Variation in Pathogenicity and Genetic Structure in the *Eutypa lata* Population of a Single Vineyard

J.-P. Péros, G. Berger, and Florence Lahogue

Station de Recherches Viticoles, Institut National de la Recherche Agronomique, BP13, 34751 Villeneuve-les-Maguelone Cedex, France, and UFR de Viticulture, ENSAM-INRA, 2, place Viala, 34060 Montpellier Cedex 01, France.

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ABSTRACT

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Fifty-five isolates of *Eutypa lata* were collected in 1994 from a single vineyard, each from a different vine that showed either shoot and foliar symptoms of *Eutypa* dieback or only abnormalities during the period of 1990 to 1994. These isolates showed a large variation in pathogenicity after inoculation on cuttings in the greenhouse. Variability also was observed for cultural traits and radial growth rate on potato dextrose agar (PDA) medium, but no relation was found between these characteristics and pathogenicity. The isolates were paired on PDA medium, and a barrage reaction was observed for all pairings, indicating the isolates were vegetatively incompatible. Thirty-two random amplified polymorphic DNA (RAPD) markers were used to study the genetic relatedness of the isolates and the genetic structure of the population. Fifty-five different RAPD

patterns were observed, confirming the genetic uniqueness of each isolate. Gametic disequilibrium values were calculated among all pairs of the 32 putative RAPD loci, and only one value was significant. Unweighted pair-group method with arithmetic averages analysis of distance data, as well as a heterogeneity statistic (*F*_{st}), did not indicate any population substructure. The results strongly suggest that isolates originated from a random-mating population and that the disease was propagated in this vineyard by ascospores produced from diverse outside sources. Southern hybridization performed for five markers indicated that the two-allele assumption made to interpret RAPD data may be violated for markers that are similar in size. However, the exclusion of such markers did not change the conclusions of the study.

Additional keywords: diversity, grapevine, RAPD markers, vegetative incompatibility.

Eutypa lata (Pers:Fr.) Tul & C. Tul. (= *E. armeniaca* Hansf. & M.V. Carter; anamorph *Libertella blepharis* A.L. Smith) is the causal fungus of dieback in a wide range of perennials, including grapevine (12). The disease is escalating in many viticultural areas, causing important economic losses (16,35). Numerous factors, such as climate, cultural practices, scion and rootstock cultivars, and vigor and age of vines, seem to be implicated in the large variation in disease incidence (15). If these factors explain the differences observed among vineyards, it is necessary to make other assumptions to explain variation at the vineyard level. In the same vineyard, symptom expression fluctuates from vine to vine and is very unstable from year to year (24). Variability in *E. lata* may play a role, but this role has not been studied precisely. Differences in pathogenicity have been noticed between *E. lata* isolates from different geographic origins (39,41), among single-ascospore isolates from the same perithecial stroma (13,39,44), and even among isolates from the same ascus (17). Variation in cultural traits also has been observed (17,18,39). More recently, genetic diversity in *E. lata* was identified by isozyme and RAPD (random amplified polymorphic DNA) markers (40).

The analysis of diversity at a microgeographic scale can be considered as a first step in describing the population biology in *E. lata*. Information on this aspect of its biology is particularly lacking. For instance, ascospores appear to propagate the disease (18,34), and conidia of the anamorph may act only as spermatia (12). However, germination of conidia has been observed, and it has been suggested that they can propagate the disease (6,22). The

relative importance of the asexual and sexual stages in propagation of Ascomycetes has been elucidated when diversity has been studied among isolates from restricted areas (1,10,19,20,23,29,30,33,38). We would expect to find genetically identical individuals or clonal lineages of *E. lata* in the same vineyard if self-fertilization or asexual propagation occurs, whereas the presence of genetically different individuals would indicate outcrossing.

As reviewed by Leslie (25), vegetative incompatibility has been demonstrated as an easy and powerful tool to identify individual genetic entities in natural populations of fungi. Vegetative incompatibility in Ascomycetes is heterogenic and allelic (3,7,8,32,36). Individuals with identical alleles at all of the vegetative compatibility loci form anastomoses, and their mycelia merge. Incompatibility may result in a visible rejection response that can be seen easily when cultures are paired. Such a system probably exists in *E. lata* but has never been studied.

