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Genetic structure and variation in aggressiveness in European and Australian populations of the grapevine dieback fungus, *Eutypa lata*

Jean-Pierre Péros and Gilles Berger
Unité Mixte de Recherche DGPC, Equipe vigne, Institut National de la Recherche Agronomique, Centre de Recherches de Montpellier, 2 place Viala, 34060 Montpellier Cedex 1, France (E-mail:peros@ensam.inra.fr)

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Abstract

Eutypa lata is an ascomycete fungus causing a severe dieback in grapevine. The genetic structure of populations of *E. lata* from seven regions in Australia, France, Italy and Spain was examined using 20 random amplified polymorphic DNA (RAPD) markers. In some regions, populations were subdivided and a total of 14 samples were analysed. A total of 231 RAPD haplotypes were found among the 240 isolates. Vegetative compatibility testing further demonstrated that isolates of the same haplotype were genetically distinct. Gene diversity was the highest in the population from northern Italy and lowest in the Alsace region in France. Linkage disequilibrium between pairs of putative loci was very low and most of the multilocus analyses were consistent with the hypothesis of random association of the loci. This suggests that random mating occurred in every population and that the sexual stage shapes the genetic structure of *E. lata* populations in the regions sampled. Only 6% of the total variability was attributable to differences between populations. Nevertheless, significant differences in allele frequency appeared with respect to six RAPD markers indicating some genetic differentiation between populations. This differentiation appeared attributable to differences between the Italian and Spanish populations and the other populations. We thus hypothesize that a restriction of gene flow exists within Europe. The population from Australia was genetically closer to the French and Spanish populations than to that from Italy. Genetic diversity is associated with considerable variation in aggressiveness, which was assessed on cuttings in the greenhouse in six populations. All populations included a range of isolates differing in aggressiveness, but the Italian population seemed to have more isolates with low aggressiveness.

Introduction

The ascomycete *Eutypa lata* (Pers : Fr.) Tul. & C. Tul. (anamorph *Libertella blepharis* A.L. Smith) is associated with many species of woody plants throughout the temperate regions of both hemispheres (Carter et al., 1983). As infections occur primarily through wounds (English and Davis, 1978), the fungus is favoured by the pruning of its potential hosts. Carter (1991) put forward two possibilities to explain the current status of the fungus as a plant pathogen. In the first, *E. lata* acquired its pathogenic ability in grapevine many centuries ago in Asia Minor, the region believed to be the place of origin of *Vitis vinifera*. The geographical distribution of the pathogen subsequently is thought to have expanded along with its range of potential hosts. As an alternative possibility, Carter suggested that *E. lata* might be present as a weak pathogen on different hosts worldwide, and only recently started to induce pandemics in grapevine and apricot, which are both hosts of economic importance. In grapevine, the pathogen induces a dieback that seriously impairs the longevity of vineyards and also causes significant yield losses (Dubos, 1994; Munkvold et al., 1994).

Random amplified polymorphic DNA (RAPD) markers and vegetative compatibility were used to describe the genetic diversity within *E. lata* populations sampled in France (Péros et al., 1997; Péros and

Larignon, 1998; Péros et al., 1999). The maximum level of genotypic diversity and lack of linkage disequilibrium between RAPD markers indicated that recombination occurs frequently in *E. lata*. Analyses of natural progenies further demonstrated heterothallism in this ascomycete haploid fungus (Péros and Berger, 1999). Differences in aggressiveness were also observed in a population from a single vineyard (Péros et al., 1997), as well as among single-spore isolates from the same perithecial stroma (Péros and Berger 1999). The population structure in France appeared similar to that observed in other European countries, since a very high level of genotypic diversity was found in two populations sampled in single vineyards in Italy and Germany using vegetative incompatibility testing (Cortesi and Milgroom, 2001). Outside Europe, the only available data were from California, USA, where DeScenzo et al. (1999) identified 95 *E. lata* isolates from grapevine as different haplotypes using amplified fragment length polymorphism (AFLP).

Before concluding that *E. lata* uses sexual reproduction worldwide, analysis of samples from other areas was required since only a few regions have been investigated up to now, and also because the relative importance of sexual and asexual reproduction in fungal pathogens may vary with the geographic location (Leung et al., 1993; Milgroom and Fry, 1997). In particular, a more thorough investigation appeared to be necessary in European vineyards where variation exists for climate, cultivars and cultivation practices. Furthermore, the extent to which populations are subdivided, an important aspect of population structure analysis (Brown, 1996), is poorly documented in *E. lata*. A preliminary comparison of allele frequencies at RAPD loci suggested that two populations, sampled in single French vineyards located at a distance of 390 km, were not genetically differentiated (Péros and Larignon, 1998). In natural populations, lack of genetic differentiation can be explained by high levels of current gene flow, and-or by gene flow that occurred in the past (Slatkin, 1987).

