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Genetic structure in populations of the fungus *Fomitiporia punctata* associated with the esca syndrome in grapevine

I. JAMAUX-DESPRÉAUX and J. P. PÉROS

UMR DGPC, Equipe vigne, INRA, Montpellier, France

Summary

Six populations of *Fomitiporia punctata* (Fr.) Murrill were sampled in France and Italy from vines showing symptoms of esca syndrome. Genetic variation within and among populations was studied by using 34 random amplified polymorphic DNA (RAPD) markers. All the 192 isolates analyzed were distinguished, and the haplotypic diversity was similar in each population. The hypothesis that markers were randomly associated was not rejected for 5 populations and for the total sample. Comparison of marker frequency showed significant differences among populations for only 4 markers, indicating a low level of genetic differentiation. The analysis of molecular variance (AMOVA) confirmed that most of the variance in RAPD banding patterns was present within populations (98.5 %). These data strongly suggest that the fungus spreads by means of airborne basidiospores and regularly outcrosses in nature. The prospected regions seem to form an epidemiological unit with a panmictic population of *F. punctata*. However, a very low but significant differentiation was detected between the populations in western France and those in Mediterranean locations.

K e y w o r d s: *Fomitiporia punctata*, *Vitis vinifera* L., esca syndrome, genetic diversity, RAPD, population biology.

Introduction

The esca syndrome is a major threat for grapevine (*Vitis vinifera* L.) longevity worldwide (DUBOS and LARIGNON 1988; MUGNAI *et al*. 1999). Two basidiomycetes, *Stereum hirsutum* (Willd.) Pers. and *Phellinus igniarius* (L.:Fr.) Quél., were formerly suspected of being involved in the wood decay process (VIALA 1926; CHIARAPPA 1959). However, recent investigations indicated that *Fomitiporia punctata* (Fr.) Murrill [= *Phellinus punctatus* (P. Karst.) Pilát] is the basidiomycete responsible for the white rot lesion (MUGNAI *et al*. 1996; LARIGNON and DUBOS 1997; CORTESI *et al*. 2000). *F. punctacta* is able to colonize grapevine alone (SPARAPANO *et al*. 2000) but it is accepted that pioneer decay fungi, like *Eutypa lata* (Pers:Fr.) Tul. *et* C. Tull., *Phaeomoniella chlamydospora* (W. Gams *et al*.) Crous *et* W. Gams (formerly *Phaeoacremonium chlamydosporum* W. Gams *et al*.) and *Phaeoacremonium aleophilum* W. Gams *et al*., play an important role in the development of the syndrome in vineyards (MUGNAI *et al*. 1996; JAMAUX-DESPRÉAUX *et al*. 1997; LARIGNON and DUBOS 1997). The banning of sodium arsenite, which was the only mode of chemical control in vineyards, means that now new strategies against esca have to be developed. This requires a better knowledge of the population biology of the fungal species that degrade the wood, and, in particular, of the white-rot pathogen *F. punctacta*.

The way *F. punctata* populations are established in vineyards is not known. According to VIALA (1926) and many others, the white rot lesion develops first in the pith of pruning wounds and the vine is killed before the lesion reaches the rootstock. This indicates that the *F. punctacta* infection does not start from an inoculum in the soil. The infection of wounded aerial parts may be due to the vegetative spread of the fungus from vine to vine, for instance by contaminated pruning tools. Another possibility is that airborne basidiospores spread the fungus. To address the question of how the fungus spreads, the spatial distribution of vines showing foliar symptoms has been extensively studied. Evidence for aggregation (SURICO *et al*. 1999; POLLASTRO *et al*. 2000 b) as well as for random distribution (CORTESI *et al*. 2000; SURICO *et al*. 2000) has been presented. Spreading by airborne basidiospores could lead to random distribution but aggregation could have other reasons than propagation from vine to vine. For instance, soil heterogeneity may have a considerable effect on the expression of symptoms. Moreover, as several fungi are associated with esca, considering the spatial patterns of the syndrome may be of limited value to establish the spreading mode of one of these fungi.

Assessment of the genetic diversity of *F. punctata* in vineyards could provide more information about the spreading mode of the fungus than the spatial patterns of symptomatic vines. CORTESI *et al*. (2000) described a wide diversity of somatic incompatibility types within Italian vineyards and POLLASTRO *et al*. (2000 a) found that isolates of *F. punctata* taken from adjacent vines were different Random Amplified Polymorphic DNA (RAPD) phenotypes. This high level of fine-scale genetic diversity suggests that airborne basidiospores could be important sources of inoculum and that *F. punctata* is able to outcross in nature.

Whether *F. punctacta* occurs in European vineyards as a single population or as genetically distinct subpopulations has not been documented. Addressing this question could also provide information about the spreading mode. For instance, lack of genetic differentiation among geographically distant regions may be explained by a gene flow that occurs through the movement of basidiospores. On the other hand,

Correspondence to: Dr. J. P. PÉROS, Unité Mixte de Recherche DGPC, Equipe vigne, Institut National de la Recherche Agronomique, Centre de Recherches de Montpellier, 2 place Viala, F-34060 Montpellier Cedex 1, France. Fax: +33-4-6799 2064, E-mail: peros@ensam.inra.fr

a high level of genetic differentiation may reflect the existence of intersterile populations, *i.e.* sibling species, which have been described in several basidiomycetes that cause wood decay (MCKEEN 1952; RAYNER and BODDY 1988; FISHER and BRESINSKY 1992; FISCHER, 1994; STENLID *et al.* 1994). *F. punctata* is widespread in Europe where different grapevine cultivars are cultivated in very diverse climates. *F. punctata* is also found on many living trees as well as on logs of hardwoods and conifers (WALLA 1984; RYVARDEN and GILBERTSON 1994; IPPOLITO *et al.* 1998). These different ecological niches could be occupied by different subpopulations.

