Comparison by Isozyme and RAPD Analysis of Some Isolates of the Grapevine Dieback Fungus, *Eutypa lata*

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The purpose of this study was to identify molecular markers suitable for developing studies on the genetic variation and biology of the grapevine dieback fungus, *Eutypa lata.* Using a preliminary set of 18 isolates, 21 isozyme markers were identified in nine isozyme systems and 18 random amplified polymorphism DNA (RAPD) markers were detected with six different 10-mer primers. A high level of genetic variation was found among isolates originating from different geographic locations. Polymorphism was also detected among isolates from the same stroma and the different stromas were clearly separated in the cluster analysis based on RAPD data. No association was found between the molecular patterns and either the host or the geographic proximity of the isolates. Isolates having a low pathogenicity on grapevine appeared genetically similar to other isolates. The isolate AM14 was clearly distinguished by both types of markers, and its taxonomic position is discussed. RAPDs appeared more promising than isozymes for the study of genetic variation of *E. lata* and for fingerprinting isolates with specific pathogenic traits.

KEY WORDS: *Eutypa lata,* grapevine dieback

Eutypa lata (Pers:Fr.) Tul & C. Tul. (syn. *E. armeniacae* Hansf. & M. V. Carter, anamorph *Libertella blepharis* A. L. Smith) is a worldwide pathogen affecting a large number of perennials (3). Apricot and grapevine are economically important hosts of the fungus. The ascospores of the fungus are dispersed from a perithecial stroma developing on dead wood. The ascospores enter into the vessels of fresh pruning wounds and initiate an haploid infectious mycelium (3,32). In France, the incidence of Eutypa dieback varies in large proportions among and within geographic regions (25). In California, the vineyards where perithecia could be found showed a higher incidence of the disease than vineyards without perithecia (32). Different factors such as inoculum level, climate, age and vigor of vines, pruning strategies, rootstock, and cultivar could explain the variation in disease incidence. The pathogenicity of the fungus could also be implicated since this character has been shown to vary among isolates of *E. lata* (4,39,42) even at the stroma level (11,37). In particular, some isolates induced cankers which ceased development on apricot branches (4), and others did not reproduce the stem and foliar symptoms on grapevine cuttings (37).

It would be desirable to use rapid methods to characterize pathotypes as well as the genetic variation within and between populations of *E. lata*. In addition, the knowledge of some important aspects of the biology of the fungus is very limited. In particular, the mating system is not elucidated. Molecular markers have been shown to be very useful in investigating the genetic variation and the biology of fungi (28). Starch gel electrophoresis of enzymes (27) has been used for differentiating isolates within fungal species (2,10,12,15,31,38). However, more polymorphism can be obtained with DNA markers (28). These markers can detect any variation in coding and non-coding DNA sequences, whereas the isozyme markers only reflect detectable changes in protein-coding or regulatory sequences. DNA restriction fragment length polymorphisms (RFLPs) have been widely studied in pathogenic fungi (20,21,24,29,34,47,48,51). A method to detect random amplified polymorphic DNA sequences (RAPD) was recently developed (49,50) as an application of the polymerase chain reaction (PCR) technique. RAPD markers have been used for characterizing pathotypes (8,16,23,43) and analyzing the genetic variation in fungi (9,13,18,35). The PCR-RAPD method is faster and requires lower DNA quantities than RFLP analysis (49,50). Nearly all RAPD markers are dominant (50), nevertheless, this limit has no signification when the DNA analysis is performed on haploid mycelial cultures.

The objective of this work was to compare the relative merits of isozyme and RAPD analysis for developing studies on the genetic variation and the biology of *E. lata* Some preliminary data was also obtained on the genetic relationships among isolates originating from different geographic regions and among isolates from the same stroma.

