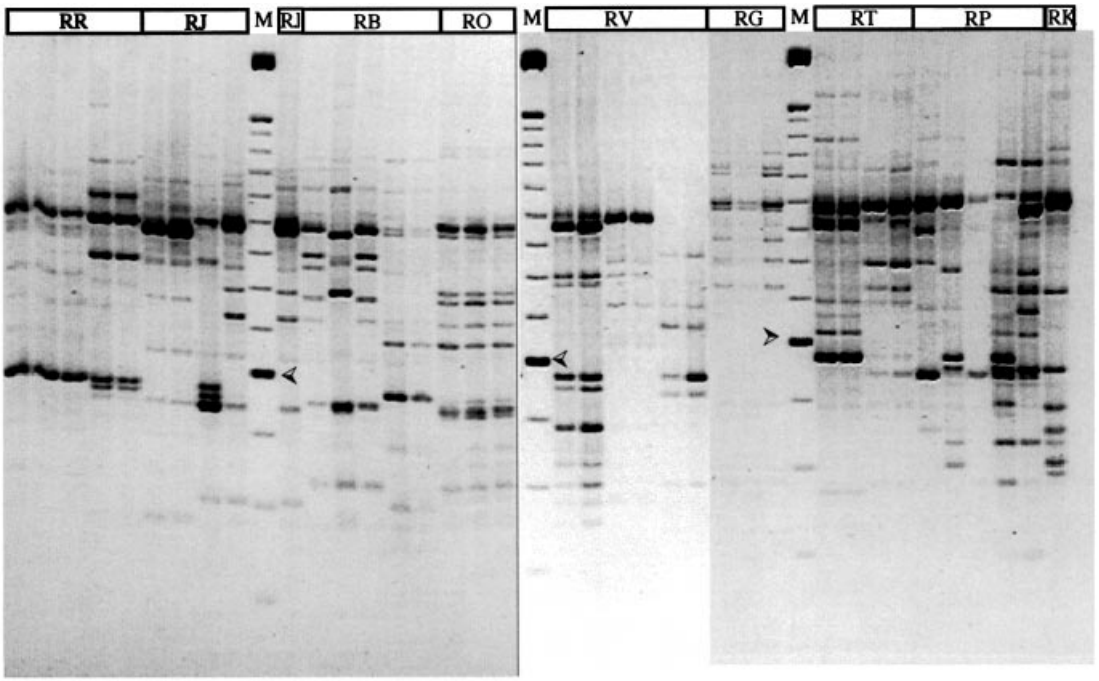

Fig. 3 DNA fingerprints of various *Laccaria amethystina* sporophores produced by random amplified microsatellite analysis (RAMS) using $(GTG)_5$ as a single primer. Lane M shows the DNA molecular size markers (100 bp-BRL ladder). Lanes 1–13 correspond to DNA fingerprints of sporophores grouped by intergenetic spacer (IGS1) type. Arrows on the gels indicate the 600-bp marker.

Table 2 Characteristics of the *Laccaria amethystina* population in the 150-year-old beech stand of Aubure

	Sampling 1 (1994)			Sampling 2 (1994)			Sampling 3 (1997)		
	Plot I	Plot II	Plot III	Plot I	Plot II	Plot III	Plot I	Plot II	Plot III
Number of sporophores sampled	127	104	109	242	438	147	32	27	28
Number of sporophores analysed:									
IGS1 analyses	119	100	102	132	86	50	32	27	28
RAMS analyses	117	94	99	120	67	48	33	27	29
Genets	63	57	39	71	53	35	28	18	24
Sporophores per genet	1.8	1.6	2.5	1.7	1.3	1.4	1.2	1.5	1.2
Average size of genets (m)	0.25	0.35	0.65	0.25	0.25	0.20	0.15	0.20	0.20
Estimated number of genets per ha		5200			5266			2266	

IGS1, intergenetic spacer; RAMS, random amplified microsatellite analysis.

identified seven alleles (*a* to *g*; Table 1 and Fig. 2B), among which alleles *a* and *b* were those most frequently found (Table 1).