The objective of this study was to determine the genetic structure of *F. punctata* populations obtained from esca-diseased vines in France and Italy. We used Random Amplified Polymorphic DNA (RAPD) markers to assess the level of diversity within populations and to determine if distinct subpopulations of *F. punctata* exist.

Material and Methods

Fungal isolates: The 192 *F. punctata* isolates studied were collected in different geographical areas in France and Italy (Tab. 1, Fig. 1). Isolates from Languedoc-Roussillon (region F1) were separated in two groups: F1-A (N = 46) corresponded to vineyards in the Pyrénées-Orientales and Aude districts (eastern part of the region) whereas F1-B (N = 63) corresponded to those in the Hérault and Gard districts (western part of the region). Isolates from southwestern France (region F2) were also divided in two zones: F2-A (N = 23) from the Charentes region and F2-B (N = 28) from the Aquitaine and Midi-Pyrénées regions. The F2-A population was sampled on a single grapevine cultivar (cv. Ugni blanc) in a rather homogenous area, whereas the F2-B population corresponded to several cultivars cultivated in diverse environmental conditions (Tab. 1). P. LARIGNON (INRA, Bordeaux, France) provided isolates from F2-A and F2-B populations. Italian isolates (IT) originated from two different zones in northern Italy separated by approximately 150 km: IT-A (N = 13) from near lake of Garda and IT-B (N = 19) from Tuscany. Italian isolates were provided by M. MINERVINI (University of Milan, Italy).

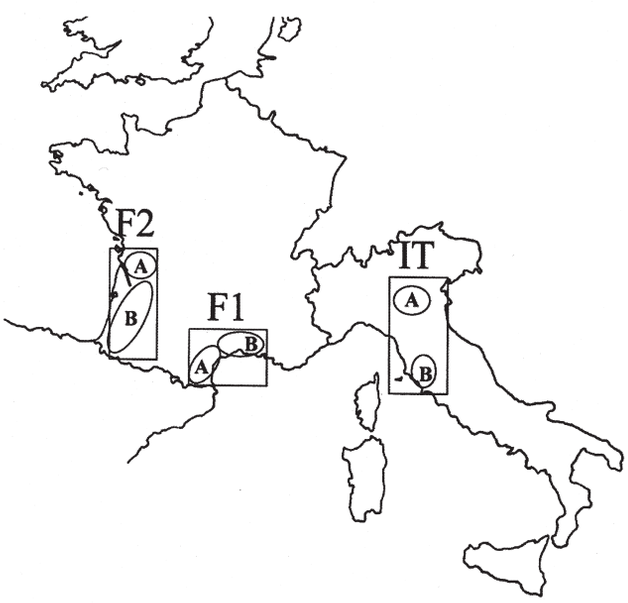

Fig. 1: Location of the *Fomitiporia punctata* populations sampled in this study from southern France (F1), south-western France (F2) and northern Italy (IT). Each region was separated in two areas, A and B.

Isolates were generally sampled in different vineyards but isolates from different plants were also taken within the same vineyard (Tab. 1). To isolate the fungus in the F1 region, we chose vines with external symptoms of infection, *i.e.* either leaf discoloration or dieback. Trunks were then cut into pieces to look for white-colored soft lesions in the wood. Pieces of wood (10 x 5 x 5 mm) were taken at the lesion margin and disinfected in a 3 % active chlorine solution. The pieces were washed twice in sterile water and put in Petri plates containing Potato Dextrose Agar (PDA, Difco) medium. The plates were incubated at 23 ±2 °C and observed every 2-3 d for two months. Isolated fungi were subcultured on PDA medium, and identification of *F. punctata* was based on its cultural characteristics. Isolates were maintained at 4 °C as culture disks placed in plastic tubes containing 1 ml of distilled water.

DNA extraction: PDA plates were inoculated with two plugs taken at the margin of fresh PDA cultures and incubated for two weeks at 23 °C. Mycelium was scraped using a tip and immediately ground with a chilled mortar and pestle in liquid nitrogen. The mycelium powder was put in a microtube. After addition of 0.5 ml of extraction buffer: 2 % cetyldimethylethyl ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, the mixture was incubated for 1-2 h at 65 °C. One volume of chloroform was added to the tube, which was then centrifuged at 12,000 *g* for 15 min at 16 °C. The supernatant was transferred into another microtube containing 0.1 ml of 10 % CTAB and 0.4 ml of chloroform. After homogenization and centrifugation, the supernatant was transferred and nucleic acids were precipitated using one volume of cold isopropanol. The tube was kept at -20 °C overnight, then gently agitated and centrifuged. Isopropanol was discarded and the pellet washed with 70 % ethanol. The pellet was dried and dissolved in 0.5 ml of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. The DNA was quantified on 0.8 % agarose gel stained with ethidium bromide by visual comparison with different quantities of lambda DNA (Life Technologies, Illkirch, France).