Materials and Methods

Fungal isolates: Eighteen isolates corresponding to mass isolates from wood or different single spore isolates from several stromas were used (Table 1). These isolates originated from 10 different geographic locations and were obtained as previously described

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(37). The morphology of isolate AM14 on potato dextrose agar media (PDA; Difco Laboratories, Detroit, USA), was characteristic of the anamorph at the beginning of the culture. Afterwards, the mycelium became white, orange, and more fluffy in comparison with other isolates. In addition, the cultures of isolate AM14 never melanized and did not produce pycnidiospores. These latter characteristics were also observed for AD30-1, MA26-1, VL11-3, and VL11-5 isolates. The pathogenicity of the isolates was previously assessed on grapevine cuttings using a rapid method (37). Some isolates were not able to cause the foliar symptoms of the disease, *i.e.,* smaller and necrotic leaves (Table 1).

The isolates were stored in distilled water at 4°C. Liquid cultures were initiated by transferring mycelial plugs, cut from actively growing subcultures on PDA, in 100-mL Erlenmeyer flasks containing 50 mL of a modified MS medium (33) with macronutrients reduced to 50%, 20 g • L^{-1} sucrose, and no growth regulators. The cultures were grown for 21 days at 25°C in a 12 hour light/12 hour dark cycle with constant shaking (80 rpm). The mycelium was collected on GF/A glass microfibre filters (Whatman, Maidstone, England) by vacuum filtration, lyophilized and then ground to a fine powder using a glass rod. Aliquots of 25 or 50 mg were stored at -20°C in 1.5 mL microtubes until further use.

Isozyme analysis: The soluble proteins were extracted at 4°C by adding 0.3 mL of the buffer defined by Arulsekar and Parfitt (1) to a microtube containing 50 mg of lyophilized mycelium. After homogenization with a plastic grinder for one minute, the extract was centrifuged at 6000 × *g* for 15 minutes and then at 12000 × *g* for 10 minutes. The supernatant of each sample was absorbed into 4 × 9-mm paper wicks cut from filter paper (No. 3, Whatman). The wicks were deposited at 5 cm from the cathode side of a 12% starch gel. Electrophoresis was performed at 87 mA using a refrigerated apparatus constructed in the laboratory. Starch and tools used for slicing the gels were purchased from Génome, Montpellier, France.

Four electrophoresis buffer systems and 19 enzymes were initially screened (Table 2) using isolate BX1-10 as our reference and AM14 because of its particular characteristics of growth; VL11-4 and VL11-12 were chosen arbitrarily. The optimal buffer system and the enzymes showing sufficient activity were then selected to study and compare all the isolates. The entire experiment from enzyme extraction to electrophoresis was repeated three times.

DNA analysis: DNA was extracted using a modification of the method of Rogers and Benedich (40). A 0.5 mL volume of extraction buffer (2% CDAB [cetyldimethylethyl ammonium bromide], 1.4 *M* NaCl, 100 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA) was added to a microtube containing 25 mg of lyophilized mycelium. After homogenization with a plastic grinder for one minute, the tube was incubated at 65°C for 30 minutes. One volume of chloroform was added to the tube which was then agitated. After centrifugation at 12000 × *g* for 10 minutes, the supernatant was transferred into another microtube. Then, 0.1 mL of 10% CDAB in water and 0.4 mL of chloroform were added. After homogenization and centrifugation, the supernatant was transferred and nucleic acids were precipitated using one volume of cold isopropanol. The tubes were placed on ice for 15 minutes, then gently agitated and centrifuged. Isopropanol was discarded and the pellet washed with 70% ethanol. The pellet was dried under vacuum and dissolved in 0.3 mL of 10 m*M* Tris-HCl, pH 8, 0.1 m*M* EDTA. The DNA was quantified on 0.8% agarose gel stained with ethidium bromide by visual comparison with known quantities of lambda DNA (Boehringer Mannheim, Mannheim, Germany).

A modification of the method described by Williams *et al.* (50) was adopted to perform the amplification reactions. Each reaction volume of 25 μL included 0.4 units of Taq DNA polymerase (Stehelin, Basel, Switzerland), 1 × buffer [10 m*M* Tris-HCl, pH 9.0, 50 m*M* KCl, 0.01 % (w/v) gelatin, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100] provided with the polymerase, 200 μM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 30 ng of primer and approximately 25 ng of template DNA. The reaction was overlaid with a drop of mineral oil. A Trio-

Table 1. *Eutypa lata* isolates used for isozyme and RAPD analyses.

