Populations of ectomycorrhizal *Laccaria amethystina* and *Xerocomus* spp. show contrasting colonization patterns in a mixed forest

Anne-Marie Fiore-Donno[1,2] and Francis Martin[1]

[1]UMR 1136 INRA–UHP Interactions Arbres/Micro-Organismes, Centre INRA de Nancy, 54280 Champenoux, France; [2]Université de Lausanne, Institut d'Ecologie, CH-1015 Lausanne, Switzerland

Author for correspondence:
Francis Martin
Tel: +33 3 83 39 40 80
Fax: +33 3 83 39 40 69
E-mail: fmartin@nancy.inra.fr

Received: *9 May 2001*
Accepted: *13 August 2001*

Summary

- The knowledge of temporal and spatial structure of populations of ectomycorrhizal fungi, together with the origin and maintenance of their genetic variation, is critical to understanding how populations of these fungi establish, evolve and disappear at different stages of development of forest ecosystems.
- Identification and spatial delimitation of genets in populations of the ectomycorrhizal basidiomycetes, *Laccaria amethystina*, *Xerocomus chrysenteron* and *X. pruinatus* were inferred from the polymorphism of two codominant genetic loci, the nuclear rDNA internal transcribed spacers (ITS) and intergenic spacers (IGS), and anonymous dominant RAPD markers from basidiocarps collected in a mixed mature forest in the fungal reserve of La Chanéaz (Switzerland).
- The *L. amethystina* population showed numerous small, short lifespan genets; most closely spaced basidiocarps were genetically unique. Our results confirmed that sexual spore propagation is important in the life history of *L. amethystina* in undisturbed mature forests. By contrast, we found a single genet for each of the boletoid species colonizing a nearby plot indicating that clonal growth dominated.
- In La Chanéaz forest, the intrinsic biological features of the investigated species appear to play a higher role in colonization strategy than the features of local habitat.

Key words: ectomycorrhiza, genets, *Laccaria amethystina*, *Xerocomus chrysenteron*, *Xerocomus pruinatus*, population structure, RAPD, rDNA.

© *New Phytologist* (2001) **152**: 533–542

Introduction

Within forest soils, that host a large and diverse community of microbes that compete and interact with each other and with plant roots, ectomycorrhizal fungi are almost ubiquitous (Taylor *et al.*, 2000). The mutualistic ectomycorrhizal symbiosis allows trees to grow efficiently in suboptimal environments in boreal, temperate and some subtropical forest ecosystems (Smith & Read, 1997).

In a forest ecosystem, roots are exposed to an assemblage of several different species of ectomycorrhizal fungi (Taylor *et al.*, 2000; Dahlberg, 2001). Each species exists as a population of many individuals between which there is almost invariably some genetic variation (Debaud *et al.*, 1995). The fact that the host plants are exposed to mycobiont populations which are genetically diverse is a crucial consideration in forest ecology. Individual symbionts vary in their ability to colonize different genotypes of host-plant, their capacity to utilize mineral and organic soil nutrients, and in their adaptation to abiotic factors, such as soil pH and drought. Each of these abiotic and biotic factors might affect the course of a beneficial symbiosis and the establishment and subsequent dissemination of an individual fungal symbiont. Questions about the temporal and spatial structures of communities and populations of ectomycorrhizal fungi, together with the origin and maintenance of their genetic variation, are therefore critical when trying to understand how populations of these symbiotic fungi evolve and disappear at different stages of development of forest ecosystems (Egger, 1994; Dahlberg & Stenlid, 1995; Dahlberg, 2001). By implication, such a knowledge can

help in the understanding of biogeochimical cycles in forest ecosystems (Taylor *et al.*, 2000).

When and how genetic individuals of a given species (also referred to as genets) arrive at a site and how interactions between them evolve with maturation of fungal and host-plant populations are key questions in the assessment of dynamics in populations of ectomycorrhizal fungi. To understand the dispersal, establishment, and evolution of ectomycorrhizal populations, it is necessary to analyse these populations over several seasons and compare them at different successional stages of forest ecosystems at different habitats. The source and maintenance of genetic diversity within these ectomycorrhizal populations have been questioned in previous studies (Dahlberg & Stenlid, 1990, 1994; De La Bastide *et al.*, 1994; Gryta *et al.*, 1997, 2000; Bonello *et al.*, 1998; Selosse *et al.*, 1998, 1999; Gherbi *et al.*, 1999; Sawyer *et al.*, 1999; Zhou *et al.*, 1999, 2001; Guidot *et al.*, 2001; Redecker *et al.*, 2001). From these investigations, conclusions about the relative role of vegetative spreads vs spore establishment of ectomycorrhizal basidiomycete can be drawn (Egger, 1994; Dahlberg, 2001).