RAPD amplifications: PCR conditions described by WILLIAMS *et al.* (1990) were used with some modifications. Each reaction volume of 25 µl included 1.5 units of Taq DNA polymerase (Appligene, France), 1x buffer (10 mM Tris-HCL, pH 9, 50 mM KCl, $MgCl_2$ 1.5 mM, 0.1 % tritonX100, 0.2 mg.ml^{-1} BSA) provided with the enzyme, 120 µM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany), 30 ng of primer (Life Technologies, Illkirch, France) and approximately 25 ng of template DNA. The reaction was overlaid with a drop of mineral oil. A thermocycler (Biometra, Göttingen, Germany) was programmed for one step of 4 min at 94 °C, followed by 36 cycles of 1 min at 93 °C, 1 min at 38 °C, 1 min at 72 °C, and a final step of 6 min at 72 °C. Amplified products were analyzed by electrophoresis in 1.6 % agarose gel at 5 V per cm for 4 h, along a molecular size marker (1-kb ladder, Life Technolo-

Table 1

List of isolates of *Fomitiporia punctacta* used in this study with their geographical origin, vine cultivar origin, and date of sampling in three European regions in France (F1, F2) and Italy (IT), each region being subdivided into two areas (A and B)

Identification[a]	Pop.	Locality[b]	Vine cultivar	Sampling date
LR6 to LR8	F1-A	Antugnac (11)	Mauzac	Sept. 1996
LR9 to LR13	F1-A	Couiza (11)	Mauzac	Sept. 1996
LR14 to LR18	F1-A	Montréal (11)	Cinsault	Sept. 1996
LR96 and LR98	F1-A	St Laurent Cabrerisse (11)	Grenache	Sept. 1996
LR104 to LR106	F1-A	St Laurent Cabrerisse (11)	Carignan	Sept. 1996
LR112	F1-A	Ribaute (11)	Carignan	Sept. 1996
LR114	F1-A	Laure-Minervois (11)	Cinsault	Sept. 1996
LR118	F1-A	Durban (11)	Grenache/Carignan	Sept. 1996
LR140	F1-A	Narbonne (11)	Cinsault	Oct. 1996
LR143	F1-A	Treilles (11)	Muscat à petits grains	Oct. 1996
LR80 and LR81	F1-A	Montesquieu (66)	Cinsault	Sept. 1996
LR82 to LR84	F1-A	Montesquieu (66)	Grenache	Sept. 1996
LR85 to LR89	F1-A	Ortoffa (66)	Grenache	Sept. 1996
LR90 to LR94	F1-A	Tour de France (66)	Mourvèdre	Sept. 1996
LR99 to LR103	F1-A	Banyuls del Aspres (66)	Muscat à petits grains	Sept. 1996
LR131	F1-A	Maury (66)	Grenache	Oct. 1996
LR144	F1-A	Le Boulou (66)	Macabeu	Nov. 1996
LR146	F1-A	Le Boulou (66)	Macabeu	Nov. 1996
LR1 to LR5	F1-B	Beauvoisin (30)	Carignan	Sept. 1996
LR34 to LR38	F1-B	Les Tavernes (30)	Cinsault	Sept. 1996
LR39	F1-B	Monteils (30)	Cinsault	Sept. 1996
LR43 to LR47	F1-B	Cannes et Clairan (30)	Carignan	Sept. 1996
LR76	F1-B	St Nazaire (30)	Syrah	Sept. 1996
LR126	F1-B	Montaren-St Médiers (30)	Cinsault	Oct. 1996
LR123	F1-B	Villeneuve les Maguelone (34)	Carignan	Sept. 1996
LR19 to LR23	F1-B	Assignan (34)	Grenache	Sept. 1996
LR24 to LR27	F1-B	Assignan (34)	Sauvignon	Sept. 1996
LR69	F1-B	Adissan (34)	Bourret	Sept. 1996
LR48 to LR 51	F1-B	Puissalicon (34)	Carignan	Sept. 1996
LR52 to LR55	F1-B	Puissalicon (34)	Cinsault	Sept. 1996
LR57, L58 and LR60	F1-B	Pinet (34)	Cinsault	Sept. 1996
LR61	F1-B	Pinet (34)	Alicante Bouschet	Sept. 1996
LR63 to LR65	F1-B	St Christol (34)	Grenache	Sept. 1996
LR66 to LR68	F1-B	St Christol (34)	Grenache blanc	Sept. 1996
LR124	F1-B	Villeneuve les Maguelone (34)	Carignan	Sept. 1996
LR70 to LR72	F1-B	Vendargues (34)	Alphonse Lavallée	Sept. 1996
LR107 to LR111	F1-B	Villeveyrac (34)	Cabernet-Sauvignon	Sept. 1996
LR30	F1-B	Leyrac (34)	Cinsault	Sept. 1996
LR79	F1-B	Octon (34)	Cinsault	Sept. 1996
LR73 to LR75	F1-B	Vendargues (34)	Cinsault	Sept. 1996
LR134	F1-B	St Mathieu de Tréviers (34)	Mourvèdre	Oct. 1996
LR137	F1-B	Montpellier (34)	Unknown	Oct. 1996
PC8	F2-A	Pérignac (16)	Ugni blanc	Dec. 1996
PC9	F2-A	St Laurent de Cognac (16)	Ugni blanc	Dec. 1996
PC11	F2-A	Montguyon (16)	Ugni blanc	Dec. 1996
PC14	F2-A	La Couronne (16)	Ugni blanc	Dec. 1996
PC15 and PC17	F2-A	Vaux-Rouillac (16)	Ugni blanc	Dec. 1996
PC18	F2-A	Monchaude (16)	Ugni blanc	Dec. 1996
PC20 to PC22	F2-A	St Preuil (16)	Ugni blanc	Dec. 1996
PC23	F2-A	Angeac-Charente (16)	Ugni blanc	Dec. 1996
PC25 to PC27	F2-A	Graves (16)	Ugni blanc	Dec. 1996
PC28	F2-A	Julienne (16)	Ugni blanc	Dec. 1996