Isolate	Host	Source	Geographic origin	Pathogenicity[a]
AD30-1	Apricot	Stroma 1	Adelaïde, Australia	-
AM14	Grapevine	Necrotic wood	Aigues-Mortes, France	-
BR8-1	Grapevine	Stroma 2	Bram, France	+
BR8-2	Grapevine	Stroma 2	Bram, France	-
BR8-3	Grapevine	Stroma 2	Bram, France	+
BX1-2	Grapevine	Stroma 3	Bordeaux, France	+
BX1-5	Grapevine	Stroma 3	Bordeaux, France	+
BX1-10	Grapevine	Stroma 3	Bordeaux, France	+
GI33-1	Grapevine	Stroma 4	Gilly, Switzerland	-
GI33-6	Grapevine	Stroma 4	Gilly, Switzerland	+
MA26-1	Apricot	Stroma 5	Martigny, Switzerland	-
MB25	Grapevine	Necrotic wood	Montreuil-Bellay, France	+
NA32	Grapevine	Necrotic wood	Naussa, Greece	+
RH24	Grapevine	Necrotic wood	Roschwihr, France	+
VL11-3	Grapevine	Stroma 6	Villeneuve-les-Mag., France	-
VL11-4	Grapevine	Stroma 6	Villeneuve-les-Mag., France	+
VL11-5	Grapevine	Stroma 6	Villeneuve-les-Mag., France	-
VL11-12	Grapevine	Stroma 6	Villeneuve-les-Mag., France	+

[a](37; J.-P. Péros and G. Berger, unpublished data, 1993). Isolates which did not produced clear foliar symptoms (*i.e.,* small necrotic leaves) on grapevine cuttings in the greenhouse were rated - for pathogenicity.

Table 2. Enzymes, buffer systems[a], and stain recipes used to compare the isolates of *Eutypa lata*.

Enzyme	Abbreviation	E.C. no.[b]	Stain recipe[c]	No bands
Aspartate aminotransferase	AAT	2.6.1.1	1	3
Acid phosphatase	ACP	3.1.3.2	1	1
Alcohol dehydrogenase	ADH	1.1.1.1	1	1
Alkaline phosphatase	AKP	3.1.3.1	1	2
Catalase	CAT	1.11.1.6	1	-[d]
Esterase	EST	3.1.1.1	1	7
Glutamate dehydrogenase	GDH	1.4.1.3	1	-
Glucose-6-phosphate dehydrogenase	G-6-P	1.1.1.49	1	-
ß-D-glucosidase	ß-GLU	3.2.1.21	2	-
Isocitrate dehydrogenase	IDH	1.1.1.42	1	-
Lactate dehydrogenase	LDH	1.1.1.27	2	1
Malic enzyme	ME	1.1.1.40	1	-
Malate dehydrogenase	MDH	1.1.1.37	1	5
Mannitol dehydrogenase	MADH	1.1.1.67	2	-
Peroxydase	PO	1.11.1.7	3	-
6-Phosphogluconate dehydrogenase	6-PG	1.1.1.43	1	-
Phosphoglucomutase	PGM	2.7.5.1	2	4
Phosphoglucose isomerase	PGI	5.3.1.9	1	3
Shikimate dehydrogenase	SDH	1.1.1.25	1	-