In open forest communities, genet recruitment from spore germination occurs massively during a short period early in the development of the population, leading to the development of numerous small genetically different individuals. Many early colonizers, such as *Hebeloma cylindrosporum* and *Laccaria* spp., appear to have relatively small nonpersistent genets (De La Bastide *et al.*, 1994; Gryta *et al.*, 1997, 2000; Selosse *et al.*, 1998). For some *Suillus* species, it is believed that no further genet recruitment from spores occurs after the initial colonizing phase and vegetative growth through hyphal networks appears to dominate in closed fungal communities (i.e. older forests). These communities are claimed to be resistant to genet establishment from spores, unless small disturbances in favourable sites create 'windows of opportunity' for local new genet recruitment (Dahlberg & Stenlid, 1995). Once thought to be dominated by a few, large (20–40 m) and long lasting genets, undisturbed closed forests can host populations of ectomycorrhizal basidiomycetes having high levels of genetic diversity characterized by small, short life-span genets (Gherbi *et al.*, 1999; Redecker *et al.*, 2001). Some ectomycorrhizal fungal populations found in undisturbed mature forest settings could therefore propagate by intense fruiting and spore germination. Whether population structure is related to microsite heterogeneity (Zhou *et al.*, 1999, 2001; Guidot *et al.*, 2001) or the biological/genetic features of the species is unknown. A way to validate the above mentioned models on the processes driving the evolution of genetic structure in populations of ectomycorrhizal fungi is to determine the genetic structure of a given species in different habitats differing by their ecological characteristics (Redecker *et al.*, 2001).

Laccaria amethystina is found in both recently established plantations and mature forests. We have investigated the genetic structure of *L. amethystina* populations in a 150-yr-old-monospecific beech (*Fagus sylvatica*) forest in the Vosges range in north-eastern France (Gherbi *et al.*, 1999). Surprisingly, a high density (> 5000 genets per ha) of small size, short life-span genets was found indicating an important role for sexual reproduction in this mature forest ecosystem. To assess whether this feature can be influenced by the habitat, we used the random amplified polymorphic DNA (RAPD) technique to estimate DNA variation within a population of *L. amethystina* in the mixed forest of La Chanéaz (Swiss Mittelland). *Xerocomus chrysenteron* and *X. pruinatus*, two boletoid species were also analysed for comparative analysis.

Materials and Methods

Study site

The study was conducted in mixed (conifers and deciduous trees) stands in the mycological reserve of La Chanéaz in Swiss Mittelland (Fribourg canton) (46°48′ N 7°00′ E). The 75 ha-forest is located at an altitude of 575 m above sea level (asl) on a plateau made up of fresh water molasse, and the soil is mainly an acidic moder. It is a typical mixed forest, with deciduous and coniferous trees species, such as *Fagus sylvatica*, *Picea abies*, *Pseudotsuga menziesii*, *Pinus sylvestris*, and *Abies alba*. The mean annual temperature is 9.4° and the mean annual precipitation is 900–1000 mm. The reserve was set up in 1979 by the Swiss Federal Institute for Forest, Snow and Landscape Research and mushroom harvesting is not allowed except for scientific investigations. Many studies on fungal ecology and protection were carried out on the site (Egli *et al.*, 1990). The climatic data, ectomycorrhizal communities and fungal species richness of the fungal reserve were described by Egli & Ayer (1997) and Straatsma *et al.* (2001).

Sampling plots

Within the study site, two sampling areas were established. Plot 59 (10 × 5 m) was used for sampling *Laccaria amethystina* Cooke basidiocarps, whereas those of *Xerocomus chrysenteron* (Bull.) Quél. and *X. pruinatus* (Fr. and Hök) Quél. were collected in the nearby plot 47 (10 × 10 m). Each of these areas was chosen to have mature trees at their edges. Plot 59 comprised two 140-yr-old *Pinus sylvestris* and two 40-yr-old *Fagus sylvatica* and several scattered young ecto- and endomycorrhizal trees (*F. sylvatica*, *Corylus avellana*, *Acer pseudoplatanus*, *Sorbus aucuparia*, and *Prunus padus*) (Fig. 1). Trees present on plot 47 comprised four 140-yr-old *Picea abies* and three understorey 40-yr-old *Fagus sylvatica* (Fig. 2). Plot 47 is surrounded by monospecific closed beech stands (F. Ayer, pers. comm.). Investigated plots were surrounded by 2 m high fences to avoid all inconvenient influences by mushroom pickers and large forest animals. The plot areas were gridded using pegs planted every meter and location of collected basidiocarps