(Table 1, continued)

Identification[a]	Pop.	Locality[b]	Vine cultivar	Sampling date
PC1 to PC 3	F2-A	Les Touches de Périgny (17)	Ugni blanc	Dec. 1996
PC4 and PC5	F2-A	Les Eglises d'Argenteuil (17)	Ugni blanc	Dec. 1996
PC6	F2-A	Saintes (17)	Ugni blanc	Dec. 1996
PC7	F2-A	Le Chay (17)	Ugni blanc	Dec. 1996
PC12	F2-A	Pouillac (17)	Ugni blanc	Dec. 1996
AQ15	F2-B	Montbazillac (24)	Sauvignon	Sept. 1996
AQ18	F2-B	St Avit St Nazaire (24)	Sauvignon	Sept. 1996
AQ19	F2-B	St Méard de Gurçon (24)	Sauvignon	Sept. 1996
AQ21	F2-B	Fougueyrolles (24)	Cabernet-Sauvignon	Sept. 1996
AQ4	F2-B	Léognan (33)	Cabernet-Sauvignon	Oct. 1996
AQ5	F2-B	St Emilion (33)	Cabernet-Franc	Sept. 1996
AQ6	F2-B	St Emilion (33)	Merlot	Sept. 1996
AQ7	F2-B	Grézillac (33)	Sauvignon	Sept. 1996
AQ8	F2-B	Tauriac (33)	Cabernet-Sauvignon	Sept. 1996
AQ9	F2-B	Labarde (33)	Cabernet-Sauvignon	Sept. 1996
AQ10	F2-B	Pessac (33)	Cabernet-Sauvignon	Sept. 1996
AQ12	F2-B	Bouqueyran (33)	Cabernet-Sauvignon	Sept. 1996
AQ13	F2-B	Bouqueyran (33)	Cabernet-Franc	Sept. 1996
AQ14	F2-B	St Julien Beychevelle (33)	Cabernet-Sauvignon	Sept. 1996
AQ23	F2-B	St Christophe des Bardes (33)	Cabernet-Sauvignon	Sept. 1996
AQ33 to AQ37	F2-B	Naujan et Postiac (33)	Cabernet-Sauvignon	Sept. 1996
AQ27	F2-B	La Bastide d'Armagnac (44)	Baco 22A	Oct. 1996
AQ29	F2-B	Arthez d'Armagnac (44)	Folle Blanche	Oct. 1996
AQ28	F2-B	Aydie (64)	Tannat	Oct. 1996
AQ30	F2-B	Moncaup (64)	Cabernet-Franc	Oct. 1996
AQ31	F2-B	Monein (64)	Gros Manseng	Oct. 1996
AQ32	F2-B	Jurançon (64)	Gros Manseng	Oct. 1996
AQ43	F2-B	Moncaup (64)	Cabernet-Franc	Oct. 1996
MP1	F2-B	Soublecause (65)	Cabernet-Franc	Oct. 1996
VE3	IT-A	Lazise, Custoza	Corvina	May 1996
VE6	IT-A	Lazise, Custoza	Trebbiano Toscano	May 1996
VE7	IT-A	St Giorgio in Salici, Custoza	Trebbiano Toscano	May 1996
VE10	IT-A	St Giorgio in Salici, Custoza	Corvina	Mar. 1996
VE11	IT-A	St Giorgio in Salici, Custoza	Trebbiano di Lugana	Sept. 1995
VE12 andVE13	IT-A	St Giorgio in Salici, Custoza	Trebbiano di Lugana	Aug. 1995
VE17	IT-A	Lazise, Custoza	Corvina	May 1995
VE23 and VE31	IT-A	St Benedetto di Lugana, Lugana	Trebbiano di Lugana	May 1996
VE26	IT-A	St Benedetto di Lugana, Lugana	Trebbiano di Lugana	Oct. 1996
EM1	IT-A	Imola, Emilia	Sangiovese	Jun. 1995
LO1	IT-A	Broni, Oltrepo Pavese	Barbera	Oct. 1995
TO1	IT-B	St Andrea, Chianti	Sangiovese	Nov. 1996
TO3	IT-B	St Andrea, Chianti	Sangiovese	Oct. 1996
TO4	IT-B	Villa Branca, Chianti	Sangiovese	Oct. 1996
TO5	IT-B	Villa Branca, Chianti	Sangiovese	Oct. 1996
TO6	IT-B	St Andrea, Chianti	Sangiovese	Oct. 1996
TO8	IT-B	St Cristina, Chianti	Sangiovese	Oct. 1996
TO9	IT-B	St Cristina, Chianti	Sangiovese	Oct. 1996
TO11	IT-B	St Teresa, Chianti	Sangiovese	Sept. 1996
TO17, TO19 and TO21 to TO28	IT-B	Valledoro, Chianti	Sangiovese	Mar. 1997