The use of electrophoretic markers provides more detailed knowledge about genetic diversity and population structure of fungal pathogens (28). Among these markers, RAPDs increasingly are used. The fact that RAPDs generally are dominant is not a drawback for haploid fungi (28). Moreover, evidence that RAPDs detected in fungi generally can be interpreted as alleles segregating in a 1:1 ratio has been reported (14,27,38,47). The availability of markers interpreted as loci allows precise analysis of the genetic structure of fungal populations. For instance, the statistical measure of associations among alleles at different loci, i.e., measure of gametic disequilibrium, has been undertaken to determine the likelihood of outcrossing (10,11,31,33,38).

We examine here the pathogenicity and cultural characteristics of 55 *E. lata* isolates, each collected from a different vine in a single vineyard. The genetic relatedness of these isolates also is investigated, using vegetative compatibility and RAPDs. This work was undertaken to determine the variability found at the micro

Corresponding author: J.-P. Péros; E-mail address: peros@ensam.inra.fr

TABLE 1. Code, vine origin, growth rate in culture, and pathogenicity in the greenhouse of 55 *Eutypa lata* isolates from a single vineyard

Isolate	Vine origin ^a	Growth rate (mm/day)	Pathogenicity in greenhouse ^b		
			Symptoms (%)	Abnormalities (%)	Colonization ^c
VL136	–	5.7	0.0	25.0	+
VL140	–	2.5 ^d	0.0	25.0	+
VL175	+	4.9	0.0	32.5	+
VL125	–	6.1	0.0	32.5	+
VL126	–	4.7	0.0	32.5	+
VL127	+	5.6	0.0	35.0	+
VL143	+	4.2	0.0	35.0	+
VL172	–	4.7	0.0	35.0	+
VL166	–	5.5	0.0	37.5	+
VL128	–	5.1	0.0	40.0	+
VL148	+	5.6	0.0	42.5	+
VL141	–	5.6	0.0	42.5	+
VL134	–	5.8	0.0	47.5	+
VL137	–	5.4	0.0	47.5	+
VL124	+	5.6	0.0	52.5	+
VL132	–	5.5	0.0 ^e	32.5	
VL146	–	5.3	0.0 ^e	35.0	
VL135	–	6.2	0.0 ^e	42.5	
VL174	–	5.9	0.0 ^e	42.5	
VL167	+	5.5	2.5	37.5	
VL177	+	4.7	2.5	40.0	
VL160	–	4.3	2.5	40.0	
VL139	–	5.0	2.5	42.5	
VL173	+	5.3	2.5	45.0	
VL154	–	5.4	2.5	55.0	
VL176	–	5.9	2.5	55.0	
VL133	–	5.8	5.0	22.5	
VL151	–	4.8	5.0	40.0	
VL164	+	5.4	5.0	42.5	
VL157	–	4.1	5.0	45.0	
VL168	–	5.3	5.0	55.5	
VL155	–	4.3	7.5	40.0	
VL138	+	4.5	7.5	42.5	
VL130	–	4.7	7.5	45.0	
VL152	–	5.0	7.5	50.0	
VL142	–	5.2	10.0	50.0	
VL158	+	4.2	10.0	52.5	
VL159	+	5.0	10.0	60.0	
VL149	–	5.4	12.5	52.5	
VL163	–	6.4	12.5	52.5	
VL161	–	5.4	12.5	65.0	
VL145	+	5.3	15.0	65.0	
VL162	–	6.2	15.0	65.0	
VL153	+	5.4	15.0	67.5	
VL147	+	5.0	15.0	87.5	
VL178	+	4.8	15.0	90.0	
VL150	–	4.6	17.5	47.5	
VL169	+	5.1	20.0	85.0	
VL165	+	5.0	22.5	67.5	
VL170	–	5.8	22.5	72.5	
VL144	–	5.3	25.0	65.0	
VL131	–	5.7	25.0	75.0	
VL156	–	3.4 ^d	25.0	85.0	
VL171	–	4.9	27.5	65.0	
VL129	+	4.8	30.0	62.5	
Mean ± SE		5.2 ± 0.1	7.8 ± 1.2	49.9 ± 2.2	
Control			0.0	22.5	–

^a Isolates originated from vines showing (+) or not showing (–) characteristic symptoms at least during 2 years from 1990 to 1994.

^b Percents were calculated with pooled data of replicates 1 through 3 for a total of 40 inoculated cuttings.