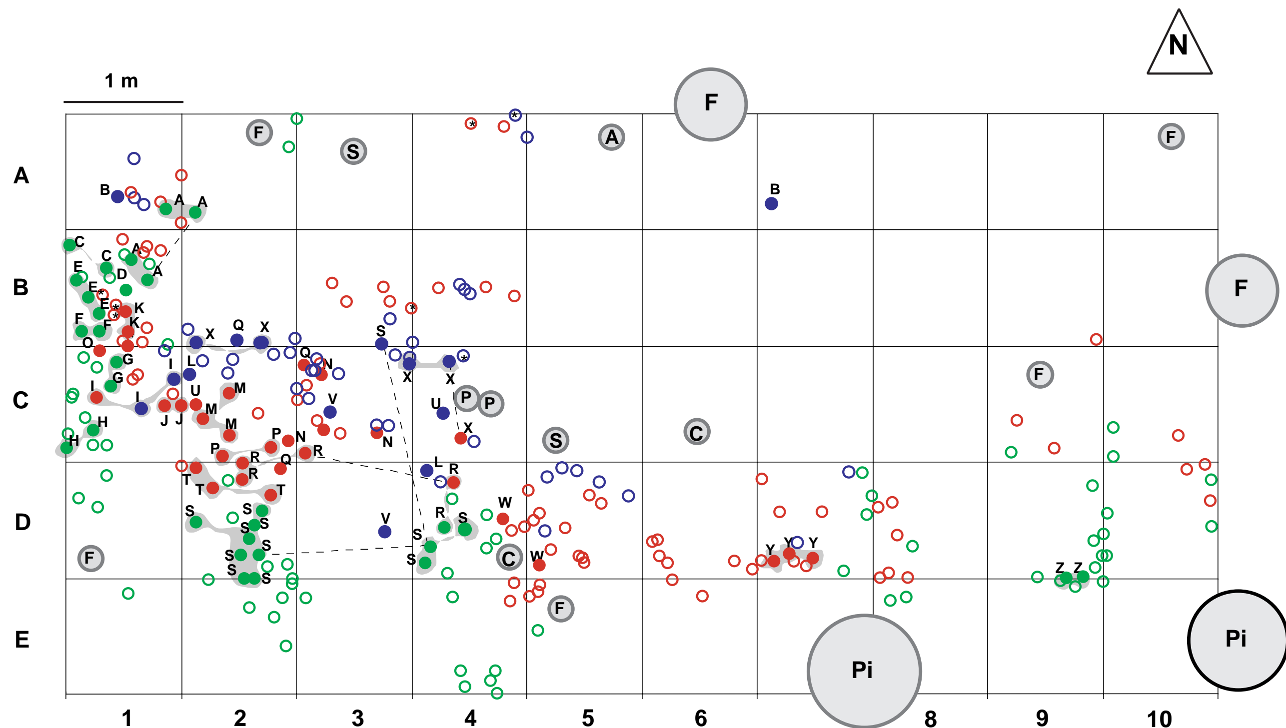

Fig. 1 Schematic depiction of relative positions of *Laccaria amethystina* basidiocarps and genets plotted on the reference mapping of plot 59 (5 × 10 m) (La Chanéaz forest). Standing trees are represented by grey closed circles, diameters to scale. Pi, *Pinus sylvestris*; F, *Fagus sylvatica*; C, *Corylus avellana*; A, *Acer pseudoplatanus*; S, *Sorbus aucuparia*; P, *Prunus padus*. Each blue (1997), red (1998) or green (1999) circle identifies a basidiocarp. Basidiocarps belonging to the same genet are represented by closed circles and are indicated with bold letters. The shaded zones encompass adjacent basidiocarps belonging to the same genets. Dotted lines connect different patches (ramets) of the same genet. Basidiocarps from neighbour-joining grouping A are indicated by an asterisk; they showed a 39–59% similarity of their RAPD patterns.

were recorded with a precision of a few centimetres (< 5 cm). Only fresh specimens without any signs of decay were used.

Survey of basidiocarps

In 1997–99, the fruiting period of *L. amethystina* was from early October to early November, whereas fruiting of *X. chrysenteron* and *X. pruinatus* was from mid-August to mid-November. The *Xerocomus* species were morphologically identified according to Binder & Fischer (1997) and their identification was confirmed by sequencing their nuclear ribosomal internal transcribed spacer (ITS) and part of the large 25S rDNA subunit (GenBank accession # AF402139 and AF402140). The sites were visited up to twice a week to ensure an exhaustive sampling. In 1997, 1998, and 1999, we sampled a total of 280 *L. amethystina* basidiocarps (Table 1). The collection of *X. chrysenteron* totalized 146 basidiocarps (Table 1). *Xerocomus pruinatus* basidiocarps were rare and only 10 were collected (Table 1). After recording the coordinates of collected basidiocarps on a sampling grid, they were placed in a labelled plastic bag, transported to the laboratory and stored at −20° within 2 h.

Molecular methods

Total DNA was extracted directly from basidiocarp tissues using the hexadecyltrimethylammonium bromide (CTAB)/proteinase K protocol as described by Henrion *et al.* (1994). PCR amplifications of the nuclear rDNA ITS and intergenic spacer 1 (IGS1) were carried out as described in Henrion *et al.* (1992, 1994), except that a BIOMETRA Unothermocycler system was used. RAPD was performed using primers 174 and 157 at high annealing temperature (50°C) according to Selosse *et al.* (1998). Amplification products were separated by electrophoresis using 1.4% (w/v) Sigma Wide Range agarose/0.8% agarose gel electrophoresis for 5 h (120 V h^{-1}) in a 1X TBE buffer system. Gels were stained with ethidium bromide and photographed with a digital camera.