The objectives of this study were to analyse the genetic structure of *E. lata* populations sampled in different vine-growing regions in Europe and Australia and to study the genetic differentiation between them. RAPD markers were used to perform multilocus analysis of each population and RAPD allele frequencies were compared between populations. In addition, some populations were assessed for aggressiveness to describe the population diversity with respect to this crucial phenotypic trait.

Materials and methods

Sampling

The fungus was sampled from seven vine-growing regions, four in France, one in Spain and Italy (Figure 1), and one in Australia. In Europe, the grape cultivars from which isolates originated were very diverse with major differences between regions (Table 1). In South Australia, sampling was performed in the Clare and Barossa Valleys and in Adelaide and surroundings; Four isolates came from other hosts than grapevine (Table 1). In Spain, isolates were sampled in two areas in the Catalogne region separated by a distance of approximately 30 km. In northern Italy, most of the isolates were obtained from around lake Garde and others were from Tuscany. In some regions, it was possible to obtain larger numbers of isolates than in others. We thus performed sub-sampling to maximize the geographic diversity within regions. In these cases, each of the samples included isolates from the entire

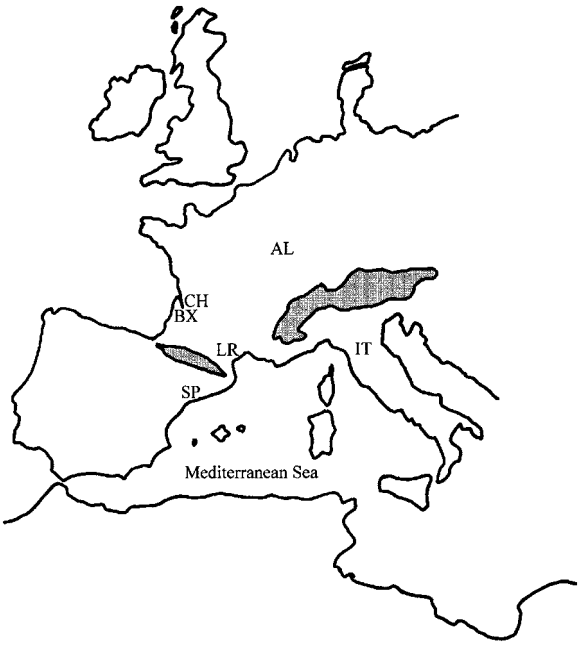

Figure 1. Map of *E. lata* populations sampled in Europe. The shaded areas correspond to mountains above 1000 m.

Table 1. Characteristics of *E. lata* populations sampled for this study

Population[a]	*N* (*S*)[b]	Country, region	Year	Host (number of isolates)
AUS	18	Australia, South Australia	[c]	Unknown grape varieties (13), apricot (2), viburnum (1), hawthorn (1)
LR	54 (3)	France, Languedoc-Roussillon	1996	Cinsault (19), Grenache (10), Carignan (5), Macabeu (4), Cabernet-Sauvignon (3), Muscat (2), Mauzac (2), Mourvèdre (2), other grape varieties (9)
AL	53 (3)	France, Alsace	1997	Pinot (15), Gewurztraminer (12), Chasselas (9), Sylvaner (5), Auxerrois (6), Riesling (5), Muscat (1)
BX[d]	16	France, Bordelais	1996	Cabernet-Sauvignon (all)
CH	36 (2)	France, Charentes	1996	Ugni blanc (all)
SP	36 (2)	Spain, Catalogne	1999	Sauvignon (13) Cabernet-Sauvignon (10) Chenin (6) Gewurztraminer (7)
IT	27 (2)	Italy, Lombardy and Toscana	1995–96	Ugni blanc (7), Trebbiano di Luguana (6), Sangiovese (6), Merlot (3), other grape varieties (5)

[a]French isolates were obtained by the authors with the help of I. Jamaux-Despréaux for the (Region Languedoc-Roussillon) populations and P. Larignon for the BX (region Bordelais) and CH (region Charentes) populations, G. Blaszczyk and G. Cloquemin provided infected vines from the Alsace region (AL); the authors performed single-ascospore isolation from perithecial stromata provided by M.V. Carter from Australia (AUS); isolates from Italy (IT) were obtained by M. Minervini; Spanish isolates (SP) were obtained by the authors from infected vines collected in vineyards belonging to Torres SA.
[b]Total number of isolates *N* and the number of samples *S* within population when subdivided. The size of samples is given in Table 3.
[c]Isolated from grapevine in 1997 (11) and 1992 (2), apricot in 1991 and unknown year, viburnum in 1984, and hawthorn in 1991.
[d]Including the isolate BX1-10, which served as a standard in RAPD analyses and pathogenicity tests.

region. A total of 14 samples was compared (Table 1), most of them comprised of 18 isolates. This strategy enabled us to obtain population genetic statistics from samples having similar numbers of isolates. In addition, the reproducibility of the results can be evaluated as several statistical values were obtained within and between certain regions.