[a] Isolate code includes the geographical origin (LR, Languedoc-Roussillon; PC, Poitou-Charentes; AQ, Aquitaine; MP, Midi-Pyrénées; VE, Venetia; EM, Emilia Romagna; TO, Tuscany) and the order of isolation for each region.

[b] Numbers in parentheses indicate official French district codes, as follows: 11, Aude; 16, Charente; 17, Charente-Maritime; 24, Dordogne; 30, Gard; 33, Gironde; 34, Hérault; 44, Landes; 64, Pyrénées-Atlantiques; 65, Hautes-Pyrénées and 66, Pyrénées-Orientales.

gies, Illkirch, France). Fragments were detected by staining with ethidium bromide and gels were photographed under UV light. A total of 120 decamer primers were initially screened using one DNA sample to identify primers that gave scorable patterns. Twenty primers were then evaluated to find polymorphism among 18 isolates from Languedoc-Roussillon (region F1). Finally, 9 primers that produced strongly amplified, polymorphic and reproducible bands were selected for full analysis. These primers were A08 (5'GTGACGTAGG), A11 (5'CAATCGCCGT), B18 (5'CCACAGCAGT), E11 (5'GAGTCTCAGG), E15 (5'ACGCACAACC), E16 (5'GGTGACTGTG), E18 (5'GGACTGCAGA), P15 (5'GGAAGCCAAC) and P17 (5'TGACCCGCCT). Analyses were repeated twice from extraction to electrophoresis of amplification products. A negative control reaction with no DNA template and a positive control reaction were included in each run.

Data analysis: To analyze RAPD patterns, it is commonly assumed that each band represents the phenotype at a single *locus* with two alleles. Without a segregation analysis to validate this hypothesis for each marker, allele frequencies cannot be estimated with precision. For instance, if the absence of the band is due to several genetic events, the diversity will be underestimated. The dominant nature of RAPDs introduces another bias since the marker is present both in homozygotes and heterozygotes of the positive allele. According to Fischer (1996), hyphal segments of *F. punctata* contain 2-8 nuclei that were presumed to be diploid. Therefore, if *F. punctata* is heterothallic, the dominance of RAPDs would also lead to a false estimation of the gene diversity. Another important bias concerns the analysis of the differentiation between populations. Studies in conifers have shown that estimates of population differentiation derived from dominant RAPD fingerprints were inflated compared to those obtained from haploid tissues (Isabel *et al*. 1995; Szmidt *et al*. 1996; Isabel *et al*. 1999). Although statistical methods have been implemented to reduce the bias (Lynch and Milligan 1994), we analyzed data in terms of differences in RAPD patterns without attempting to estimate allele frequencies.

Only polymorphic bands were considered and scored as 1 present, or 0 absent. To describe the diversity in populations, we first calculated the number of marker differences (d_i) for each pair of isolates within the population i using a program written in C++ language. The values were averaged over all comparisons to obtain D_i. For each RAPD marker and each population, the haplotypic diversity (Nei and Tajima 1981) corrected for small population sizes was also calculated as $h_i=N_i(1-(p_i^2+q_i^2))/(N_i-1)$ where, for the population i, N_i was the number of isolates, p_i the frequency of isolates showing the band and q_i the frequency of isolates not showing the band. The values were averaged over all markers to represent the diversity (H_i) within each population. *H* corresponded to the probability of observing a difference in markers between two isolates taken at random. This quantity might thus also be obtained by dividing the mean number of differences (D_i) by the number of markers. The relationships among isolates from each population were studied by cluster analyses applying the unweighed pair group method with arithmetic averages (UPGMA) based on pair-wise differences. For this purpose, we used the procedures Neighbour of PHYLIP Version 3.5c (J. Felsenstein, Department of Genetics, University of Washington).

The hypothesis that recombination might occur in each population was tested using the method developed by Brown *et al*. (1980). In the original method, the observed variance in the number of *loci* at which two individuals from a population of *N* individuals have different alleles was calculated over $N(N-1)/2$ pairs and compared to the variance expected under the assumption of random association. Our markers were not genetically characterized, but we applied the same reasoning to the presence/absence of markers. In each population, the observed variance in the number of marker differences (V_D) was compared to the variance expected (V_E) for random association of the markers. The null hypothesis H_0: $V_D=V_E$ was tested by simulation using the Monte Carlo procedure (Souza *et al*. 1992). Variances, critical value for V_D and the probability of rejecting by chance only the null hypothesis were obtained using LIAN (Haubold and Hudson 2000).

To study the genetic differentiation, we compared the observed numbers of isolates showing or not each RAPD marker with the numbers expected under the null hypothesis that no difference occurred between populations. An unbiased estimate of the P-value of a log-likelihood ratio (G^2)-based exact test was performed for each marker and combined over markers using Fisher's method. These calculations were carried out using GENEPOP Version 3.2 (Raymond and Rousset 1995).