[a]Buffer recipes from Clayton and Tretiack (7) [electrode buffer = 0.04 *M* citric acid adjusted to pH 6.1 with N-(3-aminopropyl)-morpholine, diluted 1:10 for gel buffer], from Cheliak and Pitel (5) [electrode buffer = 0.125 *M* Tris adjusted to pH 7 with citric acid; gel buffer = 0.05 *M* histidine-HCl, 0.0014 EDTA, adjusted at pH 7 with 1 *M* Tris], from Pasteur *et al. (35)* [electrode buffer = 0.05 *M* lithium hydroxyde, 0.19 *M* boric acid, adjusted to pH 8.1 with lithium hydroxide; gel buffer = 90% (V/V) of 0.046 Tris, 0.007 *M* citric acid and 10 % (V/V) of electrode buffer, adjusted to pH 8.3 with Tris], and from Ridgway *et al.* (42) [electrode buffer = 0.06 *M* lithium hydroxyde, 0.3 *M* boric acid, adjusted to pH 8.1 with lithium hydroxide; gel buffer = 99% (V/V) of 0.03 *M* Tris, 0.005 *M* citric acid, adjusted to pH 8.5 with Tris, and 1% (V/V) of electrode buffer].

[b]Enzyme commission (EC) number according to the Nomenclature Committee for the International Union of Biochemistry.

[c]Stain recipes from 1: Pasteur *et al.* (35), 2: Micales *et al.* (26), and 3: Arulsekar and Parfitt (1).

[d]No activity detected.

Thermobloc thermocycler (Biometra, Göttingen, Germany) was programmed for one step of four minutes at 94°C, followed by 36 cycles of one minute at 93°C, one minute at 38°C, one minute at 72°C, and a final step of six minutes at 72°C. Amplified products were analyzed by electrophoresis in 2% agarose gel at 5 V per cm for four hours, along with a molecular size marker (1-kb ladder, BRL, Gaithersburg, USA). Fragments were detected by staining with ethidium bromide and gels were photographed under UV light.

A total of 50 decamer primers (Operon, Alameda, USA or identical primer sequence from Bioprobe, Montreuil, France) were initially screened. This screening was made using the reference isolate BX1-10, an isolate from the same stroma (BX1-2) and an isolate from another stroma (BR8-1) to have a first appraisal of the polymorphism. The primers which gave banding patterns with a few intense bands were selected to study and compare all the isolates. The entire experiment from mycelial culture to DNA amplification was repeated twice. For each amplification, DNA of isolate BX1-10 from a third extraction was included as a positive control. A negative control reaction with water instead of DNA was also included.

Data analysis: Intense and reproducible bands appearing on starch and agarose gels were scored 1 for presence and 0 for absence and reported in binary matrices. Two coefficients of similarity (CS) and the corresponding distances (D = 1 - CS) were calculated. The first one was Jaccard's coefficient (22)

$$CSJ = a / (a + b + c)$$

where a is the number of markers shared by the two individuals, and b and c the number of markers observed in only one of each individual. The second one was Sokal and Michener's coefficient (45)

$$CSSM = a + d / (a + b + c + d)$$

where d is the number of markers absent for both individuals. The distance matrices were analyzed by the NEIGHBOR program of PHYLIP (14) using the unweighted paired group method of arithmetic averages (UPGMA) (44). Phylogram trees were generated by the DRAWGRAM program of PHYLIP.

Results and Discussion

Identification of isozyme markers: The Ridgway's buffer system was chosen, as it was found to allow faster migration (8 cm in 3 hr) and better resolution of more enzymes than the three other buffer systems tested. Nine enzymes out of the 19 assayed had sufficient activity to be reliably scored; the number of bands per enzyme ranged from one to seven (Table 2). No polymorphism was detected for AAT, ACP, and AKP. For the six other enzymes, 21 bands were reproducible and displayed polymorphism (Table 3).

We did not assign genotypes (*i.e.*, allele frequencies) on the basis of isozyme patterns, since the assignment requires segregation analysis through controlled sexual crosses (6). However, such crosses are not available for *E. lata* because the production of perithecia in culture is not currently possible. The analysis of the isozyme data was done according to the hypothesis of the independence of markers. Since alleles were not identified, different markers could be allelic forms of the same enzyme hence reducing the number of independent markers.