^c Reisolation was only attempted for isolates that did not induce symptoms and for the control. For five cuttings per treatment, a few chips of wood were cut from a transverse section (2 cm from the inoculation site) and placed in a potato dextrose agar plate. + indicates *E. lata* was recovered from at least one chip.

^d Mycelial growth of these isolates ceased in the second replicate, and they were excluded from the mean calculation.

^e Isolates induced symptoms in replicate 4, which was performed with isolates that caused no symptoms in replicates 1 through 3.

geographic scale to describe the genetic structure of an *E. lata* population and clarify the propagation mode of the fungus.

MATERIALS AND METHODS

Origin and culture of isolates. The 55 *E. lata* isolates studied (Table 1) were collected in 1994, each from a different vine, in a trial at the INRA Experimental Station located at Villeneuve-les-Maguelone (48.00 N, 1.69 E) near Montpellier, France, in the littoral Mediterranean area. This vineyard was planted in 1979 to analyze the effect of six rootstocks and two *Vitis vinifera* scion cultivars on growth, yield, and vine quality. The trial plot, 90 × 180 m, was arranged in a split-plot design, with scion as the main plots and 6 replicates of 75 vines per treatment. The trial plot was surveyed during late May and early June throughout the period from 1990 to 1994 for the presence of dieback. We chose to collect the *E. lata* isolates into six plots of cv. Gramon (Grenache × Aramon) grafted onto 41B. In addition to vines with shoot and foliar symptoms, i.e., dwarfed shoots with smaller and necrotic leaves, vines with arm mortality and reduced growth were observed. Two-thirds of the isolates analyzed originated from such vines; one-third originated from vines with shoot and foliar symptoms. The vineyard also was inspected carefully for the presence of the sexual stage of the fungus, but no perithecial stromata were found on dead parts of vines. Isolation, culture, and storage of the fungus were performed, using methods described previously (39).

Measure of radial growth rate. Plugs, 5 mm diameter, taken from 7- to 10-day-old potato dextrose agar (PDA) cultures were placed in the center of 9-cm PDA dishes and incubated at 25 ± 1°C under cool-white fluorescent light (16-h photoperiod, 100 µE m⁻² s⁻¹). There were three replicate plates per isolate. Colony diameters were measured along two lines intersecting at right angles after 3 and 6 days, and the daily radial growth rate was calculated. The experiment was repeated twice. The nonparametric Mann-Whitney's test (46) was used to determine whether the growth rate in culture differed according to the origin of isolates, i.e., vines with or without shoot and foliar symptoms.

Pathogenicity test. Canes of cv. Cabernet-Sauvignon (clone 191) were collected during December 1994 and stored at 4°C. One-bud unrooted cuttings were inoculated from March to July 1995 as previously described (39). Briefly, a plug (5 mm diameter) of a fresh mycelial culture on PDA medium was inserted in a hole drilled 4 cm under the bud. The inoculation site was covered with a plastic film, and inoculated cuttings were placed in rockwool cubes saturated with tap water. The experiment was repeated three times, using 8, 16, and 16 cuttings, respectively. Isolates that did not induce shoot and foliar symptoms in any of the three replicates were inoculated to an additional set of 16 cuttings. The control cuttings were treated as described above, except that plugs consisted of PDA medium alone. The number of cuttings with shoot and foliar symptoms (*S*) was recorded 8 weeks after inoculation. The total number of abnormal cuttings (*A*) was obtained by adding to *S* the number of cuttings with no bud break, a stem with poor growth, or a necrosed tip. The Mann-Whitney's test was used to determine whether the growth rate in culture of the isolates that did not produce symptoms in the greenhouse was different from the growth rate of isolates producing symptoms. The same test was used to determine whether pathogenicity in the greenhouse differed according to the origin of the isolates, i.e., vines with or without shoot and foliar symptoms. For the isolates that did not induce shoot and foliar symptoms in any of the replicates, reisolation was attempted 2 cm from the inoculation site as previously described (39), using five cuttings for each isolate and for the control.

Test for vegetative compatibility. Isolates were grown for 7 to 10 days in PDA plates at 25°C. Cubes of mycelium and agar with sides of about 1 mm were transferred to fresh plates of PDA. Inocula of paired isolates were placed 1 cm apart and incubated under the conditions described above for 2 to 4 weeks. We then

determined whether the mycelia intermingled (compatible reaction) or showed some form of antagonism (incompatible reaction).