The analysis of molecular variance (AMOVA) developed by Excoffier *et al*. (1992) was implemented to study the genetic differentiation between the 6 populations in more detail. The analysis was performed using ARLEQUIN Version 2.000 (Schneider *et al*. 2000) based on the number of differences between all pairs of RAPD phenotypes. The total variation for these differences was partitioned in hierarchical components (among regions IT, F1 and F2, among populations areas A and B within regions, and within populations). ARLEQUIN also gave the pair-wise distance between populations (ϕ_{st}) and computed by permutation procedures the significance levels for variance component estimates and for ϕ_{st}.

Results

From the multiple-banding patterns produced with the 9 primers, we selected a total of 34 reproducible and clearly scorable bands that were polymorphic across the whole sample (Tab. 2). These markers allowed us to distinguish each isolate as a distinct RAPD phenotype, the number of marker differences between isolates ranging from 1 to 22 over all populations. The diversity appeared similar within the 6 populations; the haplotypic diversity ranged from 0.292 to 0.322 whereas the mean number of marker differences ranged from 9.9 to 11.2 (Tab. 3). The pattern of clustering of isolates to be expected if geographical proximity structured each population was not observed. In particular, isolates derived from the same vineyard were not grouped in the cluster analysis performed for each population (example in Fig. 2 for F2-A

Table 2

Frequency of 34 Random Amplified Polymorphic DNA (RAPD) markers in 6 populations of *Fomitiporia punctata*. For details: Tab. 1

	Population							
RAPD	F1-A *N*=46	F1-B *N*=63	F2-A *N*=23	F2-B *N*=28	IT-A *N*=13	IT-B *N*=19	All populations[a] *N*=192	Pr[b]
A8-1910	0.565	0.460	0.391	0.357	0.384	0.473	0.458	0.557
A8-1370	0.717	0.825	0.696	0.536	0.615	0.895	0.734	0.032*
A8-870	0.348	0.175	0.261	0.357	0.154	0.211	0.255	0.273
A8-670	0.217	0.349	0.261	0.321	0.462	0.263	0.302	0.552
A11-2300	0.152	0.222	0.087	0.143	0.154	0.105	0.161	0.689
A11-2130	0.348	0.270	0.217	0.214	0.385	0.211	0.276	0.680
A11-1110	0.326	0.270	0.087	0.107	0.385	0.316	0.250	0.063
A11-910	0.848	0.794	0.783	0.964	0.077	0.947	0.844	0.212
A11-740	0.652	0.714	0.870	0.679	0.769	0.421	0.688	0.056
B18-1230	0.152	0.111	0.087	0.107	0.154	0.158	0.125	0.959
B18-470	0.696	0.698	0.913	0.964	0.462	0.473	0.724	0.001*
E11-1030	0.261	0.079	0.348	0.250	0.154	0.105	0.188	0.039*
E15-1270	0.217	0.317	0.348	0.393	0.385	0.263	0.307	0.630
E15-930	0.000	0.016	0.043	0.038	0.077	0.053	0.052	0.288
E15-730	0.022	0.127	0.174	0.071	0.000	0.053	0.083	0.159
E15-560	0.565	0.556	0.391	0.536	0.615	0.421	0.526	0.627
E15-330	0.804	0.825	0.739	0.750	1.000	0.842	0.813	0.465
E16-1580	0.761	0.667	0.435	0.643	0.692	0.632	0.656	0.185
E16-1060	0.043	0.063	0.130	0.036	0.231	0.053	0.073	0.452
E16-840	0.609	0.778	0.696	0.750	0.692	0.684	0.708	0.564
E18-1780	0.196	0.222	0.087	0.143	0.538	0.263	0.214	0.096
E18-1470	0.717	0.746	0.739	0.536	0.615	0.421	0.667	0.061
E18-670	0.239	0.127	0.174	0.214	0.154	0.053	0.167	0.440
E18-590	0.065	0.095	0.043	0.107	0.077	0.053	0.078	0.946
E18-490	0.087	0.159	0.087	0.143	0.077	0.105	0.120	0.863
E18-410	0.848	0.730	1.000	0.964	1.000	1.000	0.869	0.001*
P15-1590	0.109	0.095	0.087	0.036	0.000	0.105	0.083	0.663
P15-1160	0.826	0.746	0.783	0.821	0.846	0.684	0.781	0.772
P15-670	0.174	0.222	0.304	0.321	0.154	0.211	0.229	0.675
P15-460	0.174	0.095	0.174	0.036	0.154	0.263	0.135	0.262
P15-290	0.239	0.317	0.130	0.214	0.308	0.263	0.255	0.593
P17-1590	0.217	0.175	0.174	0.143	0.077	0.105	0.167	0.804
P17-1160	0.087	0.127	0.130	0.071	0.154	0.052	0.104	0.871
P17-970	0.152	0.127	0.261	0.107	0.154	0.158	0.151	0.791

[a] Mean allele frequencies for each RAPD locus calculated for the total sample.

[b] Probability to observe the likelihood ratio (G^2) under the null hypothesis that frequency did not differ among populations, asterisk shows probability below the 0.05 level of significance.

population). The null hypothesis that markers were randomly associated was tested in each population and for the total population. A significant value that indicated deviation from random association was detected only in the F2-B population (Tab. 3).