The amplified nuclear ribosomal DNA ITS and IGS were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, USA), following the manufacturer's instructions. Reactions were then electrophoresed on either an ABI 373XL (at the Institut d'Ecologie de l'Université de Lausanne) or an ABI Genotyper 310 (at INRA-Nancy) automated sequencer (Applied Biosystems Inc.). Gels were tracked using the ABI Prism Sequencing

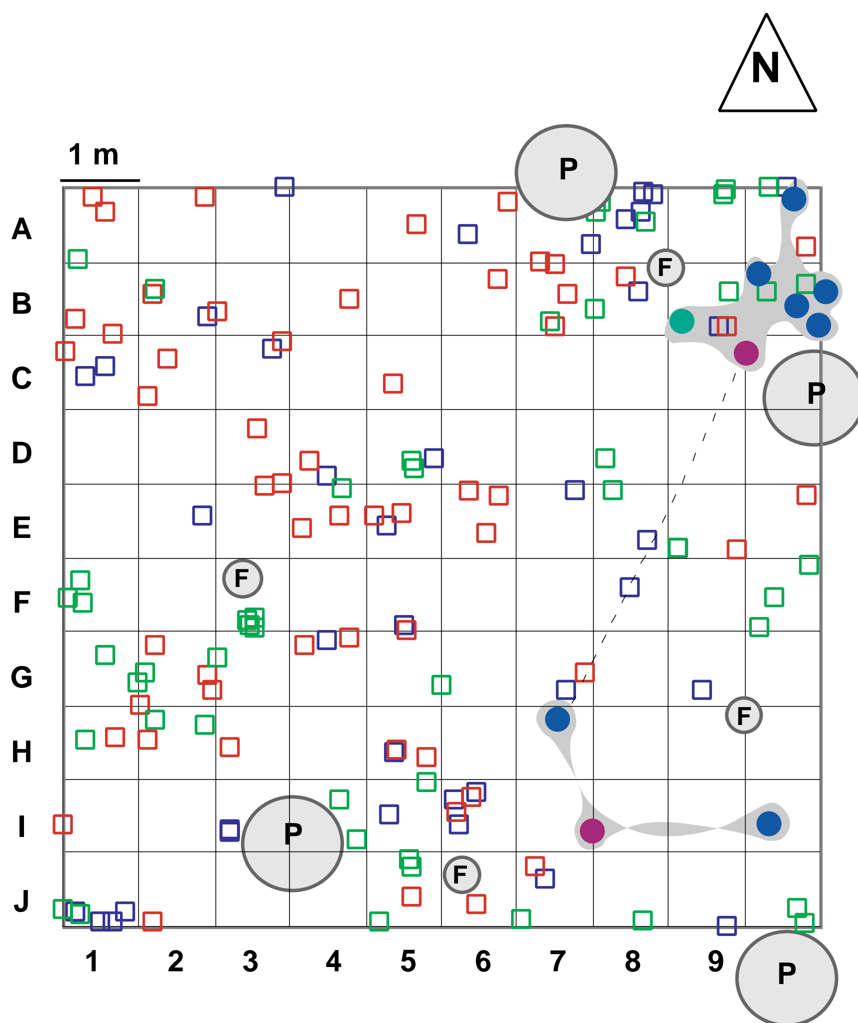

Fig. 2 Schematic depiction of relative positions of basidiocarps and genets of *Xerocomus chrysenteron* and *X. pruinatus* in plot 47 (10 × 10 m) (La Chanéaz forest). Standing trees (P, *Picea abies*; F, *Fagus sylvatica*) growing in the plot are represented by grey closed circles, diameters to scale. Basidiocarps of the single *X. chrysenteron* genet are represented by blue (1997), red (1998) or green (1999) open squares. Basidiocarps of the single *X. pruinatus* genet are represented by closed circles (dark blue, 1997; purple, 1998; dark green, 1999). The shaded zones encompass basidiocarps belonging to *X. pruinatus* large genet.

programme and raw sequence data were edited using the Sequence Analysis (Applied Biosystems Inc.) or Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) programmes.

Data and statistical analyses

The different *L. amethystina* basidiocarps were initially analysed by ITS and IGS1 typing and genets were distinguished by their RAPD patterns. We selected primers yielding both reproducible and polymorphic variations with well defined and darkly stained bands. RAPD were performed twice and only the bands that reproduced consistently were scored for each individual basidiocarp. Similar or identical samples were checked against each other by comparing their electrophoregrams side by side. Basidiocarps having identical ITS, IGS1 and RAPD banding patterns were regarded as belonging to the same genet and were mapped according to the distribution of collected basidiocarps. The area between adjacent basidiocarps sharing identical DNA patterns was assumed to represent the genet surface and the basidiocarps at the edge of this area were used to draw the borders of genets.

RAPD was scored as presence (1) or absence (0) of a band and a matrix of RAPD phenotypes was assembled. This rectangular matrix of individual × RAPD marker containing the band scoring information was transformed to resemblance matrices using four distance and similarity coefficients: simple matching, Sorensen and Jaccard similarity coefficients, and Euclidean distance for binary data (SIMIL in the R package 4.0d3; Casgrain & Legendre, 2001) with similar results. A dendrogram was constructed by unweighted pairgroup method with arithmetic mean (UPGMA) from the similarity matrices as a graphic representation of the relationships between samples using the R package (Casgrain & Legendre, 2001). The binary data matrix was also used for UPGMA and neighbour-joining (NJ) analyses in PAUP*4.08b (PPC/Altivec) (Swofford, 1999). The effect of spatial separation on genetic structure was tested by a regular Mantel test (Mantel & Valand, 1970) on the resemblance (RAPD pattern similarity) matrix and a Euclidean matrix of metric distance