RAPD analysis. The extraction of DNA from freeze-dried mycelium and the amplification reactions were done according to methods described previously (40), except that the nucleotides were used at 120 μ M, and *Taq* DNA polymerase was purchased from Appligène-Oncor (Illkirch, France). Seven random 10-mer primers (Bioprobe, Montreuil, France) were used (Table 2). Analysis was repeated twice from extraction to electrophoresis of amplification products.

Polymorphic, reproducible, and intense bands were scored as separate putative loci with two alleles. The presence and absence of the amplification product corresponded to the positive (1) and null (0) allele, respectively. The matrix containing the binary data was used to calculate the Jaccard's distance (21) between all paired isolates. To investigate the relationships between the isolates, a phenogram was constructed from the distance matrix by the unweighted pair-group method with arithmetic averages (UPGMA) of PHYLIP software (version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle). The correlation between the distance matrix obtained from the whole set of markers and the distance matrix obtained from a subset of markers, as described later in this paper, was calculated according to Mantel's procedure (26).

For each RAPD locus (*i*), the frequency (*p*) of the positive allele was determined, and Nei's allelic diversity (*h*) was calculated as $h = 1 - \sum p_i^2$ (37). The mean allelic diversity within the population was calculated over all RAPD loci. The significance of nonrandom association (gametic disequilibrium) among all pairs of RAPD loci was tested by GENEPOP software (version 2.1; M. Raymond and F. Rousset, Institut de Sciences de l'Evolution, University at Montpellier II, Montpellier, France). To study the even-

tual genetic differentiation within the population, two subdivisions were successively made: (i) two subpopulations corresponding to isolates from vines with and without shoot and foliar symptoms in the vineyard and (ii) two subpopulations corresponding to the ability of isolates to produce foliar symptoms in the greenhouse. GENEPOP software was used to calculate an unbiased estimator of *F*_{st} and to test its significance among subpopulations for each locus and the combination of all loci. The excess of significant tests at *P* = 0.05 due to multiple comparisons was overcome by Bonferroni's sequential test procedure (42) for both gametic disequilibrium tests and *F*_{st} calculated for each locus.

Southern hybridization. The five RAPD markers analyzed (Table 2) were recovered from 2% low-melting agarose gel (Appligène-Oncor) in 1× Tris-acetate-EDTA buffer (45) and purified by Wizard polymerase chain reaction (PCR) Preps kit (Promega, Madison, WI). The fragments were ligated in pGEM-T vector (Promega) according to the manufacturer's instructions. Competent cells of *Escherichia coli* strain DH5 α were transformed with the ligated plasmids (45). A selection of the transformants was performed directly on white colonies by PCR amplification with universal primers SP6 and T7. Plasmids with fragments of the correct size were purified according to Sambrook et al. (45), and inserts were reamplified.

The reamplified DNA were labeled with 20 μ Ci (α -³²P)dNTP with the Megaprime kit (Amersham, Arlington Heights, IL) and were used as probes on Southern blots. The original PCR products were separated on 1.6% agarose gel, partially denatured for 10 min in 0.25 N HCl, and transferred to Hybond N+ membranes (Amersham) with 0.4 N NaOH for 1 h with a vacuum blotter (Appligène-Oncor). Blots were rinsed with 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) and air-dried. Prehybridizations were carried out for 1 h at 65°C with 100 μ g of

TABLE 2. Sequence of the random amplified polymorphic DNA primers used, size of putative loci, frequency of positive allele, and allelic diversity in a population sample (*n* = 55) of *Eutypa lata* isolates from a single vineyard