Methods of isolation

Each isolate was sampled from an individual plant. Isolates were either mass-hyphal isolates obtained from wood lesions or single-ascospore isolates obtained from a perithecial stroma. In the case of mass-hyphal isolates, vines showing dieback symptoms were collected. The external symptoms were those that characterize Eutypa dieback, i.e., short stems with small, necrotic leaves, except for the vines from Languedoc–Roussillon (population LR) and Charente (population CH) and some vines from Bordelais (population BX). In these cases, the vines showed either the mild or acute form of the esca syndrome, which is another important dieback of grapevine (Dubos and Larignon, 1988). *E. lata* is considered to be a pioneer fungus in esca (Mugnai et al., 1996; Larignon and Dubos, 1997). Vines were transversely cut and isolation of *E. lata* was performed at the margin of brown, sectorial wood lesions according to Péros and Berger (1994). Small pieces of wood were taken at the margin of the lesion, disinfected in calcium hypochlorite, rinsed and placed onto potato-dextrose agar (PDA, Difco Laboratories, Detroit, USA) plates. All isolates from Australia (population AUS) and some from population BX originated from single ascospores. This method of isolation was chosen to facilitate the sampling of the Australian population and because it was not always possible to perform destructive sampling in the Bordelais region. An ascospore suspension was prepared from a few perithecia in water and was plated onto PDA plates. After 24 h at 25 ± 2 °C, individual germinated spores were removed under a dissecting microscope (120× magnification) and transferred to PDA plates. All isolates were stored as culture plugs (5 mm diameter) in distilled water at 4 °C.

RAPD analysis

DNA extraction was performed on freeze-dried mycelia using a CTAB method (Péros et al., 1996). The polymerase chain reaction with random 10-mer primers was undertaken according to Williams et al.

(1990) with some modifications. Amplification reactions (25 µl final volume) consisted of one unit of Taq polymerase (Appligene, Illkirsch, France), the buffer purchased with the enzyme, 120 µM of each dNTP, 30 ng of primer, and 25–50 ng of template DNA. Amplifications were carried out in a Trio-Thermobloc thermocycler (Biometra, Göttingen, Germany). Each run included a reaction without DNA (negative control) and a reaction with DNA from the standard isolate BX1-10. This single-ascospore isolate was obtained from a perithecial stroma collected near Bordeaux, France, in 1990. Amplification products (8 µl) were analysed by electrophoresis on 1.6% agarose gels and, visualized by UV fluorescence following ethidium bromide staining. All isolates from a given sample were compared on the same gel along with the standard isolate, the negative control and a molecular size marker (1 kb ladder, Invitrogen, Lyon, France).

The five RAPD primers A15, C13, E15, P06 and P14 (Invitrogen, Lyon, France) were selected from previous studies (Péros et al., 1996; Péros et al., 1997; Péros and Berger, 1999). They produced clear, multiple-banded patterns from which a total of 20 reproducible and polymorphic fragments (Table 2) were scored for presence or absence. These markers had a Mendelian segregation in natural progenies (Péros and Berger, 1999). Each marker was considered to reflect a putative locus with two alleles. In the following, markers are named by the primer code followed by the size of the polymorphic amplicon. Estimates of the gene diversity within a given sample (H_S) and of the total gene diversity (H_T) were calculated over all putative loci using the formulas given by Nei and Chesser (1983).

Two methods were used to test for random mating. First, the probability associated with the coefficient of linkage disequilibrium for each pair of putative loci was estimated by using Fisher's exact test and the Markov chain method as implemented in Genepop (Raymond and Rousset, 1995) at default settings. Two putative loci were considered in linkage disequilibrium when the associated probability was ≤ 0.05. Second, the method described by Brown et al. (1980) in a study of population structure in the wild diploid barley, *Hordeum spontaneum*, was used. The observed variance (V_D) in the number of loci at which two individuals from a sample of N individuals have different alleles was calculated over $N(N\text{-l})\text{-}2$ pairs and compared to the variance expected (V_E) under the assumption of random association. The null hypothesis H_0: $V_D = V_E$ was tested by simulation using a Monte Carlo procedure (Souza et al., 1992). Variances, the critical value for V_D and the probability of rejecting the null hypothesis by chance alone were obtained using LIAN (Haubold and Hudson, 2000).