Isolate AM14 was responsible for the variation detected in ADH, GPI, LDH, and MDH and represented the most particular electrophoretic type (ET). The other isolates displayed variation for EST and PGM only and were classified into 11 ETs (Table 3). The differentiation of these isolates was mainly due to the polymorphism for ESTe and ESTf. In addition to

Table 3. Electrophoretic types (ET) detected among *Eutypa lata* isolates using the Ridgway's buffer system. Markers were scored 1 for presence and 0 for absence and Rf is indicated.

		Isozyme, polymorphic bands																				
		ADH		EST							GPI		LDH		MDH				PGM			
		a	b	a	b	c	d	e	f	g	a	b	a	b	a	b	c	d	a	b	c	d
	Rf[x]	0.17	0.26	0.27	0.49	0.53	0.56	0.59	0.62	0.65	0.18	0.25	0.17	0.24	0.18	0.26	0.40	0.46	0.25	0.26	0.49	0.55
Isolate	**ET**																					
AD30-1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
AM14	2	1	0	0	1	0	1	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0
BR8-1	3	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
BR8-2	3	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
BR8-3	4	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1	0	0	1	0	1
BX1-2	5	0	1	1	1	0	1	1	0	1	0	1	0	1	0	1	1	0	0	1	0	1
BX1-5	6	0	1	1	1	0	0	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
BX1-10	5	0	1	1	1	0	1	1	0	1	0	1	0	1	0	1	1	0	0	1	0	1
GI33-1	7	0	1	1	1	0	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1
GI33-6	3	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
MA26-1	8	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1
MB25	7	0	1	1	1	0	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1
NA32	9	0	1	1	0	0	1	1	0	1	0	1	0	1	0	1	1	0	0	1	0	1
RH24	10	0	1	1	1	0	1	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
VL11-3	10	0	1	1	1	0	1	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
VL11-4	11	0	1	0	1	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	0	1
VL11-5	3	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
VL11-12	12	0	1	1	1	0	1	0	0	1	0	1	0	1	0	1	1	0	0	1	1	0

[x] Mean for 3 replicates.

Table 4. RAPD types detected among *Eutypa lata* isolates using six primers. Markers were scored 1 for presence and 0 for absence and size of the fragment is indicated.

		Primer, segment in base pairs																	
	RAPD	A10			A18				A20			B02		B10	C09				
Isolate	**type**	1230	370	310	960	920	450	240	2000	460	360	1650	870	960	1230	820	760	620	310
AD30-1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	0	1	1	1
AM14	2	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
BR8-1	3	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1
BR8-2	4	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1
BR8-3	3	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1
BX1-2	5	0	1	0	0	1	1	0	0	1	1	1	1	1	0	0	1	1	0
BX1-5	6	0	1	0	0	1	1	0	0	0	1	1	0	1	0	0	1	1	1
BX1-10	7	0	1	0	0	1	1	0	0	0	1	1	1	1	0	0	0	1	0
GI33-1	8	1	1	0	1	0	1	1	1	0	1	1	1	1	0	0	1	1	0
GI33-6	9	1	1	0	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1
MA26-1	10	1	1	0	1	0	1	0	0	1	1	1	1	1	0	0	1	0	1
MB25	11	1	1	0	1	0	1	1	0	1	0	1	1	1	0	0	1	1	1
NA32	12	0	1	0	1	0	1	1	1	0	1	0	1	0	0	1	0	0	1
RH24	13	0	1	0	1	0	1	1	0	1	1	1	0	0	0	0	1	1	0
VL11-3	14	0	1	1	1	0	0	1	1	1	0	1	0	1	0	0	1	0	1
VL11-4	15	0	1	0	0	1	1	0	1	1	0	1	1	1	0	0	0	1	1
VL11-5	16	0	0	1	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1
VL11-12	17	0	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	1	1