Genotyping of L. amethystina *using RAMS*

Further molecular typing was carried out using RAMS to ascertain the genetic identity of *L. amethystina* sporophores collected. For each sporophore, $(GTG)_5$-primed PCR displayed five to 20 amplification products within the range 0.3–1.5 kb pairs (Fig. 3). The banding patterns obtained using the $(GTG)_5$ (Fig. 3) and $(CCA)_5$ (data not shown) microsatellites were highly polymorphic, but a few markers (e.g. bands at 570 and 980 bp) were shared by several sporophores. Up to 388 different banding patterns were identified among the 634 sporophores analysed from the three sampling plots in 1994 and 1997. The average number of sporophores per genet was 1.6 (ranging from 1.2 to 2.5) (Table 2). Genetic similarity between genets was assessed by principal component analysis (Fig. 4); no clustering of genotypes was revealed between and within plots.

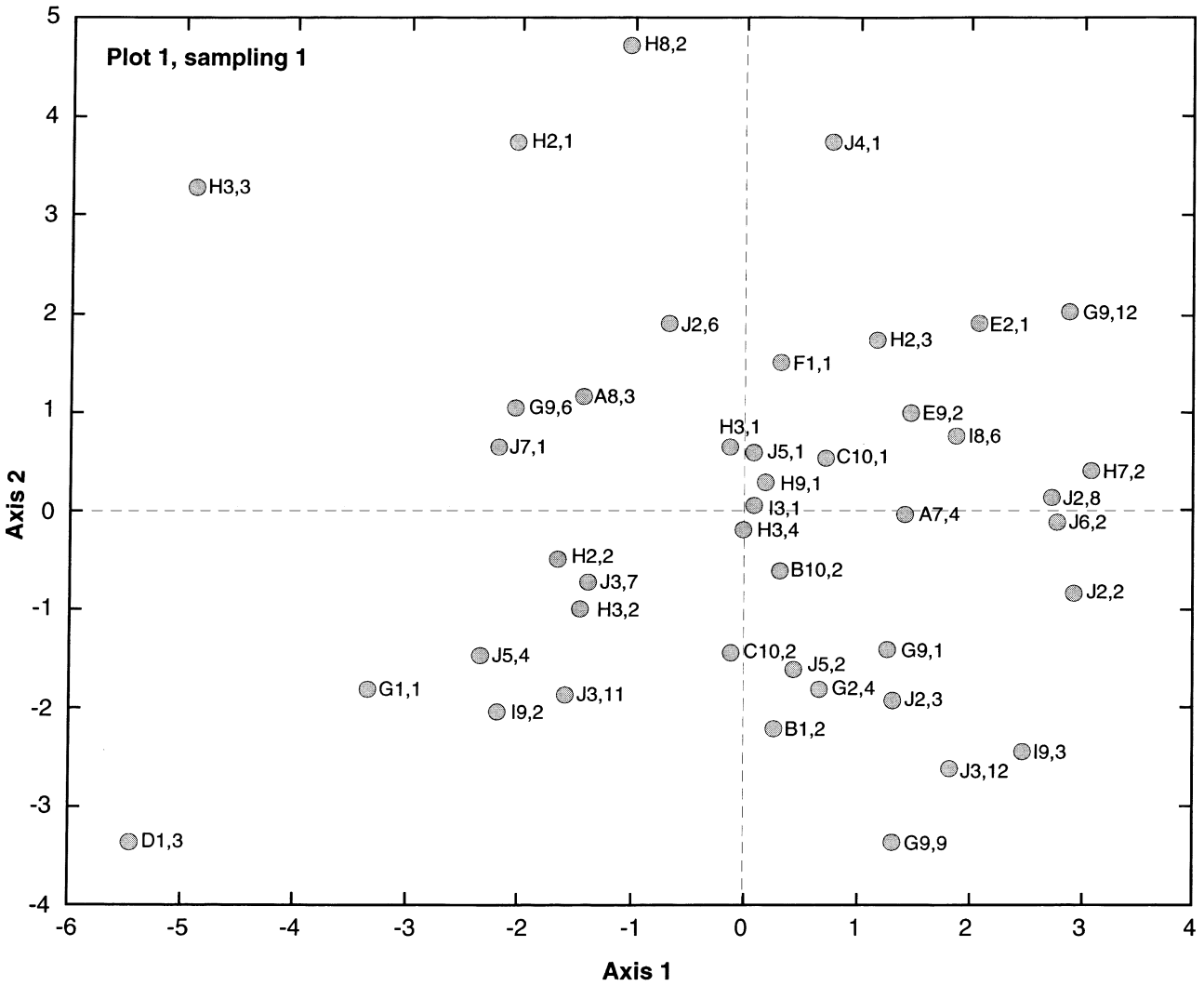

Fig. 4 Principal component analysis constructed from a matrix of similarity based on random amplified microsatellite analysis (RAMS) data of the *Laccaria amethystina* sporophores collected in plot I in early September 1994 (sampling 1). The combination of letters and numbers on the scatterplots encode the coordinates of the sporophores on Fig. 5. The inertia of the model, explained by the first two axes of the plots, is 14.2%. Scatters factor maps for other plots and UPGMA dendrograms for the three plots are available at: http://mycor.nancy.inra.fr/Aubure/Aubure.html.