Primer code	Sequence (5' to 3')	Fragment size (bp)	Putative locus	Frequency of positive allele	Allelic diversity
A10	GTGATCGCAG	1,240	A10-1240 ^a	0.218	0.341
A10	GTGATCGCAG	420	A10-420	0.164	0.274
A10	GTGATCGCAG	410	A10-410 ^b	0.655	0.452
A15	TTCCGAACCC	1,060	A15-1060 ^a	0.509	0.500
A15	TTCCGAACCC	910	A15-910 ^a	0.346	0.453
A15	TTCCGAACCC	680	A15-680	0.018	0.035
A15	TTCCGAACCC	620	A15-620	0.128	0.223
A15	TTCCGAACCC	560	A15-560	0.018	0.035
A15	TTCCGAACCC	490	A15-490	0.018	0.035
A15	TTCCGAACCC	380	A15-380 ^a	0.054	0.102
A15	TTCCGAACCC	360	A15-360 ^{a,b}	0.254	0.379
B02	TGATCCCTGG	1,660	B02-1660 ^a	0.710	0.412
B02	TGATCCCTGG	860	B02-860	0.272	0.396
B02	TGATCCCTGG	830	B02-830 ^b	0.764	0.361
C20	ACTTCGCCAC	1,280	C20-1280 ^a	0.036	0.069
C20	ACTTCGCCAC	1,070	C20-1070 ^a	0.036	0.069
C20	ACTTCGCCAC	580	C20-580 ^a	0.036	0.069
D18	GAGAGCCAAC	1,340	D18-1340 ^a	0.128	0.223
D18	GAGAGCCAAC	1,030	D18-1030 ^a	0.036	0.069
D18	GAGAGCCAAC	720	D18-720 ^a	0.090	0.164
D18	GAGAGCCAAC	560	D18-560 ^a	0.018	0.035
D18	GAGAGCCAAC	530	D18-530 ^{a,b}	0.964	0.069
D18	GAGAGCCAAC	330	D18-330 ^a	0.272	0.396
E03	CCAGATGCAC	1,120	E03-1120	0.182	0.298
E03	CCAGATGCAC	1,060	E03-1060	0.036	0.069
E03	CCAGATGCAC	570	E03-570 ^a	0.290	0.412
E03	CCAGATGCAC	420	E03-420 ^a	0.490	0.500
P05	CCCCGGTAAC	830	P05-830	0.164	0.274
P05	CCCCGGTAAC	780	P05-780 ^b	0.472	0.498
P05	CCCCGGTAAC	620	P05-620 ^a	0.310	0.428
P05	CCCCGGTAAC	440	P05-440 ^a	0.364	0.463
P05	CCCCGGTAAC	330	P05-330 ^a	0.890	0.196

^a Used in second data analysis.

^b Used as probes in Southern hybridization analysis.

salmon sperm DNA per ml in buffer containing 5× Denhardt's solution, 5× SSC, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Labeled probes were added, and hybridizations were conducted overnight at 65°C. Blots were washed at 65°C for 15 min successively in each of the following solutions: 2× SSC and 0.1%

SDS; 1× SSC and 0.1% SDS; and 0.1× SSC and 0.1% SDS. Labeled RAPDs were detected by autoradiography with XAR-Kodak (Eastman Kodak Co., Rochester, NY) film.

RESULTS

Growth and characteristics in culture. Large variations were observed in the aspect of the cultures, especially after aging (Fig. 1). The color of cultures varied from white to gray, some isolates had a cottony aspect, and others formed black droplets. No conidial formation was observed. The radial growth rate varied from 4.1 to 6.4 mm per day among the isolates (Table 1). Isolates VL140 and VL156 had limited growth in the second replicate, and their values were not taken into account. On average, the growth of isolates from vines with shoot and foliar symptoms did not differ from the growth of isolates from vines without foliar symptoms, according to Mann-Whitney's test at $P = 0.05$ (5.1 versus 5.3 mm per day).

Variation in pathogenicity. The percentage of plants with shoot and foliar symptoms (S) and the percentage of abnormal plants (A) varied widely among the isolates (Table 1). The control had no plants with shoot and foliar symptoms, but 22.5% of the plants were abnormal. This large percentage of abnormalities within the control mainly corresponded to failure in bud break. Isolates with low or high pathogenicity were observed regardless of origin (Table 1). On average, the pathogenicity of isolates obtained from vines with shoot and foliar symptoms in the vineyard ($S = 9.1\%$ and $A = 54.9\%$) was not significantly different from that of the other isolates ($S = 7.2\%$ and $A = 48.7\%$), according to Mann-Whitney's test at $P = 0.05$.

When inoculated to a total of 40 cuttings (pooled data of the first three replicates), 19 isolates did not induce shoot and foliar symptoms. However, four of these isolates produced shoot and foliar symptoms in a small number of plants inoculated in the additional replicate (Table 1). All isolates that did not produce shoot and foliar symptoms in the greenhouse had the ability to colonize cuttings, because they were reisolated at a distance from the inoculation site (Table 1). The isolates that did not produce shoot and foliar symptoms in the greenhouse were isolated either from vines that showed shoot and foliar symptoms in the vineyard or from vines that did not exhibit shoot and foliar symptoms (Table 1).