Table 2. Sequence of the RAPD primers and sizes of selected markers used in this study

Primer	Sequence (5′–3′)	Marker size in base pairs
A15	TTCCGAACCC	1440, 1170, 1060, 910
C13	AAGCCTCGTC	1980, 1100, 630, 490, 430
E15	ACGCACAACC	1040, 990, 540, 270
P06	GTGGGCTGAC	1460, 1290, 1230, 1050, 450
P14	CCAGCCGAAC	1700, 1140

To determine whether or not allele frequencies differed significantly between samples, an unbiased estimate of the probability associated with the likelihood ratio chi-square (G^2) was calculated with exact tests by using the Markov chain method in Genepop at default settings. The differentiation across all loci was also tested for all sample pairs using Fisher's test in Genepop at default settings. In addition, a global G_{ST} was determined as $G_{ST} = 1 - H_S/H_T$ (Nei, 1973). To explore the genetic relatedness among populations, Nei's unbiased genetic distance (Nei, 1987) was calculated between all pairs of samples using Popgene (Version 1.31, Yeh FC, Yang R and Boyle T, University of Alberta and Centre for International Forestry Research, Canada). Significance testing for genetic differentiation between sample pairs was performed using an exact test provided by Genepop at default settings.

Vegetative compatibility testing

Isolates with the same RAPD patterns were paired onto PDA plates to determine if they were vegetatively compatible. Plugs of the stored culture of each isolate were directly placed 1 cm apart in the center of PDA plates (9 cm diameter). After a 2–4 wk incubation period at $25 \pm 2^\circ$ C, the isolates that produced a barrage zone were considered as vegetatively incompatible (Péros et al., 1997).

Assessment of aggressiveness

The variation in aggressiveness was assessed in six populations: AUS, LR (sample LR1), AL (sample AL1), BX, CH (sample CH1) and IT (sample IT1).

Inoculation of grapevine cuttings of *Vitis vinifera* cv. Cabernet-Sauvignon was performed as described in detail by Péros and Berger (1994). Briefly, a 5-mm-diameter plug was cut from the margin of an actively growing culture on PDA plates and placed mycelium-side down in a wound drilled 4 cm above the bud of one-bud cuttings prepared from one-year-old branches of Cabernet-Sauvignon vines. Plastic film was wrapped around the inoculation site. Each sample was tested independently. The standard isolate BX1-10 and a negative control, which consisted of non-colonized PDA plug applied to wounded cuttings, were included in each experiment. The number of cuttings showing abnormalities, i.e., no bud break, dead shoot, shoot with bad development, or the characteristic symptoms of Eutypa dieback, i.e., shoots with small necrotic leaves, was scored eight weeks after inoculation. Each experiment included three blocks of eight cuttings inoculated per isolate and was replicated three times in successive years in the spring of 1997–1999. Proportions of plants showing abnormalities were subjected to angular transformation prior to analysis of variance using Proc GLM in SAS (SAS Institute, Gary, USA). The mean percentages were separated using Dunnett's test with standard isolate BX1-10 as control.

Results

The RAPD patterns obtained for the standard isolate BX1-10 were constant over all runs and were identical to those obtained in previous studies (Péros et al., 1996, 1997; Péros and Berger, 1999). The analysis with 20 RAPD markers detected 231 haplotypes among the 240 isolates. One haplotype was observed in three isolates and seven haplotypes were detected in two isolates each. The pairing of isolates with the same RAPD patterns revealed only vegetative incompatibility. Thus, all isolates had distinct genotypes.

Gene diversity varied from 0.25 in the AL2 sample to 0.44 in the IT2 sample (Table 3). This variation appeared larger than the variation due to sampling since the estimates for different samples within a given population were similar. The percentage of significant tests for linkage disequilibrium between putative locus pairs was very low, ranging from 0% to 3.2% (Table 3). In addition, multilocus analysis showed that the observed variance was less than the upper confidence limit of the variance expected under the null hypothesis of no association (Table 3). Analyses performed on the seven regional populations or on all 14 samples led to the same conclusions with one exception. The analysis performed on the combined population of 54 isolates collected in LR (LR1, LR2 and LR3 were pooled) led to the rejection of the null hypothesis. This combined population had the largest observed variance in the number of differences in alleles. In addition, the null hypothesis was rejected for the global sample of 240 isolates (Table 3).

The gene diversity within a given sample averaged 0.34 and total gene diversity (H_T) was 0.36. The global G_{ST} value was therefore 0.06. This means that only 6% of the genetic variability occurred between samples compared with 94% within them. However, allele frequency varied significantly among the 14 samples with respect to six of the 20 RAPD markers (Table 4). Genetic differentiation was assessed for all pairs of samples combining data for all loci (Table 5). Estimates of Nei's unbiased distance among the 10 samples from France and Australia varied from 0.01 to 0.06 and none of the comparisons between these samples indicated significant differentiation. Higher values, ranging from 0.04 to 0.08, were obtained when these populations were compared to the Spanish population. Four of twenty comparisons showed significant differentiation at the 5% level. The highest values were observed when the 10 samples from France and Australia were compared to the two Italian samples, the genetic distance varying from 0.07 to 0.18. Seventeen out of the 20 comparisons showed a significant differentiation at the 5% level. Only the French samples from LR (LR1, LR2 and LR3) were not distinguished from the Italian sample IT2 (Table 5). Genetic distance between Spanish and Italian samples varied from 0.02 to 0.07 and no significant differentiation was evidenced. The analysis also demonstrated that differentiation between regional populations might appear either significant or not depending on the sample. For instance for the Alsace region, the AL1 sample evidenced differentiation with Italy but not with Spain, whereas the AL2 sample displayed differentiation with both Italian and Spanish populations.