Spatial distribution of L. amethystina *genets*

Mapping of the sporophores with identical RAMS patterns revealed a high number of small-sized genets (Fig. 5). Samples taken from a patch of sporophores were usually genetically identical (e.g. sporophores in coordinates C6, G9 and H2 in Fig. 5). However, two sporophores less than 2–5 cm apart from each other could belong to different genets as a result of intermingling genets (e.g. coordinates J2 and H2; Fig. 5). Of the 270 sporophores, typed by RAMS, on plot I in 1994 and 1997, 162 different genotypes were identified (Fig. 5; grey zones and circles), whereas 128 and 98 genets were found on plots II and III (data not shown), respectively, indicating a high density of genets—131 different genets were observed over an area of 100 m^2 during the autumn of 1994. The largest genet covered ≈ 1 m^2 and comprised 21 sporophores (J2, J3; Fig. 5), but most genets comprised a single sporophore. The average size of genets (maximal distance between outermost sporophores with the same genotype) ranged between 0.15 and 0.65 m (Table 2). As a result, the three plots showed no common genets. The number of genets per plot (between 18 and 71) was not affected by the placement of the sampling area along the 120 m transect crossing the stand.

The sporophore surveys revealed obvious differences among weeks and years in the composition of genets. Although three genets (≈ 5%) (e.g. coordinates B9 and