Fig. 1. Examples of cultural traits and vegetative compatibility of isolates of *Eutypa lata* on potato dextrose agar. Isolate VL139 (section 3) was gray and formed black droplets; isolate VL154 (section 5) had a cottony aspect. Isolate VL124 (section 1), placed in the plate center, was paired with itself and seven other isolates. Mycelia only merged (M) when self-pairing; antagonism, indicated by a barrage zone (B), was observed for all other pairings. The pairing between isolates VL156 (section 6) and VL158 (section 7) resulted in a marked barrage zone with partial lysis of VL156 (L), whereas a normal barrage zone was obtained when this isolate was paired with VL154 (section 5). Other isolates studied include VL133 (section 2), VL146 (section 4), and VL168 (section 8).

Fig. 2. Agarose gel showing random amplified polymorphic DNA banding patterns from isolates of *Eutypa lata* generated by seven primers. The patterns were chosen to display the 32 detected markers (indicated by arrowheads). Lanes 1 and 2, primer A10, isolates VL163 and VL169, respectively; lanes 3 through 7, primer A15, isolates VL149, VL133, VL175, VL140, and VL124, respectively; lanes 8 and 9, primer B02, isolates VL151 and VL152, respectively; lanes 10 through 12, primer C20, isolates VL170, VL158, and VL164, respectively; lanes 13 through 16, primer D18, isolates VL160, VL153, VL145, and VL133, respectively; lanes 17 and 18, primer E03, isolates VL150 and VL173, respectively; lanes 19 and 20, primer P05, isolates VL178 and VL155, respectively. Lane M, molecular-weight size markers (1-kb ladder), indicated in base pairs on the left.

The growth rate in culture of isolates that caused shoot and foliar symptoms in the greenhouse did not significantly differ from the growth rate of isolates that did not induce symptoms, according to Mann-Whitney's test at $P = 0.05$ (5.2 versus 5.3 mm per day).

Vegetative compatibility. A compatible reaction was observed only when cultures of the same isolate were paired (Fig. 1). An incompatible reaction, obtained when different isolates were paired, was indicated by the development of a barrage zone that varied in intensity according to the pairing (Fig. 1). The barrage zone gen-

erally became dark and depressed and sometimes showed formation of pycnidia.

RAPD markers. The seven primers identified 32 polymorphic amplicons (Fig. 2; Table 2). Each isolate had a specific RAPD profile. The Jaccard's distance between isolates ranged from 0.167 (2 band differences and 10 bands in common out of 32 total bands scored) to 0.909 (10 band differences and 1 band in common out of 32 total bands scored), the average genetic distance was 0.636. According to UPGMA cluster analysis (Fig. 3), the isolates ob-

Fig. 3. Relationships among 55 isolates of *Eutypa lata* as revealed by unweighted paired group method using arithmetic averages cluster analysis of the matrix of Jaccard's distance. Data were obtained from 32 random amplified polymorphic DNA markers generated by seven primers. The top scale shows the Jaccard's distance. ■ and □ indicate isolates that originated from vines that did or did not have foliar symptoms in the vineyard, respectively; ● and ○ indicate isolates that did or did not induce symptoms in the greenhouse, respectively.

tained from vines with shoot and foliar symptoms were not separated from the other isolates; similarly, the isolates that did not produce foliar symptoms in the greenhouse were not distinguishable from the other isolates. In addition, no marker was specific to an isolate's ability to produce these symptoms (data not shown).

The frequency of the positive allele varied from 0.018 to 0.964, and allelic diversity varied from 0.035 to its maximum 0.500 (Table 2). The allelic diversity averaged 0.259 over all 32 RAPD markers. Of 496 tests for gametic disequilibrium between pairs of markers, there were only 13 (2.6%) that were significant at $P = 0.05$. After application of Bonferroni's sequential procedure, only the value for the A10-420 and A10-410 pair was significant. This value reached the maximum due to the lack of a gametic class, i.e., both markers were present. The overall estimate of F_{st} between the subpopulations, defined according to the presence of shoot and foliar symptoms in the vineyard, was very small ($+0.001$), indicating no genetic differentiation ($P = 0.984$), according to Fisher's exact test. The F_{st} value ($+0.091$) was significant ($P = 0.017$) for only one locus (A15-910). In the case of the subdivision made according to the production of shoot and foliar symptoms in the greenhouse, a nonsignificant overall estimate of F_{st} ($+0.002$) was obtained ($P = 0.942$), and only the F_{st} value for A15-360 ($+0.073$) was significant ($P = 0.045$). The application of Bonferroni's procedure gave nonsignificant values for F_{st} at the locus level.