The majority of markers were detected in all the populations and their frequency varied from 0.052 to 0.869 over all populations (Tab. 2). The null hypothesis that the number of isolates having or not having the marker was the same in each population was rejected for only 4 markers indicating a low level of genetic differentiation. The P value obtained with Fisher's method over all markers was significant (P=0.0088). AMOVA analysis clearly confirmed that most of the genetic variation was present within populations (Tab. 4). A significant level of genetic differentiation was detected between the three regions, but there was no difference between the two populations within each region (Tab. 4). Pair-wise ϕ_{st} revealed that this differentiation was due to some differences between populations sampled in western France and those taken in Mediterranean areas (Tab. 5).

Discussion

Our study revealed that *F. punctata* had a high degree of genetic variation. All isolates within populations were

Table 3

Haplotypic diversity, number of marker differences between pairs of isolates and marker association for 34 Random Amplified Polymorphic DNA (RAPD) markers in 6 populations of *Fomitiporia punctata*. For details: Tab. 1

Population	*N*	H_i[a]	Number of differences[b] D_i	Range	Marker association[c] V_D	V_E	L_{MC}	*P*
F1-A	48	0.317	10.8	4-20	7.48	6.78	7.93	0.148
F1-B	63	0.315	10.9	4-20	6.40	7.00	8.01	0.828
F2-A	23	0.304	10.4	4-20	7.59	6.57	8.47	0.175
F2-B	28	0.292	9.9	3-18	7.98	6.19	7.78	0.032
IT-A	13	0.322	11.2	4-19	8.28	6.50	9.27	0.117
IT-B	19	0.304	10.3	5-16	5.56	6.36	8.33	0.779
All	192	0.315	10.7	1-22	7.24	6.93	7.53	0.194

[a] Haplotypic diversity.
[b] The number of differences between pairs of isolates (out of 34 markers) was averaged (D_i) in each population and was also calculated for the total population.
[c] V_D = observed variance in number of differences; V_E = expected variance assuming random association of markers; L_{MC} = simulated 5 % critical value for V_D (obtained using a Monte Carlo procedure with 1000 resamplings); *P* = probability of rejecting by chance alone the null hypothesis that $V_D = V_E$.

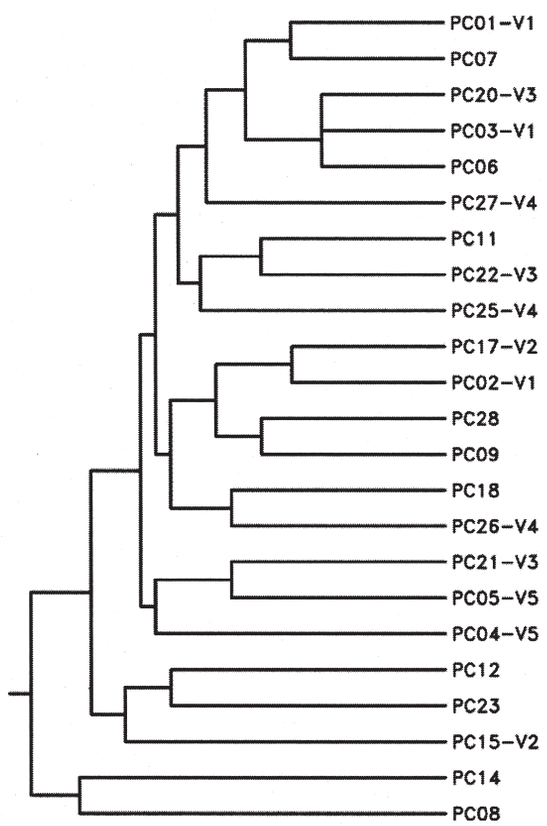

Fig. 2: Relationships among isolates from a *Fomitiporia punctata* population sampled in south-western France (population F2-A) revealed by UPGMA cluster analysis of the matrix of pair-wise differences for presence of 34 RAPD markers. In 10 vineyards only one isolate was taken whereas in 5 other vineyards (V1 to V5), 2-3 isolates were collected from different vines.

Table 4

Analysis of molecular variance (AMOVA) of Random Amplified Polymorphic DNA (RAPD) phenotypes for 6 populations of *Fomitiporia punctata*. For details: Tabs 1 and 2

Source of variation	df	Variance component	% total	*Pr*
Among regions	2	0.084	1.56	0.000
Among populations within regions	3	-0.012	-0.04	0.462
Within populations	186	5.307	98.48	0.002

Table 5

Pair-wise ϕ_{st} between 6 populations of *Fomitiporia punctata*. For details: Tab. 1

Population	F1-A	F1-B	F2-A	F2-B	IT-A
F1-B	0.0046	-			
F2-A	0.0155*	0.0173*	-		
F2-B	0.0116	0.0179**	-0.0073	-	
IT-A	0.0017	-0.0017	0.0301	0.0248	-
IT-B	0.0064	0.0095	0.0356*	0.0284**	-0.0059

*, **: significant at P=0.05, P=0.01

distinguished and no RAPD phenotype was shared between populations. The same high level of diversity was observed within a given vineyard, thus confirming at this spatial scale the findings of Cortesi *et al*. (2000) and Pollastro *et al*. (2000 a). Furthermore, independent markers appeared to be randomly assorted. This suggests that spreading of *F. punctata* is achieved by airborne basidiospores within outcrossing populations. A low level of fine-scale genetic

diversity would indeed be expected if the fungus was propagated either by mycelium or by basidiospores produced by a homothallic mating system.