In the aggressiveness tests, the percentage of abnormal cuttings in the control averaged 25% (Table 6). This means that approximately one quarter of the cuttings did not develop at all or displayed bad development. However, no control cuttings showed the symptoms characteristic of the disease, i.e., shoots with small, necrotic leaves. In contrast, the percentage of abnormal cuttings averaged 73% for the standard

Table 3. Genetic structure of seven populations of *E. lata* sampled in Australia (AUS), France (LR, CH, BX, AL), Italy (IT) and Spain (SP) based on data from 20 RAPD (RAPD) putative loci

Sample	No. haplotypes	H_S[a]	% LD[b]	Multilocus association[c]			
				V_D	V_E	L_{MC}	Pr
AUS ($N = 18$)	18	0.30	0.8	2.71	3.53	4.58	0.96
LR ($N = 54$)	54	0.36	3.2	5.00	4.34	4.87	0.02
LR1 ($N = 18$)		0.40	1.6	4.84	4.45	5.55	0.27
LR2 ($N = 18$)		0.36	2.1	5.35	4.18	5.29	0.05
LR3 ($N = 18$)		0.35	2.1	4.49	4.23	5.54	0.35
AL ($N = 53$)	52	0.28	0.5	3.35	3.62	4.17	0.80
AL1 ($N = 18$)		0.28	1.5	2.33	3.35	4.33	0.98
AL2 ($N = 17$)		0.26	0.0	2.44	3.29	4.51	0.92
AL3 ($N = 18$)		0.32	1.1	4.07	3.82	5.01	0.33
BX ($N = 16$)	16	0.32	0.0	3.91	3.88	5.35	0.48
CH ($N = 36$)	36	0.34	1.1	4.32	4.09	4.78	0.21
CH1 ($N = 18$)		0.34	0.5	4.30	4.08	5.28	0.34
CH2 ($N = 18$)		0.35	1.3	4.11	4.04	5.19	0.42
SP ($N = 36$)	36	0.35	0.5	4.28	4.16	4.91	0.38
SP1 ($N = 16$)		0.36	0.5	4.31	4.21	5.49	0.42
SP2 ($N = 20$)		0.33	0.5	3.71	4.00	5.01	0.69
IT ($N = 27$)	26	0.42	1.6	4.49	4.60	5.41	0.58
IT1 ($N = 15$)		0.41	1.8	4.05	4.47	5.72	0.75
IT2 ($N = 12$)		0.44	0.0	4.15	4.59	6.18	0.75
All ($N = 240$)	231	0.36	0.0	5.11	4.34	4.59	0.00

[a]Gene diversity calculated according to Nei and Chesser (1983) for small sample sizes.
[b]Linkage disequilibrium (LD) is given as the number of disequilibrium values significant at $P \leq 0.05$ over the total number of pairwise comparisons between polymorphic RAPD loci.
[c]V_D = observed variance in number of marker 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); Pr = probability of rejecting by chance alone the null hypothesis that $V_D = V_E$.

isolate BX1-10. Among these cuttings, 24% expressed characteristic symptoms (data not shown). Variation in aggressiveness was observed in each of the six populations tested (Table 6). In each case, the analysis of variance showed a highly significant effect of the isolate on the percentage of cuttings showing abnormalities. Some isolates were not distinguished from the standard isolate using the Dunnett test at the 5% level, others had similar percentages to the negative control. The characteristic symptoms were not observed in 26 isolates, each inoculated to a total of 72 cuttings. Most of the Italian isolates did not induce characteristic symptoms and only a few of them had the same level of aggressiveness as the standard isolate.