Table 1 Characteristics of the populations of *Laccaria amethystina*, *Xerocomus chrysenteron*, and *X. pruinatus* of the fungal reserve La Chanéaz

	Samplings			
	1997	1998	1999	Total
Laccaria amethystina				
Basidiocarps sampled	59	116	105	280
IGS1 and RAPD analyses	44	90	75	209[a]
Genets detected	36	69	52	157
Basidiocarps per genet	1.22	1.30	1.44	1.33
Proportion of distinguishable genets	0.82	0.77	0.69	0.75
Genets per ha per year	7200	13 800	10 400	10 467
Xerocomus chrysenteron				
Basidiocarps sampled	39	56	51	146
IGS1 and RAPD analyses	39	56	51	146
Genets detected	1	1	1	1
Basidiocarps per genet	39	56	51	48
Proportion of distinguishable genets	0.02	0.01	0.02	0.02
Genets per ha per year	10	10	10	10
Xerocomus pruinatus				
Basidiocarps sampled	7	2	1	10
IGS1 and RAPD analyses	7	2	1	10
Genets detected	1	1	1	1
Basidiocarps per genet	7	2	1	3.3
Proportion of distinguishable genets	0.14	0.5	1	0.3
Genets per ha per year	10	10	10	10

[a]As a result of PCR failures, some of the basidiocarps produced no or poor results.

between individual basidiocarps (MANTEL module in the R Package). The significance of the correlation between the two matrices was tested by 999 random permutations of the similarity matrix to generate a null distribution of correlation coefficients (z-values). A significant result was inferred if ≥ 95% of the randomly generated statistics were greater than the observed value. Tests for goodness of fit to Hardy–Weinberg equilibrium were computed in POPGENE (Yeh *et al.*, 1997).

Results

Genetic structure and spatial distribution of *Laccaria amethystina*

Most of the basidiocarps were clustered in the westward part of the plot (Fig. 1). Genetic variability of the *L. amethystina* population was initially assessed by investigating the size polymorphism of the rDNA ITS and IGS1, regions showing intraspecific variability within *Laccaria* spp. (Henrion *et al.*, 1992; Selosse *et al.*, 1996, 1998; Gherbi *et al.*, 1999). No size polymorphism was found in the ITS (data not shown). By contrast, three different IGS1 patterns, differing by the number and size of the amplification products, were identified from among the 209 basidiocarps analysed. IGS1-type *AA*, presenting a single 700 bp DNA fragment, was detected in 113 basidiocarps (54%). Type *aa*, having a single DNA band at 760 bp, was found in 24 basidiocarps (12%). The remaining 72 analysed basidiocarps (34%) displayed a heterotype *Aa* with two bands at 700 and 760 bp. Sequencing of the two IGS1 types revealed a low divergence (0.8%) for most of the sequence, but a 47-bp-indel similar to the one observed in *L. amethystina* in the beech forest of Aubure (Gherbi *et al.*, 1999). The IGS1 types were treated as codominant biallelic markers in a test for Hardy–Weinberg equilibrium expectation in the 209 isolates. Isolates which possessed a single band (700- or 760-bp) were treated as homozygotes (*AA*, *aa*) and isolates with two bands (700- and 760-bp) were treated as heterozygotes (*Aa*). A slight heterozygote deficit was observed in the IGS1 locus (expected and observed heterozygotes: 0.41 and 0.35, respectively; expected and observed homozygotes: 0.58 and 0.64, respectively). A Chi-square test returned the genotype distribution in the IGS1 locus to be significantly different from the genotype distribution under Hardy–Weinberg equilibrium (4.4%; $P < 0.05$). The heterozygote deficit in the IGS1 locus suggests that inbreeding may occur, although this process should be limited by mating types. However, the significant level of heterozygosity indicates that outcrossing, heterothallic reproductive mode was taking place in this population.

Further molecular typing was carried out using RAPD to ascertain the DNA diversity amongst collected *L. amethystina* basidiocarps. The two chosen primers yielded 59 scorable markers that ranged in size from 180 to 1200 bp. Up to 157 unique RAPD banding patterns were identified indicating the existence of a large DNA variation in the analysed population.

Most of the DNA bands were only detected in a few basidiocarps. Only two DNA fragments, generated by primer 157, were found in all DNA samples. The mean 'proportion of distinguishable genets' (i.e. total number of unique RAPD patterns divided by the sample number) from this sample of the population of La Chanéaz forest was 0.75 (Table 1). We found a similar high value (0.61) in *L. amethystina* populations surveyed in a beech forest in the Vosges mountains (north-eastern France) (Gherbi *et al.*, 1999).

Basidiocarps belonging to the same genet were not necessarily collected on the same day. The persistence of basidiocarps belonging to a discrete genet varied from 5 to 22 d. Only six genets (< 4%) were detected for more than 1 yr (Fig. 1). Genets I, Q, U, and X were detected in both 1997 and 1998, whereas a single genet (i.e. R) was found in 1998 and 1999 samplings. The greatest distance separating two basidiocarps belonging to the same genet and collected in two consecutive years ranged from 0.3 (genet I) to 2.2 m (genet Q & U). Basidiocarps of the largest genet, so-called S, were sampled in 1997 and 1999 (Fig. 1).