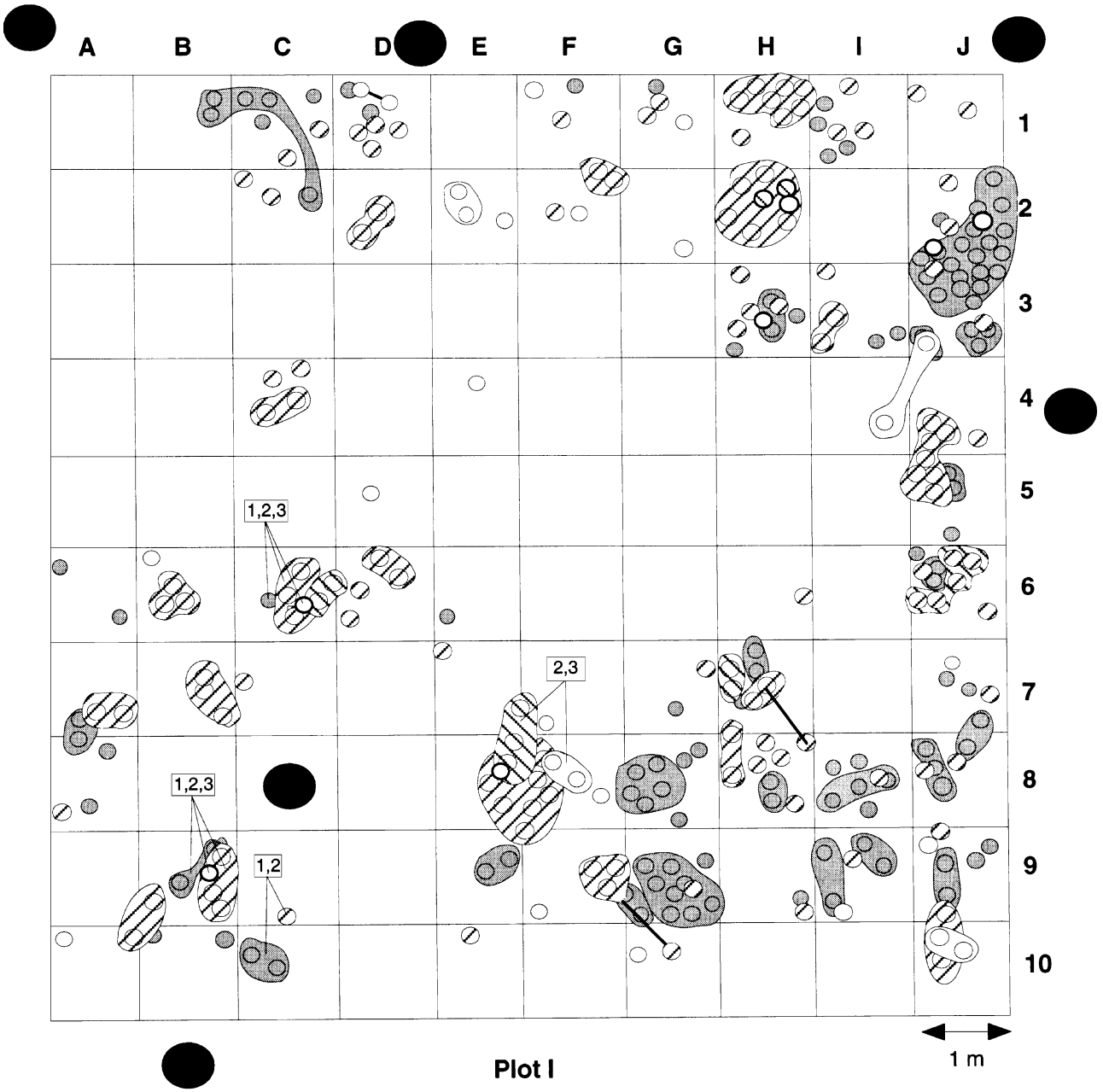

Fig. 5 Spatial distribution of the various *Laccaria amethystina* genets found on plot I in early September 1994 (sampling 1) (grey areas and circles), at the end of September 1994 (sampling 2) (hatched areas and circles) and at the end of October 1997 (sampling 3) (white areas and circles). Sporophores are represented by small unfilled circles, whereas beech trees are represented by large filled circles. Grey, hatched and white zones encompass sporophores belonging to the same genet, as determined by random amplified microsatellite analysis (RAMS). Boxes numbered 1, 2 or 3 indicate the few persisting genets found at different sampling dates. Fingerprints of genets linked by a line displayed a similarity higher than 95%.

C6, plot I; Fig. 5) identified in early September 1994 were still producing sporophores at the end of September 1994, most genets were not observed 2 weeks later (Fig. 5). Conversely, several genets (e.g. coordinates C4, D2, EF7–8, plot I; Fig. 5) were not fruiting in early September, whereas their sporophores were identified at the end of September in the same year.

Molecular typing of 89 sporophores collected in 1997 identified 70 genets on the three plots (Table 2). Although most of them were novel genets, six genotypes were sampled in both 1994 and 1997 at a similar ground location (e.g. B9, C6, plot I; Fig. 5). The lack of detection of some genets may be attributed to the low fruiting observed in 1997.