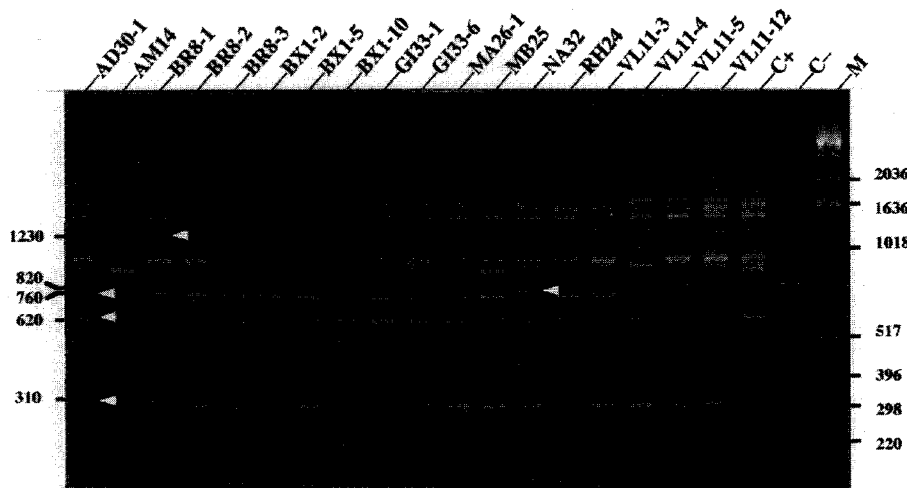

Fig. 1. RAPD patterns generated by primer C09 for 18 isolates of *Eutypa lata*. A positive control (C+) containing DNA from a third extraction of isolate BX1-10 and a negative control (C-) containing water instead of DNA were included. The fragments selected as markers are labeled with white arrows and their size in base pairs are indicated on the left. The sizes of the fragments of the 1 kb molecular weight marker (M) are indicated on the right.

the markers specific for isolate AM14 (12 out of 21), two other markers were isolate-specific; marker ESTc was only present in isolate MA26-1, and marker ESTb was only absent in isolate NA32.

Identification of RAPD markers: Based on patterns with well separated fragments of high intensity, the primers A10, A18, A20, B02, B10, and C09 were selected among the 50 tested primers. No difference in the same PCR run was observed between pattern of isolate BX1-10 and that of the positive control (a different extraction of isolate BX1-10), and a few differences among replicates (different DNA extraction and different PCR run for the same isolate) were encountered. In the absence of template DNA, some primers gave rise to amplification products which have been considered as artifacts (50). These fragments were not observed when the fungal DNA was included in the reaction mixture.

Only reproducible and intense bands were considered as potential markers. Isolate AM14 yielded very peculiar patterns, and the numerous fragments specific for this isolate were not included. A total of 18 polymorphic bands were selected as markers from the patterns obtained for the remaining isolates (Table 4). For instance, five fragments amplified using primer C09 were selected (Table 4, Fig. 1). The 18 isolates were classified into 17 different RAPD types (Table 4). The isolates BR8-1 and BR8-3 exhibited the same RAPD pattern. These isolates had different isozyme patterns, indicating some genetic differences which would surely be detected by increasing the number of RAPD markers. The absence of the marker A10-370 was specific for the isolate VL11-5, and the presence of the marker C09-820 was specific for the isolate NA32.

The RAPD markers were identified with the assumption that bands of the same size correspond to the same sequence. Since our analysis was performed at the intraspecific level, this assumption is mostly true (46), and the bands of the same size probably correspond to the same sequence for the different isolates.

Distance and cluster analysis: DSMs were lower than DJs (Table 5) since DSM contrary to DJ took into account in pair-wise comparisons the number d of markers which were absent in both individuals. Including d in the calculation may result in underestimating the distance, because the lack of a marker may result from different events (mutation, deletion, lack of the sequence). The clustering obtained with the different methods was identical for isozyme data (Fig. 2A and 2B) and very similar for RAPD data (Fig. 2C and 2D). This could indicate that the double absence of a marker has the same significance than its double presence, probably because our analysis was performed within a species.

The distance between isolate AM14 and the other isolates was very large (Table 5), and this isolate was on a separate branch in all phenograms (Fig. 2). Isolate AM14 was more clearly distinguished with isozyme analysis than with RAPD analysis (Table 5), probably because the patterns of isolate AM14 were considered for the choice of isozyme markers and not for the RAPD markers. The uniqueness of isolate AM14 indicated that it could be a variant isolate of *Eutypa lata,* another species of *Eutypa,* or a fungus from a different genus. Glawe and Rogers (17) outlined the difficulty to distinguish the different species of *Eutypa* in culture.