In contrast to the evidence we obtained for a high level of recombination, *F. punctata* has been described as a homothallic species after the pairing of mycelia from single spores from the same basidiocarp (FISCHER 1996). Six basidiocarps were analyzed that were not collected from grapevine; two were from *Salix hindsinia* in the United States, two from *Salix caprea* and *Salix fragilis* in Germany, one from *Corylus avellana* in Germany, and one from *Sorbus aucuparia* in Estonia (FISCHER, pers. comm.). The possibility that the mating system in *F. punctata* populations differs depending on the geographical origin or on the origin of the host could explain the conflicting information on the mating system. Such a phenomenon is not uncommon in wood-decaying basidiomycetes where the mating system may differ in various subpopulations. For instance, species of the genus *Stereum* contain both outcrossing and non-outcrossing subpopulations (RAYNER and BODDY 1988). The sexual system of *F. punctata* could be investigated by analyzing the segregation of molecular markers within natural progenies from different locations and hosts. However, a limiting factor for this approach is the poor germination of basidiospores in the laboratory (FISCHER, pers. comm.).

In outcrossing basidiomycetes, a germinating basidiospore produces a homokaryotic, haploid mycelium that grows until another sexually compatible mycelium is encountered to form a heterokaryotic mycelium. Infection of grapevine by *F. punctata* may be initiated by basidiospores with fusion of different homokaryotic mycelia taking place either externally or within the wood. It is generally assumed that homokaryons of *basidiomycetes* have limited development in the wood substrate. However, homokaryons of *Phellinus weirii* performed as well as heterokaryons in degrading inoculated wood strips although not all homokaryons developed successfully (HANSEN 1979). Since *F. punctata* enters through pruning wounds (VIALA 1926), basidiospore infections are expected to take place at scattered positions in the same vine. Using pairing tests, CORTESI *et al*. (2000) obtained some evidence that two different mycelia could be recovered from the same vine but more frequently only one somatic incompatibility type was found. The infrequent occurrence of basidiocarps in vineyards (FISCHER 2000; CORTESI *et al*. 2000) may be partly explained by the rare contact between sexually compatible homokaryons. It would be of great interest to analyze in more detail the composition of *F. punctata* mycelia established within the same vine and to determine how long the homokaryotic condition lasts. However, in hymenochaetales, the order to which *Phellinus* and *Fomitiporia* species belong, it was difficult to distinguish between homokaryons and heterokaryons because both can have oligonucleate cells, and heterokaryons lack clamp connections (HENNON and HANSEN 1987; RIZZO *et al*. 1995). Thus, molecular markers may provide a convenient way to compare the different mycelia of *F. punctata* recovered within and among vines in relation with the presence or absence of basidiocarps.

The low level of genetic differentiation among the populations suggests that *F. punctata* basidiospores can spread over large distances. In other wood-inhabiting, vast numbers of spores were shown to have been transported by air and spores were detected as far as 320 km from extensive sources (RISHBETH 1959). Gene flow may also be due to transport of infected material or to insects carrying spores and mycelial fragments. Only a few immigrants are sufficient to prevent the genetic differentiation of populations (SLATKIN 1987). Another possible explanation for the low level of genetic differentiation is that the populations recently derived from the same population and had no time to diverge by mutation or genetic drift. The low level of differentiation appears to be due to differences between the Mediterranean populations and those sampled in western France. This is surprising since the Alps and the Mediterranean Sea may constitute abrupt geographic barriers to the transport of basidiospores, whereas there is no such barrier between the two French regions. Although RAPD markers are supposed to be neutral, hitch-hiking selection of a few markers might cause some differentiation. Selection may occur, for instance, because of the marked difference between the oceanic climate (western France) and the Mediterranean climate (southern France and Italy).

Isolates of *F. punctata* analyzed in this study originated all from a few European vineyards whereas the fungus is present worldwide (DUBOS and LARIGNON 1988) on several other hosts (WALLA 1984; RYVARDEN and GILBERTSON 1994; IPPOLITO *et al*. 1998). It is therefore possible that subdivision in *F. punctata* occurs at another geographical scale or through a host effect. This possibility is supported by two observations, first, as discussed above, the existence of both outcrossing and non-outcrossing populations would explain the conflicting observations regarding the mating system, and, second, the RFLP (Restriction Fragment Length Polymorphism) analysis of an amplified ITS (Internal Transcribed Spacers) DNA region separated 12 isolates in three different groups: isolates from grapevine in Italy, isolates from *Salix hindsiana* and *Salix lucida* in the United States and isolates from *Salix caprea*, *Rhamnus cathartica* and *Sorbus aucuparia* in northern Europe (FISCHER 2000). Additional locations and other hosts therefore need to be sampled to determine 1) whether the same, highly diverse population of *F. punctata* is widely present in vineyards and 2) whether or not alternative hosts contribute to the epidemiology of Esca in grapevine. The markers identified in our study could help to address these questions.

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