We analyzed five RAPD fragments by Southern hybridization. For all of them, the isolates scored as 1 gave a signal with the corresponding probe. For primer A10, the probe obtained with A10-410 also hybridized to A10-420 and to a 400-bp fragment that was absent when either A10-410 or A10-420 was present. In the case of primer P05, the scoring of patterns was difficult, because four bands ranged in size from 780 to 900 bp. Despite reproducible polymorphisms for the two other fragments (800 and 900 bp, respectively), only P05-780 and P05-830 were selected. The probe obtained with P05-780 gave a hybridization signal for isolates having any of the four fragments (Fig. 4). For primer B02, the probe obtained with B02-830 gave one hybridization signal for the isolates scored as 1 for B02-830 and B02-860. However, the isolates scored as 1 for both bands showed a signal only for B02-830. For primers A15 and D18, the probes obtained with A15-360 and D18-530 did not hybridize with the similar sized A15-380 and D18-560 fragments, which also were selected as markers. Figure 5 shows the hybridization pattern of probe A15-360.

We performed a second analysis with a subset of 20 putative loci (Table 2). The Jaccard's distance between isolates ranged from 0.000 (zero band differences and four bands in common) for isolates VL126 and VL151 to 1.000 (eight band differences and no

bands in common) for isolates VL146 and VL178; the average genetic distance was 0.610. The comparison of the two distance matrices constructed from all markers versus the subset of 20 markers yielded a significant correlation coefficient of $r = 0.804$. The allelic diversity averaged 0.267 over the 20 RAPD markers. Overall estimates of F_{st} based on the 20 putative loci were similar to those calculated based on the whole set of markers (data not shown).

DISCUSSION

Phenotypic markers, i.e., pathogenicity and cultural traits, as well as genetic markers, i.e., vegetative incompatibility and RAPDs, revealed considerable variation in the *E. lata* population of a single vineyard. Such a level of variability strongly indicates that the disease was propagated in this vineyard by ascospores that originated from sexual outcrossing. This hypothesis is supported by important genetic arguments: (i) each isolate had a specific RAPD profile, (ii) each isolate was vegetatively incompatible with the others, and (iii) no gametic disequilibrium was found, using RAPD markers as putative loci. In a random-mating population, gene reassortment at each sexual generation could produce a large number of individuals with unique combination RAPD and vegetative incompatible loci. In addition, we obtained evidence that the system of vegetative compatibility in *E. lata* is governed by several genes, as in other Ascomycetes (3,7,8,32,36). The variation in the intensity of the barrage zone may be attributed to allelic differences, as has been suggested previously (2).

The fact that we did not find evidence of association between the different types of markers provides further evidence of random mating. The observation of *E. lata* cultures on PDA medium and measurement of their growth appeared valueless in predicting pathogenicity. Similarly, UPGMA analysis of distance data, as well as F_{st} calculation, did not distinguish the isolates according to pathogenicity. As emphasized by McDonald et al. (31), because gametic equilibrium is reached quickly in random-mating fungi, we are unlikely to find associations between different markers. For instance, no consistent relationship between RAPD patterns and genetic variation in fungicide resistance was found in sexually reproducing *Pyrenophora teres* (38), whereas a correlation between genetic similarity and toxin production has been identified in asexually reproducing *Aspergillus flavus* (5).

We demonstrated that the subdivision of isolates based on the presence of shoot and foliar symptoms in the vineyard did not correspond to qualitative differences in pathogenicity. The large variation in pathogenicity could partially explain the differences observed in the vineyard among infected vines both for the appearance of shoot and foliar symptoms and the progress of die-

Fig. 4. Homology tests for random amplified polymorphic DNA fragment P05-780 from *Eutypa lata*. **A**, Amplification products stained with ethidium bromide; fragment P05-780 is indicated by arrowheads. **B**, Corresponding hybridization pattern. The probe also hybridized to a 800-bp fragment (lane 3), a 830-bp fragment (lanes 1 and 4), and a 900-bp fragment (lane 5). Lanes 1 through 8 were amplified from isolates VL170, VL169, VL168, VL167, VL166, VL165, VL164, and VL163, respectively.