Although not detected by isozymes (Fig. 2A and 2B), some isolates could be clustered according to their stomatal origin using RAPD markers (Fig. 2C and 2D). However, the isolate VL11-4 from stroma 6 clustered with the BX1 isolates of stroma 3. A marker variability was observed within the stromas. Larger distances were obtained among isolates of stroma 6 in compari-

Table 5. Range of the Sokal and Michener's (DSM) and Jaccard's (DJ) distances calculated from isozymes and RAPD data obtained by analysing isolates of *Eutypa lata*.

	Isozymes		RAPD	
Isolate comparison	**DSM**	**DJ**	**DSM**	**DJ**
Between AM14 and others	0.62-0.86	0.76-0.95	0.44-0.78	0.73-1.00
Between others	0.00-0.33	0.00-0.50	0.00-0.67	0.00-0.80
Within stroma 2	0.00-0.05	0.00-0.08	0.00-0.06	0.00-0.08
Within stroma 3	0.00-0.10	0.00-0.15	0.11-0.17	0.20-0.30
Within stroma 6	0.05-0.33	0.08-0.50	0.17-0.44	0.25-0.57

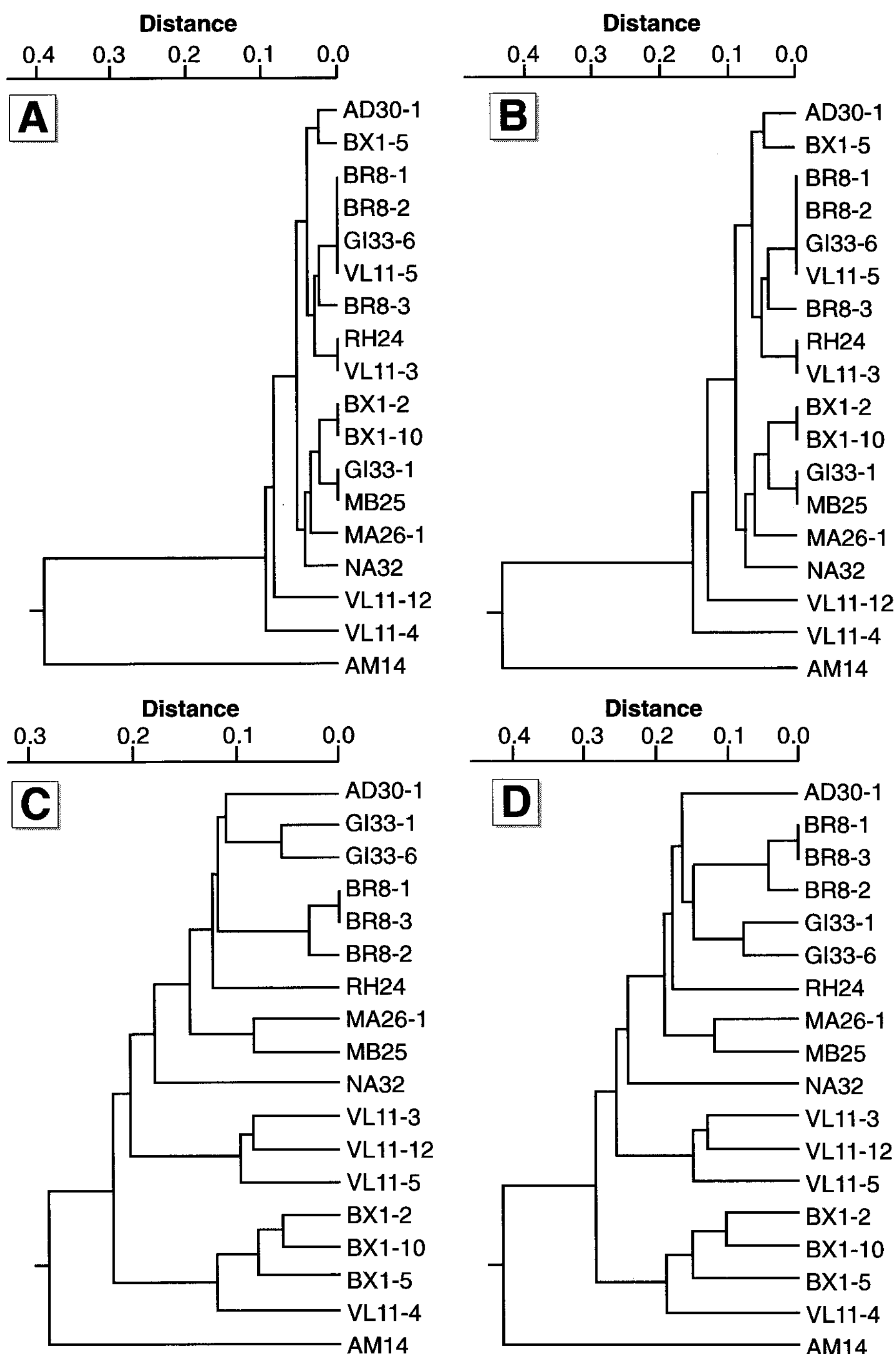

Fig. 2. Relationships among *Eutypa lata* isolates revealed by UPGMA (unweighted paired group method using arithmetic averages) cluster analysis of Sokal and Michener's distances calculated from isozymes (A) and RAPD (C) data and of Jaccard's distances calculated from isozymes (B) and RAPD (D) data.

son to the other stromas (Table 5) indicating that the level of variability was different among the stromas.

Isolates did not group according to geographic origin. Based on RAPD data (Fig. 2C and 2D), the Australian isolate AD30-1 clustered with the Switzer isolates from stroma 4, and the Switzer isolate MA26 was closely related to the isolate MB25 originated from Maine-et-Loire in France. The isolates from Aude (BR8) were more closely related to foreign isolates (NA32, AD30-1, GI33) than to the isolates from Hérault (VL11), a nearby location in France.

The two apricot isolates were not closely related and clustered with some grapevine isolates (Fig. 2). This indicated the high genetic relatedness of isolates from these two hosts. However, the analysis of a larger number of isolates from different hosts is needed to evaluate the hypothesis of non-host specificity.