The limits of *L. amethystina* intermingling individuals in plot 59 were often blurred (Fig. 1). The average number of basidiocarps per genet was 1.33 (Table 1). Even in areas of high sampling density (e.g. coordinates B1, C1 and C2), most genets were found only once. The largest genet (S) comprised 13 basidiocarps, all having the IGS1-type *AA*, and its two main patches covered an area of *c.* 0.75 m^2 (coordinates D2/D4). The average size (largest detectable distance across a genet) of the 26 genets comprising more than one basidiocarp was 1.1 m, ranging from a few cm (genet J, coordinates C1) to 5.40 m (genet B, coordinates A1 to A7). Similarity between genets was assessed by UPGMA (not shown) and NJ (Fig. 3) analyses of RAPD patterns. These statistical analyses produced the same tree topology. Genets comprising at least three basidiocarps formed easily distinguable clusters (labelled shaded boxes in Fig. 3). However, the dendrogram illustrate well the limited number of individuals sharing similar RAPD phenotypes. As an example, the similarity of RAPD patterns clustering at the top of the NJ tree (the so-called grouping A including individuals C714, C807, B731, B817, B824, C802 and C810) ranged from 0.39 to 0.59. These statistical analyses confirmed the lack of a close genetic relationship between most basidiocarps and genets, although breeding occurred as suggested by the significant proportion of heterozygotes at the IGS1 locus (see above). Some of the most divergent RAPD patterns originated from adjacent basidiocarps (see coordinates B1 and C1). The Mantel's test showed no significant correlation between the spatial distance matrix and the similarity matrix of RAPD patterns ($r = -0.04$, $P = 0.13$) suggesting that spatial distance between basidiocarps plays a marginal role in structuring this population.

Spatial distribution and DNA typing of *Xerocomus chrysenteron* and *X. pruinatus*

Basidiocarps of *X. chrysenteron*, harvested from 1997 to 1999, were scattered over plot 47 (Fig. 2), as it was recorded since 1992 (F. Ayer, pers. comm.). By contrast, basidiocarps of *X. pruinatus* were restricted to a surface of 40-m^2, less than half of the plot (coordinates H7 to A10). Amplification of the ITS from *X. chrysenteron* and *X. pruinatus* generated a single 740 bp-band (data not shown). By contrast, the size of the amplified IGS1 showed a great difference between the two species. *X. chrysenteron* had a single IGS1 product of 500 bp, whereas *X. pruinatus* had two bands of 800 and 900 bp.

RAPD typing and spatial distribution of genets

RAPD produced identical patterns, totalling 13 reliable markers, for all *X. chrysenteron* basidiocarps collected over the 3 yr survey. This genetic homogeneity suggests the presence of a single long-lasting individual in the plot covering a surface as large as 100 m^2 (Fig. 3). An attempt to define the edges of this individual was made during the autumn of 2000 by collecting five basidiocarps outside plot 47: four in the northward adjacent plot (up to 50 m), and one 60 meters south-eastward. An additional 10 basidiocarps were collected inside the plot (data not shown). The same IGS and RAPD patterns were found for all basidiocarps. The maximum distance recorded between two basidiocarps from this single genet was, thus, *c.* 110 m.

All *X. pruinatus* basidiocarps collected inside plot 47 over the 3 yr belonged to the same genet. The surface covered by this individual was at least 40 m^2 and it was restricted to the eastern part of the plot (Fig. 2). Five basidiocarps, collected outside the plot in the autumn of 2000, belonged to four different genets (data not shown) suggesting that this large individual did not extend outside plot 47.

Discussion

Genet size in ectomycorrhizal fungi can reveal features of colonization strategy and growth. Fruiting body and ectomycorrhiza surveys have shown that the size of the genets from ectomycorrhizal fungi may vary with the forest age. It is widely

Fig. 3 Dendrogram constructed by neighbour-joining analysis showing the RAPD relationships among *Laccaria amethystina* basidiocarps collected in plot 59 (5 × 10 m) of La Chanéaz forest. Analysis was conducted using the distance search algorithm of PAUP 4.0b8. Scale represents 0.01 changes. The letters indicate the IGS1 type (A, type *AA*; B, type *aa*; and C, type *Aa*), the following number stands for the sampling year (7, 1997; 8, 1998; 9, 1999) and the remaining numbers indicate the sample number. Genets comprising more than three basidiocarps are indicated by shaded boxes. Grouping A which includes individuals showing a 39–59% similarity of their RAPD pattern is shown as a shaded box at the tree top. The shaded box at the tree top indicates the grouping A referred to in Figure 1.