Fig. 5. Homology tests for random amplified polymorphic DNA fragment A15-360 from *Eutypa lata*. **A**, Amplification products stained with ethidium bromide; fragment A15-360 is indicated by arrowheads. **B**, Corresponding hybridization pattern. A signal was obtained only when the fragment was present (lanes 1 through 4 and 6). Lanes 1 through 8 were amplified from isolates VL167, VL172, VL175, VL124, VL138, VL141, VL125, and VL126, respectively.

back. Numerous other factors, such as localization of infection in the vine and vine response to infection, also may intervene in the vineyard and could explain why the pathogenicity of *E. lata* in the greenhouse cannot be predicted based on the shoot and foliar symptoms observed in the vineyard. However, differences in environmental conditions between the greenhouse and vineyard, changes in the fungus characteristics after culture on artificial media, and isolation of an isolate with low pathogenicity from a vine yielding other more aggressive isolates may be other possible causes of the absence of a relationship between greenhouse and vineyard observations.

Because of its simplicity and low development costs, the RAPD technique appears promising for use in further studies on the diversity and population genetics of *E. lata*. However, several drawbacks associated with RAPD markers have been reported (4,9). First, the RAPD patterns must be reproducible. Experience in our laboratory, as in others, indicated that specific fingerprints in *E. lata* can be generated with different batches of DNA and in replicate amplifications with the same template DNA. A second problem is that comigrating fragments may be nonhomologous. Current results in plants, however, suggest that homology requires more careful attention in interspecific than in intraspecific comparisons (43). We confirmed by Southern hybridization the homology of comigrating fragments for five markers. However, the results obtained with probe B02-830 indicated that a 860-bp band represented two different fragments. One of these fragments was detected by the probe and might be an allele of B02-830, whereas the other might correspond to a second locus. Homology also was demonstrated for three and four fragments amplified with primers A10 and P05, respectively. In each case, the different fragments might be alleles at the same locus. Such polymorphism may result either from insertion/deletion events or from variation in the number of tandem repeats, as described by McDermott et al. (27) in *Erysiphe graminis* f. sp. *hordei*. The occurrence of length differences between primer binding sites at the same locus constitutes a third potential shortcoming of RAPDs. A bias can be introduced in the study of relatedness because the expected random coverage of the genome is not achieved. If too many of the selected markers are allelic, an excess of significant gametic disequilibrium tests can support clonal propagation even in an outcrossing species. Moreover, because some of the markers do not fit the two-allele assumption, the calculation of genetic diversity (*h*) and *F*_{st} are biased.

In our study, the proportion of multiallelic markers cannot be estimated, because we used Southern hybridization to test only a few markers that were more prone to producing problems. Indeed, the five markers chosen were each similar in size to another marker, and one pair of these markers gave a significant gametic disequilibrium test. To avoid the bias associated with allelic RAPDs, the markers similar in size could be excluded. However, when we performed such selection the conclusions based on the whole data were not changed, i.e., lack of gametic disequilibrium and no substructure in the population. Specific primers could otherwise be developed to amplify sequence-characterized regions and to detect variation at defined loci, as proposed by McDermott et al. (27).

The existence in a single vineyard of individuals from a random-mating population suggests that ascospores from diverse sources constitute the primary inoculum. This agrees with the observations of Munkvold et al. (35) who analyzed the spatial patterns in vineyards of diseased plants with and without perithecia. When perithecia were absent, the infected vines generally were distributed at random due to the arrival of ascospores from distant sources. However, a nonrandom spatial pattern was observed in one vineyard, and the authors suggested disease was spread by means other than ascospores (35). Moreover, it has been shown that the reproductive mode may be different among populations of the same fungal pathogen. For instance, reproductive mode varies from random mating to clonal reproduction among populations of *Cryphonectria parasitica* (33). Other populations of *E. lata*, thus, must

be studied before any conclusions about the exclusive occurrence of random mating can be made. The analysis of DNA diversity in several *E. lata* populations from diverse geographic areas also would be of great interest to investigate genetic differentiation and gene flow among populations. However, to develop the analysis of *E. lata* population genetics, it appears necessary to obtain more information on the inheritance and nature of the genetic variation detected. Because the fungus does not fruit in culture, such studies must be undertaken with natural populations at the stroma or perithecia scale.

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