In this study, we were unable to group isolates according to their pathogenicity using RAPD and isozyme analysis. Genes controlling pathogenicity most likely represent a minute fraction of DNA, whereas the markers identified in this study cover a limited part of the genome. In order to find markers correlated with pathogenicity, it could be interesting to perform further RAPD analyses with isolates differing for pathogenicity but having a similar genetic background.

Conclusions

This study represents the first attempt to identify isozymes and RAPD markers for studying the genetic variation of *E. lata*. Both types of markers were detected and revealed a high level of polymorphism among a preliminary set of isolates originated from different geographic locations. RAPDs appeared more useful than isozymes to describe the variation in *E. lata,* since RAPD analysis revealed more polymorphism than isozymes. RAPD markers enabled us to cluster most of the isolates according to their stomatal origin. No marker differentiated the isolates having the ability to reproduce the foliar symptoms in a greenhouse test and the other isolates.

The existence of a high level of genetic variation is not surprising for a fungus propagated by ascospores and has two implications. First, to provide more insight into the genetic variation and the biology of *E. lata,* it seems necessary to perform the marker analysis for isolates from individual perithecia originated from different stroma collected in the same location. It has indeed been proved that the analysis of the polymorphism at the microgeographical scale was especially appropriate for studying the population genetic (26,51) and the mating system (12,30) of fungal pathogens. Second, it indicates that different popula-

tions of the pathogen probably exist and may evolve in response to environmental changes. This characteristic must be taken into account when isolates are chosen to evaluate the grape cultivar response as well as the efficacy of fungicides.

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