Table 3 *F*-statistics for *Laccaria amethystina* populations of plots I, II and III, based on the rDNA intergenic spacer (IGS1) locus. Only the 1994 sporophore sampling was used to determine these parameters

	Plot I	Plot II	Plot III
Plot I	$F_{IS} = -0.061$ ($P = 0.00001$)	$F_{ST} = 0.0081$ D = 15 m	$F_{ST} = 0.0212$ D = 45 m
Plot II		$F_{IS} = -0.040$ ($P = 0.0008$)	$F_{ST} = 0.0005$ D = 30 m
Plot III			$F_{IS} = +0.102$ ($P = 0.27$)

D, metric distance between the plot centres.

Analysis of the allelic diversity at the rDNA locus

As RAMS analysis allowed us to distinguish the genets forming the population sampled, we were able to calculate the allelic frequencies of the rDNA IGS1 locus and to study the genetic structure at this locus. The F_{IS} values were significantly negative for the populations of plots I and II (Table 3), suggesting an excess of heterozygous individuals at this locus compared with random mating; the positive F_{IS} value for the population of plot III was not significant because of a lower genet number. Calculation of F_{ST} demonstrated a low interpopulation differentiation (Table 3), with a substantial rate of migration between populations (11.5–500 migrants), but no correlation of this rate with interplot distance was found.

Discussion

To date, the population genetic structure of natural ectomycorrhizal fungi has been studied for six species of basidiomycetes: *Hebeloma cylindrosporum* (Gryta *et al*. 1997), *Laccaria bicolor* (Baar *et al*. 1994; de la Bastide *et al*. 1994; Selosse *et al*. 1998, 1999), *Suillus variegatus* (Dahlberg 1997), *S. bovinus* (Dahlberg & Stenlid 1990, 1994), *S. pungens* (Bonello *et al*. 1998) and *Pisolithus tinctorius* (Anderson *et al*. 1998). However, these surveys have mainly been carried out in conifer forests and little is known about the population structure of symbionts associated with hardwood species.

The typical size of genets varies greatly between fungal species, from a few mm in bark fungi (*Collybia fusipes*; Marçais *et al*. 1998) to more than 1 km in tree root pathogens (e.g. *Armillaria bulbosa*; Smith *et al*. 1992). The size of genets of ectomycorrhizal fungi is thought to vary with the forest age (Dahlberg & Stenlid 1995). Estimated population densities range from 30 to 5000 genets/ha, depending on the species and forest age (Dahlberg & Stenlid 1994, 1995). Populations of ectomycorrhizal species (e.g. *S. bovinus*, *S. variegatus*) in old forests mainly consist of discrete, large (up to 27 m) and old (> 150 years) genets, which do not seem to intermingle (Baar *et al*. 1994; Dahlberg & Stenlid 1994, 1995; Dahlberg 1997). Although spatial characteristics of populations of ectomycorrhizal fungi may differ between taxa, it appears that vegetative dissemination dominates over spore colonization in established forests, typified by canopy closure, lack of young trees and organic nitrogen-rich litter (Dahlberg & Stenlid 1995; Frankland 1998). In agreement with this contention, we expected a low clonal diversity within populations of *L. amethystina* in a closed, mature 150-year-old beech forest located in the Strengbach catchment. Unexpectedly, IGS1 and RAMS analyses indicated a high *L. amethystina* genet density in this stand. In September 1994, this fungus sporulated prolifically (up to 542 sporophores per 100 m^2) and exhibited a high number of genets (up to 134 genets per 100 m^2). The largest genet covered 1 m^2, but most genets were represented by a single sporophore (Table 2). Growth rate of the subterranean mycelium is 0.20–1.0 m/year for *Laccaria* spp. (de la Bastide *et al*. 1994; Selosse *et al*. 1999). Based on these estimates of annual mycelium growth and the observed spatial extent of genets, most *L. amethystina* genets would be less than 1 year old, assuming a continuous growth.