Discussion

The high level of genotypic diversity and the random association of independent putative loci suggest that random mating plays the main role in structuring *E. lata* populations from the regions sampled. The same conclusions were drawn from previous analyses performed in other French populations (Péros et al., 1997; Péros and Larignon, 1998; Péros et al., 1999) and in populations studied in other countries (DeScenzo et al., 1999; Cortesi and Milgroom, 2001). However, another scale of sampling is required to conclude that *E. lata* uses only ascospores for propagation. Sampling is biased against detection of clones when it is not performed on neighbouring vines in a vineyard. The possible role of

Table 4. Frequency of the positive allele at 20 putative RAPD loci in samples of *E. lata* populations from France (LR, AL, BX, CH), Italy (IT), Spain (SP) and Australia (AUS)

	Sample														
RAPD locus	AUS	LR1	LR2	LR3	AL1	AL2	AL3	BX	CH1	CH2	SP1	SP2	IT1	IT2	Pr[a]
A15-1440	0.28	0.39	0.28	0.39	0.44	0.41	0.44	0.25	0.28	0.39	0.25	0.25	0.40	0.42	0.96
A15-1170	0.00	0.06	0.06	0.06	0.00	0.06	0.06	0.13	0.06	0.00	0.05	0.13	0.31	0.42	0.02*
A15-1060	0.83	0.56	0.44	0.61	0.78	0.76	0.61	0.75	0.61	0.56	0.65	0.63	0.40	0.50	0.33
A15-910	0.17	0.39	0.39	0.22	0.28	0.29	0.17	0.19	0.17	0.33	0.20	0.13	0.27	0.33	0.80
C13-1980	0.22	0.28	0.11	0.11	0.17	0.18	0.28	0.06	0.11	0.17	0.25	0.06	0.33	0.50	0.31
C13-1100	0.00	0.06	0.06	0.11	0.11	0.12	0.11	0.19	0.22	0.11	0.25	0.19	0.20	0.08	0.57
C13-630	0.17	0.33	0.22	0.17	0.06	0.00	0.06	0.13	0.06	0.11	0.20	0.06	0.27	0.17	0.28
C13-490	0.00	0.17	0.11	0.11	0.11	0.00	0.11	0.00	0.11	0.00	0.10	0.19	0.40	0.17	0.03*
C13-430	0.44	0.50	0.39	0.33	0.50	0.41	0.50	0.38	0.33	0.50	0.80	0.63	0.67	0.58	0.24
E15-1040	0.78	0.56	0.56	0.50	0.56	0.71	0.50	0.69	0.50	0.61	0.55	0.38	0.47	0.42	0.67
E15-990	0.94	0.83	0.83	0.78	0.89	0.88	0.89	0.88	0.83	0.72	0.95	0.88	1.00	0.83	0.73
E15-540	0.50	0.44	0.44	0.61	0.67	0.82	0.78	0.63	0.44	0.67	0.50	0.69	0.53	0.50	0.36
E15-270	0.89	0.72	0.72	0.83	0.89	0.94	0.89	0.81	0.89	0.89	0.60	0.75	0.40	0.50	0.00**
P06-1460	0.39	0.56	0.33	0.22	0.22	0.12	0.06	0.13	0.33	0.22	0.40	0.19	0.33	0.33	0.09
P06-1290	0.33	0.39	0.17	0.17	0.33	0.06	0.22	0.31	0.17	0.17	0.40	0.31	0.60	0.50	0.07
P06-1230	0.00	0.33	0.22	0.17	0.06	0.12	0.17	0.25	0.44	0.33	0.05	0.00	0.27	0.25	0.00**
P06-1050	0.67	0.72	0.61	0.72	1.00	0.82	0.67	0.69	0.83	0.72	0.75	0.44	0.13	0.58	0.00**
P06-450	0.83	0.83	0.83	0.89	0.94	1.00	0.94	1.00	0.94	0.89	0.40	0.38	0.27	0.17	0.00**
P14-1700	0.33	0.22	0.06	0.28	0.22	0.29	0.44	0.38	0.33	0.33	0.15	0.19	0.20	0.25	0.48
P14-1140	0.22	0.11	0.22	0.17	0.00	0.12	0.17	0.13	0.22	0.28	0.15	0.06	0.07	0.08	0.57

[a]Unbiased estimate of the probability associated with the likelihood ratio chi-square at (G^2) under the null hypothesis of equal allele frequencies in populations.
*,**Indicates significant differences among populations at $\leq$0.05 and $\leq$0.01, respectively.

Table 5. Nei's unbiased genetic distance (under diagonal) and unbiased estimate of the probability for genetic differentiation (only those $\leq$0.10 are indicated above the diagonal with the corresponding distance in bold) for each pair of samples of *E. lata* populations from Australia (AUS), France (LR, AL, BX, CH), Spain (SP) and Italy (IT)