Grouping A
C714
C807
B731
B817
B819
B824
B818
B825
C802
C810
C870
C872
C871
Y
C874
C822
C823
C826
A880
A882
A883
C877
C878
A723
A745
A757
A762
A814
A749
A758
A751
A756
A8101
A8102
A8103
T
0.01 changes
A726
A729
A734
A736
A727
A831
X
A730
A849
A832
Q
A833
A837
A839
A836
A838
A840
N
A732
A850
A812
A813
A843
A844
A847
M
A724
A973
A959
A966
A956
A964
A968
A965
A967
A971
A958
A975
A963
S
A960
A728
A743
A746
A738
A737
A748
A752
A750
A735
A742
A739
A740
A747
A834
A8100
A8104
A896
A957
R
A8106
A944
C953
A982
A979
A980
A984
A8107
C801
C904
A881
C923
C924
C715
C719
C717
C803
C806
C804
C805
C821
C903
C909
C913
C905
A
C918
C811
C939
C862
C863
A885
A892
A893
A895
A897
A894
A886
A908
A915
A911
E
A914
A916
A917
A921
B907
B910
B912
B920
B922
B937
C716
A887
A890
C899
C8111
C8108
C8112
C8109
A869
C936
C721
C753
C763
C858
I
C854
C855
C856
C820
C859
C860
C927
C930
C755
C934
B969
B970
A954
A955
C879
C884
A943
A945
C926
C928
C933
C942
C809
B935
B929
C902
C940
B991
B992
B996
B998
B995
B9100
C9101
C993
A948
A977
A985
A986
C987

believed that the presence of many small genets indicates multiple spore establishment events and large genets reflects predominant vegetative spread (Dahlberg & Stenlid, 1990, 1995; Dahlberg, 2001). Estimated densities of populations range from 30 to 5200 genets ha^{-1} depending on the species and forest age (Gryta *et al.*, 1997; Gherbi *et al.*, 1999; Dahlberg, 2001; Redecker *et al.*, 2001). Populations of *Suillus bovinus* and *S. variegatus* in old conifer forests consist mostly of discrete, large (up to 27 m) and old (> 150 yr) genets which do not seem to intermingle (Dahlberg & Stenlid, 1994, 1995; Dahlberg, 2001). One of the largest genet of ectomycorrhizal fungi to date (300 m^2) has been found in *Suillus pungens* in a Bishop pine forest in coastal California (Bonello *et al.*, 1998). It was thus thought that vegetative growth and dissemination over long periods dominate over spore colonization in established forests typified by canopy closure, lack of young trees, and organic N-rich litter (Dahlberg & Stenlid, 1995). However, recent studies (Gherbi *et al.*, 1999; Redecker *et al.*, 2001) have demonstrated that undisturbed mature forests may host fungal populations characterized by numerous, short life-span genets relying mainly on spore dissemination for local colonization. Initial genet recruitment from spores followed by vegetative spread (large, old genets) and repeated recruitment from spores (small, short life-span genets) may be considered as two extremes of a continuum rather than as two clearly distinct recruitment patterns in ectomycorrhizal basidiomycetes.

As spatial structuration of populations of ectomycorrhizal fungi differs between taxa (Dahlberg, 2001), we have analysed populations of three ectomycorrhizal basidiomycetes, *Laccaria amethystina*, *Xerocomus chrysenteron*, and *X. pruinatus* in the undisturbed mixed forest of La Chanéaz. The investigated stands comprised 40-yr-old and 140-yr-old conifer and hardwood species, together with a few young, mainly endomycorrhizal, trees. Molecular typing of sampled basidiocarps revealed two constrasting genetic structures for these species. Both *X. chrysenteron* and *X. pruinatus* populations consisted of a discrete, large genet. Assuming a mean growth rate of 0.5 m per year for the underground mycelium of ectomycorrhizal fungi (Dahlberg & Stenlid, 1994; Bonello *et al.*, 1998; Selosse *et al.*, 1998), the founder of the 110 m-large *X. chrysenteron* clone probably associated with the older *Picea abies* when they established 140-yr-ago. The closely related species *X. pruinatus* also formed large clones (40 m^2) in the same plot. *X. chrysenteron* collected in the beech forest of Aubure (Vosges) (Gherbi *et al.*, 1999) also pertained to a single large genet (> 100 m^2) (H. Gherbi & F. Martin, unpublished). These results were consistent with the findings of Dahlberg & Stenlid (1994, 1995) showing that, although they produce a considerable number of spores, many boletoids extend mostly by vegetative growth and the genet establishment from spore germination progressively decreased in the ageing population. Their capacity to develop extensive hyphal cords probably enables the large genets to visit the roots of more trees and to pool more total C than species which are more common, but produce smaller genets (Bonello *et al.*, 1998).

By contrast, the *L. amethystina* population was made of numerous discrete genets (up to 13800 ha^{-1}). Among the analysed genets, only 4% of the identified genets fruited the following years suggesting a considerably large mortality of genets. The genet size varied from the mere diameter of the basidiocarp (i.e. *c.* 2–4 cm) to 5.40 m in length. The present results are in agreement with a previous survey carried out in a different habitat, a 150-yr-old monospecific beech forest situated at an altitude of 1000 m asl (Gherbi *et al.*, 1999). In the latter study conducted in the Vosges mountains (north-eastern France), *L. amethystina* populations exhibited up to 5200 short life-span, small-size genets per ha. The dramatic renewal of genets suggested a recolonization of the site by new genets each year and /or erratic fruiting of 'cryptic' below ground genets (Gherbi *et al.*, 1999). The numerous *L. amethystina* genets are likely to have arisen from repetitive establishment from basidiospores, as suggested by their small size. Large amounts of feeder roots with high turnover rates (Vogt *et al.*, 1986) give constant opportunities for ectomycorrhizal fungi to become established (Kranabetter & Wylie, 1998). The growth pattern of *L. amethystina* suggests that these new host root tips are colonized each season, the spreading of the new genets probably following the elongation of roots. In situations with strong intraspecific competition we could expect a reduced clonal diversity because fewer fitter genets would predominate. Yet, a high DNA diversity was maintained at both sites analysed (i.e. Aubure and La Chanéaz). Obviously, none of the genet produced sufficient clonal offspring to contribute to its predominance.