Like several other ectomycorrhizal fungi (Dahlberg & Stenlid 1995), sporophores of *L. amethystina* in the study site displayed a very disparate and patchy distribution. As previously supported (de la Bastide *et al*. 1995; Dahlberg *et al*. 1997), this distribution could be associated with mycorrhizal roots, and the occurrence of discontinuous clumped sporophores may indicate the proximity of active ectomycorrhizas. It may also suggest an irregular process of sporophore fructification within and amongst genets. Interestingly, we found that 95% of the genotypes identified in the first sampling of 1994 were not found less than 3 weeks after. This observation confirmed that the fructification phenotype of genets growing on a small area could be highly heterogeneous (Selosse *et al*. 1999). Nonreproductive structures (i.e. ectomycorrhizal short roots and connected extramatrical mycelium) located in the humus layer account for the predominant part of the ectomycorrhizal biomass and activity (Dahlberg *et al*. 1997). As a consequence, evidence based on the presence of sporophores above ground, without information on the all-important mycelia on the root and in soil horizons, could underestimate the number of genets and their spatial distribution. Further DNA typing of ectomycorrhizas (Henrion *et al*. 1994b; Gardes & Bruns 1996; Dahlberg *et al*. 1997) is therefore underway to obtain a better understanding of the role of root expansion in the structuration of populations of ectomycorrhizal fungi.

The high degree of genotypic spatial diversity can be explained either by: (i) frequent establishment of genets

followed by their extinction after 1 to 2 years of vegetative growth; or (ii) initial spore establishment with persistence, but not expansion, and erratic fruiting. If the former hypothesis is true, the *L. amethystina* population experienced a high extinction level owing to unknown factors (e.g. competition from other species, hard winters). This extinction would be compensated for by yearly recolonization of plots by basidiospores, as reported for the ruderal basidiomycete *H. cylindrosporum* (Gryta *et al*. 1997). However, survival of a few genets for up to 3 years (e.g. coordinates B9, Fig. 5) showed that the latter hypothesis cannot be ruled out. It should be noted that the ground location of these few lasting genets was similar in 1994 and 1997, confirming the low expansion of these genets. As shown by Baar *et al*. (1994), *L. bicolor*, a species closely related to *L. amethystina*, was not a common fruiter until litter was removed, but the size of the genets after litter removal suggested that they had been present, but not fruiting.

These data support an important role of basidiospores for establishment of populations in the Aubure beech forest. The number of migrants calculated and the excess of genets heterozygous for the rDNA in populations of plots I and II (Table 2) support a high contribution of allogamy to the current genetic structure. Any recruitment of novel genets is therefore likely to originate mainly from migrants already established in the beech stand investigated (siblings from a single or few mating events), rather than from spores from nearby forests, as most spores are deposited within a short distance (> 50 m) of the sporophores. Colonization of adjacent vacant root systems could then take place. Another source of genetic variation may be somatic mutations within clonal lineages (Gryta *et al*. 1997). However, microsatellite markers appeared to be stable through mitosis in filamentous fungi (DeScenzo & Harrington 1994) and hence somatic mutations are probably a restricted source of variation in these species. If mutations were common in the studied genets, one would expect that closely related genotypes would be spatially clustered, and they are not.

The observed population structure (prolific sporophore production, small genets) is a preferred reproductive strategy of early stage, ruderal ectomycorrhizal species occurring under young trees (de la Bastide *et al*. 1994; Selosse *et al*. 1998, 1999) or on disturbed sites, where novel genet establishment relies on small-scale disturbances (windthrown trees, animal tracks, soil movements) (Gryta *et al*. 1997). None of these disturbances have been recorded in the old Aubure beech forest. A continuous tree layer has been present for the last 150 years and the wood production yield is within the range of recorded values in northeastern France (E. Dambrine, personal communication) ruling out any obvious stresses.

In conclusion, reasons for the occurrence of numerous genets of small size in this long-established beech stand remain unknown. It suggests that the prominent role of sexual reproduction over the survival of the vegetative mycelium in the soil is not restricted to pioneer situations, but also applies to some ectomycorrhizal fungi, such as *L. amethystina*, found in stable established forest ecosystems.

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