Sample	AUS	LR1	LR2	LR3	AL1	AL2	AL3	BX	CH1	CH2	SP1	SP2	IT1	IT2
AUS												0.09	0.00	0.01
LR1	0.03											0.09	0.05	
LR2	0.04	0.02											0.01	
LR3	0.02	0.03	0.02										0.00	
AL1	0.03	0.04	0.04	0.02									0.00	0.00
AL2	0.03	0.06	0.04	0.02	0.02						0.01	0.02	0.00	0.00
AL3	0.03	0.05	0.04	0.01	0.02	0.01					0.04		0.00	0.00
BX	0.02	0.04	0.03	0.01	0.02	0.02	0.02				0.10		0.00	0.00
CH1	0.04	0.03	0.03	0.01	0.03	0.03	0.03	0.02			0.05	0.09	0.00	0.01
CH2	0.03	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.02		0.06	0.09	0.00	0.03
SP1	0.04	0.05	0.05	0.06	0.05	**0.08**	**0.07**	**0.06**	**0.07**	**0.06**				
SP2	**0.06**	**0.06**	0.05	0.05	0.06	**0.07**	0.06	0.06	**0.07**	**0.06**	0.02			
IT1	**0.14**	**0.10**	**0.10**	**0.13**	**0.16**	**0.18**	**0.13**	**0.14**	**0.16**	**0.15**	0.07	0.06		
IT2	**0.10**	0.07	0.08	0.10	**0.11**	**0.13**	**0.10**	**0.12**	**0.12**	**0.10**	0.04	0.05	0.03	

asexual spores in the dispersal of *E. lata* within some vineyards has been inferred from spatial analyses of symptomatic vines (Munkvold et al., 1993; Hughes et al., 1998). However, since soil heterogeneity may interfere with the expression of symptoms caused by a wood pathogen on a perennial host, we believe that studying the genotypes present at a fine scale could provide more conclusive information about the possible

Table 6. (Variation in aggressiveness in *E. lata* populations from Australia (AUS), France (samples LR1, AL1, BX, CH1) and Italy (sample IT1) determined after inoculation of grapevine cuttings in the greenhouse

Sample	*N*	% Abnormalities[a]			N^{b}_{WS}	$N^{c}_{=BX1-10}$
		Uninoculated	Isolate BX1-10	Range of isolates		
AUS	17[d]	26.4	79.1	26.4–79.1	5	4
LR1	18	26.4	75.0	26.4–80.5	6	8
AL1	18	27.8	69.4	34.6–79.1	1	11
BX	15[e]	30.6	62.5	27.8–58.3	3	5
CH1	18	16.7	80.6	27.8–88.9	1	9
IT1	14[d]	19.4	69.4	27.8–75.0	10	3

[a]Abnormalities were scored eight weeks after inoculation and included cuttings with no bud break, bad development or showing characteristic symptoms of Eutypa dieback (short shoots with small, necrotic leaves). Percentages are means from three experiments performed in 1997–1999, each including three blocks of eight cuttings.
[b]Number of isolates that did not induce characteristic symptoms of Eutypa dieback out of a total of 72 cuttings inoculated (WS = without symptoms).
[c]Number of isolates that were not significantly distinguished from the standard isolate BX1-10 according to Dunnett's test at ≤ 0.05.
[d]One isolate included in RAPD analysis was not tested for aggressiveness.
[e]Instead of 16 since BX1-10 from this sample was the standard isolate.

role of asexual inoculum in the spread of Eutypa dieback.

Gene diversity was very similar in most of the populations. Nevertheless, the Italian population showed a higher level of gene diversity than the French population from Alsace. The area where isolates were collected was larger in Italy than in Alsace, but we do not believe that this can explain the difference in gene diversity. First, the size of the area sampled in Alsace was similar to that sampled in other French regions. Second, the gene diversity found in the Spanish population, sampled from two small areas in the region of Catalogne, was similar to the gene diversity exhibited by populations from larger areas. The difference in gene diversity between populations from Italy and from Alsace could be due to statistical sampling (Weir, 1996) since a limited number of isolates was analysed to describe supposedly very large populations. However, the differences between samples from the same region were lower than the difference between the Italian and Alsace populations. Another explanation could be that the fungus has been present in Italy for a longer period than in Alsace.

The gene diversity of the population sampled in LR in 1996 for the present study may be compared to that of another population of 54 *E. lata* isolates sampled in the same region in 1992 but from vines showing Eutypa dieback (Péros et al., 1999). The mean gene diversity of this latter population was 0.29 for the 27 RAPD markers scored. However, only 12 of these markers were also scored for the 1996 population. We thus recalculated gene diversity for the common RAPD markers, and found 0.39 and 0.37 for the 1992 and 1996 populations, respectively. The values were therefore very similar despite the differences in sampling dates, in the localities that were sampled within the region, and in the type of dieback symptoms of infected vines (Eutypa dieback for the 1992 population and esca syndrome for the 1996 population).