Colonization by numerous small-sized genets, with high fruiting rates, is reminiscent of young natural populations of ectomycorrhizal fungi (e.g. *Suillus bovinus* (Dahlberg & Stenlid, 1990); *Laccaria bicolor* (De la Bastide *et al.*, 1994; Selosse *et al.*, 1998, 1999) and *Hebeloma cylindrosporum* (Gryta *et al.*, 1997; Guidot *et al.*, 2001)). This population structure was postulated to arise in recently established or disturbed stands (Dahlberg & Stenlid, 1995). Recent investigations have however, shown that this 'early stage' population structure is also found for ectomycorrhizal basidiomycetes in closed undisturbed forest settings (Gherbi *et al.*, 1999; Zhou *et al.*, 1999, 2001; Redecker *et al.*, 2001).

The population structure may depend on: the dispersal of the spores; the extent of growth of the monokaryotic mycelium (that germinated from the basidiospores of the previous seasons) and the opportunities for mating between compatible monokaryotic mycelia that thus occur; and the persistence of the below-ground mycelia. The spatial distribution of *H. cylindrosporum* (Guidot *et al.*, 2001) and *Suillus grevillei* (Zhou *et al.*, 2001) genets in the soil showed that the development of basidiocarps was tightly correlated with that of the extramatrical and ectomycorrhizal mycelia of the same genet. Disappearance of basidiocarps of these species at a given place

was associated with the disappearance of the corresponding subterranean mycelia within 1 yr. If this behaviour is also true for *L. amethystina*, basidiocarp analysis appears to reflect both the genetic diversity and the spatial structure of its subterranean populations.

Microsite heterogeneity which promotes the coexistence of genets through diversifying selection has been hypothesized to account for the mechanisms underlying the maintenance of a high level of genotypic diversity in fungal species (Frankland, 1998). Because *Xerocomus* species have been able to maintain a clonal structure for decades, this hypothesis alone is insufficient to explain the high genetic diversity of genets in aged forest ecosystems. The fact that the investigated stands are included in a fungal reserve protected by a fence precludes any major perturbation related to large animal and human activities.

The low similarity between RAPD patterns (Fig. 2) and the low correlation between spatial distance separating basidiocarps and the RAPD similarity matrix (Mantel's test) indicate that *L. amethystina* genets found on plot 59 did not arise from a common (or a few) colonization event. These results suggest that establishment of novel genets and colonization of host root tips are largely a stochastic process, with much spore dispersal occurring on a scale greater or equal to that of this study (50 m^2). The results of this study are somewhat at odds with the allelic analysis on *S. grevillei* (Zhou *et al.*, 2001) which has shown that the spread of alleles within and between populations might be by repeats of short-distance spore dispersal.

In conclusion, the prominent role of the sexual spore reproduction over the perennial spread of the vegetative mycelium in the soil for ectomycorrhizal species, such as *L. amethystina*, is not restricted to pioneer situations, but is found in stable established forest ecosystems. These species are able to establish novel genets from spores repeatedly over several decades without apparent perturbation of the sites and/or host plant genetic structure. This suggests a rapid alteration of the subterranean parts of *L. amethystina* genets and a constant colonization of new feeder roots. Knowing the spatial structure of the studied populations through the fruiting body location will facilitate the characterization of their genetic structure at the level of ectomycorrhizal roots and extramatrical hyphae using codominant genetic loci (Guidot *et al.*, 2001). So far, investigations on populations of ectomycorrhizal fungi have mainly been descriptive or correlative and no experimental study has yet been performed to manipulate these populations. Identification of causal processes controlling the dynamics and structure of ectomycorrhizal populations will advance our understanding of forest ecosystems.

Supplementary Material

A map showing the location of study sites, photographs of the ITS and RAPD gels, DNA sequences, distance matrices and UPGMA and NJ trees are available at the following URL: http://mycor.nancy.inra.fr/Aubure/Aubure.html

Acknowledgements

This study was part of Anne-Marie Fiore-Donno's Ph.D. project. It was partially funded by a scholarship from the University of Lausanne and the Special Found of the Swiss Federal Office for Education and Science for Switzerland-France Ph.D. joint programmes. We thank Prof. Heinz Clémençon (University of Lausanne) for his constant support; Simon Egli and François Ayer (WSL, Birmensdorf) for allowing us to establish field sites in the fungal reserve of La Chanéaz for these studies; Christine Delaruelle (INRA-Nancy) for her helpful assistance in molecular methods; François Ayer for his kind assistance and advices in fruiting body surveys; Jesus Diez for stimulating discussions; two anonymous referees for their constructive comments; and Beatrice Senn-Irlet (University of Bern) for suggesting this investigation and her support. Funding was provided by the Bureau des Ressources Génétiques and INRA to FM.

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