Although the analyses of linkage disequilibrium between pairs of loci were all consistent with random mating, the analysis of multilocus association in the global LR population and in the global sample of 240 isolates led us to reject the hypothesis of random association. With respect to the global sample, the pooling of populations with differences in allele frequencies clearly inflated the observed variance and the hypothesis of random association might not have been tested adequately. In the case of the LR population, the distribution of the number of allele differences showed skewness towards the extremes that also caused an inflated variance value. With respect to the population sampled in 1992 in the same region, we observed that 3.1% of the tests for linkage disequilibrium were significant at the 5% level (Péros et al., 1999). The analysis of multilocus association performed on this latter population indicated that the null hypothesis of random assortment could not be rejected

($V_D = 5.50$, $V_E = 5.01$, $L_{MC} = 5.77$, $Pr = 0.13$). Conflicting results in analysing multilocus association may thus be obtained from different samples taken in the same population.

Most of the genetic diversity in *E. lata* was present within populations. Only a low level of genetic differentiation among populations was detected as in the case of other outcrossing fungal species with potential for long-distance dispersal (Ennos and Swales, 1991; Boeger et al., 1993; Goodwin et al., 1993; Hamelin et al., 1994; Hamelin et al., 1995; Gagné et al., 2001). In France, these results confirmed the lack of genetic differentiation between two populations from vineyards located at a distance of 390 km (Péros and Larignon, 1998). The gene flow in France may be unrestricted due to the transport of ascospores between different regions. Liberation of ascospores from the perithecia has been shown to occur after light rainfall (Moller and Carter, 1965; Ramos et al., 1975), and wind has been shown to carry infectious ascospores for at least 50 km in Californian apricot orchards (Ramos et al., 1975). Continuous distribution of cultivated and wild hosts along with the ability for the pathogen to travel over long distances may explain our finding that the French population of *E. lata* is not subdivided. At its apogee in France, around 1870, grapevine covered an area two and a half times larger than the current area (Lachiver, 1988). Moreover, the numerous alternative hosts of *E. lata* may constitute stepping stones providing a bridge between vineyards. Although not documented, this role could be played, for instance, by hawthorn (*Crateagus* sp.), a wild host that is present in field hedges that are often trimmed.

Our study evidenced a weak but significant genetic differentiation between the European populations. Selection occurring on unlinked RAPD loci is unlikely and restriction in gene flow seems to be a better explanation. Mountain ranges (Figure 1) may constitute geographic barriers with respect to the transport of ascospores between France and Italy, and France and Spain, respectively. The lower level of genetic differentiation between Italian and Spanish populations as well as between these populations and some of those from the LR region of France could be explained by aerial transport of ascospores between vineyards bordering the Mediterranean Sea. A similar level of genetic differentiation has been measured between eastern and western populations of *Cronartium ribicola* despite the ability for this fungus to disseminate over long distances (Hamelin et al., 2000). According to these authors, the scarcity of suitable host plants on the Great Plains that separate the two sampled geographic areas is the most likely explanation for the restriction in gene flow. Historical effects may also explain part of the genetic differentiation existing in European populations of *E. lata*. Since their separation from a common ancestral population these populations might have had sufficient time to diverge by mutation and genetic drift. In fact, if *E. lata* was introduced with grapevine, its diffusion in Europe from Asia Minor would have followed the different routes that have been suspected for grapevine and that would have separated many centuries ago for France and Italy (Fregoni, 1991).

The introduction of grapevine in Australia began in the early 19th century, mainly from France and Spain (Dry and Gregory, 1988). This could explain the differentiation observed between the Australian and Italian populations of *E. lata*. Moreover, because gene diversity in this fungus is similar in Australia and in Europe, repeated introductions with grapevine and apricot, at least in the Adelaide region, may have occurred thus avoiding loss of alleles at low frequency because of the bottleneck effect (Nei et al., 1975). However, gaining more insight into the relationships between the Australian and European populations requires other molecular tools than RAPD markers. For instance, phylogenetic analysis of mitochondrial DNA sequences could be a more powerful way to trace the genealogy of an introduced organism as demonstrated for the grape pest phylloxera that was transported from North America to Europe (Downie, 2002).

The marked diversity in neutral molecular markers was associated with marked variation in aggressiveness within *E. lata* populations. Each population included a range of isolates varying from high to low aggressiveness. Isolates that did not reproduce the characteristic symptoms in cuttings may be low producers either of eutypine, a toxin that has been implicated in the development of symptoms (Fallot et al., 1997), or of hydrolytic enzymes that degrade the wood (Schmidt et al., 1999). The considerable diversity in the fungus may facilitate our understanding of the role and relative importance of eutypine and hydrolytic enzymes as components of *E. lata* pathogenicity. It will also be important to select isolates carefully when evaluating the tolerance of different vine clones to Eutypa dieback. The population from Italy showed more isolates with low aggressiveness. Whether or not some *E. lata* isolates in Italian populations display particular pathogenic abilities needs to be investigated, since

differences in aggressiveness are corroborated by the presence of unusual symptoms of Eutypa dieback in Italian vineyards (Bisiach and Minervini, 1985) as well as by the molecular analysis performed here.

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