Genetic Analysis of *Eutypa* Strains from California Supports the Presence of Two Pathogenic Species

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ABSTRACT

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Eutypa dieback is a perennial canker disease that adversely affects grape (*Vitis vinifera*) production throughout the world. The causal agent has been known as either *Eutypa armeniacae* or *E. lata*, and it has been unclear whether the two taxa are separate species. We analyzed 115 isolates of *Eutypa* and conspecific strains, including 106 from California, using amplified fragment length polymorphism (AFLP) and sequence analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence. Strains from cultivated plant species exhibited an average genetic distance

of 0.34, as calculated by the DICE coefficient (NTSYS-pc software). An unweighted pair-group method with arithmetic averages dendrogram revealed a genetically distinct (distance of 0.73) group of *Eutypa* strains from valley oak (*Quercus lobata*) and madrone (*Arbutus menziesii*) and a strain from grape. Analysis of rDNA ITS sequences strongly supported the genetically distinct cluster detected in the AFLP data. Combined data indicated the presence of two species of *Eutypa* (*E. armeniacae* and *E. lata*) in our sample population. However, both *Eutypa* species were capable of infecting native and cultivated hosts, suggesting the potential for native tree species to serve as inoculum sources for grape infection in California. Further investigations of *E. armeniacae* and *E. lata* would contribute to the development of a successful disease management strategy.

Eutypa dieback affects grape (*Vitis vinifera*) production throughout the world and poses a major economic threat to industries associated with grape production (5,6,16,33,35,39). The causal agent was first described as *Eutypa armeniacae* Hansf. & M.V. Carter (4,31, 32), but the species was later considered conspecific with *E. lata* (Pers.:Fr.) Tul. & C. Tul. (46). Most recent publications (35-39,42, 44) refer to the causal agent as *E. lata*, but some authors (14-16,30) continue to support the separation of the two species. For clarity, in our paper, *E. lata* refers to the species as circumscribed by Rappaz (46), and *E. lata sensu stricto* refers to the species as it was recognized prior to 1987 (with *E. armeniacae* as a separate species). Yield losses due to *Eutypa* dieback range from 19 to 50% in vines expressing moderate symptoms and from 62 to 94% in vines expressing severe symptoms (24,35). Eventually, infected vines must be removed and the vineyard replanted or disease-free scions grafted onto existing, healthy rootstock. Current methods of control include pruning late in the dormant season and treatment of pruning wounds with the benzimidazole fungicide benomyl (33,36,37,45,54,58). Although partially effective, these methods are difficult to implement in large commercial vineyards and seldom provide complete protection.

Infections occur primarily through pruning wounds, and the fungus colonizes plant vascular tissue, resulting in a chronic, lethal condition (10). Canker formation, vascular occlusion, and production of phytotoxic compounds result in yield reduction and, eventually, vine death (10,35,38). Previous reports suggest *Eutypa* infections occur only after ascospore deposition on a susceptible wound (4).

There is some evidence, however, that conidia can be infectious under optimal conditions (25). Epidemiological studies of the spatial patterns of diseased vines also suggest that disease spread is not solely due to ascospore dispersal (22).

E. lata has a wide host range, including at least 60 species (6), and causes dieback diseases on grape and other cultivated hosts, primarily apricot (4). Isolates obtained from many genera (e.g., *Ribes*, *Juglans*, *Prunus*, *Malus*, and *Salix*) can successfully colonize one or more host species. Previous studies on the pathogenicity of *E. lata* isolates derived from various host species indicate grape is a universal host (5,38). Although *E. lata* exhibits a wide range of virulence toward grape (42), no reports have demonstrated high levels of resistance to *E. lata* in any grape species tested. However, Munkvold and Marois (39) observed differences in cultivar susceptibility to *E. lata* related to wound response characteristics.

Eutypa dieback is a potentially serious problem in mature vineyards throughout California (35,37,39). As a result, the regional sources of inoculum and genetic structure of local *Eutypa* populations are of major interest. Although there is a high incidence of disease reported in the Central Valley of California, mature stroma (i.e., containing perithecia) are difficult to find on grape vines in the region (38). This, in combination with the ability of *E. lata* to infect multiple host species, suggests the potential for nongrape host species to serve as inoculum sources for Central Valley vineyards. Apricot, cherry, almond, walnut, olive, and peach are important agricultural crops in the Central Valley and are all potential sources of *E. lata* inoculum (6,38). Although *E. lata* has a wide host range, there are no reports of pathotype identification based on genetic analysis. However, it is difficult to distinguish between species of *Eutypa* based on morphology, and it is possible that the wide host range could be attributed to more than a single *Eutypa* species (14-16). To improve management of *Eutypa* dieback in California vineyards, it is necessary to (i) identify potential inoculum sources

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in native and cultivated plant species, (ii) determine whether there are pathotypes or multiple species of *Eutypa*, and (iii) elucidate the level of genetic diversity in the California population of *Eutypa*.

In recent years, numerous investigators, using biochemical and genetic techniques, have studied the population structure of phytopathogenic fungi. These techniques have included isozyme analysis (13,18,44,60), fatty acid profiling (12), sequencing of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence (1,3,34,40,47), restriction fragment length polymorphism (13,18,27,55,57), variable number tandem repeat (9,19), polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) (8,17,43,44,51), and PCR-based amplified fragment length polymorphism (AFLP) (17,28,29,41). PCR-based techniques are advantageous in that they are amenable to high throughput and require small amounts of template DNA (56,59). In the current study, we elected to use AFLP, because it is a robust, reproducible technique that generally produces a higher number of easily scored fragments than RAPD (17,23). In addition, we examined the rDNA ITS sequence in a subsample of isolates to elucidate genetic relationships at the DNA sequence level and to verify the identity of genetically distinct isolates, as determined by AFLP analysis.

Our investigation represents an initial survey of genetic diversity among *Eutypa* isolates from several *V. vinifera* production regions in California. This analysis should enable us to identify putative pathotypes or species in *Eutypa* isolates from several cultivated and wild host species. Additionally, we hope to gain insight into the epidemiology of *Eutypa* dieback in terms of inoculum sources and spread of the pathogen.

MATERIALS AND METHODS

Fungal strains. The isolates used were selected based on host species and geographic origin. AFLP analysis was performed on 112 strains of *E. lata* and 3 isolates of *Libertella viticola* (the anamorph of *E. lata*) from 10 host species (Table 1). Of the 115 isolates, 106 originated in California, 4 in Italy, 2 in Michigan, and 1 each in New York, Australia, and South Africa. For genetic comparison, one isolate of *Eutypella vitis*, another species in the family Diatrypeaceae, was included in the analysis. All isolates used have been deposited in the E. & J. Gallo Winery Culture Collection (Modesto, CA). Initial identification of *E. lata* isolates was based on comparison of the morphology of the anamorph (or teleomorph when available) with published descriptions (14–16).

DNA extraction. Isolates were grown for 3 weeks prior to harvest in 30 ml of liquid medium (2% malt extract and 0.2% yeast extract) in 125-ml Erlenmeyer flasks at room temperature (20 to 25°C) with light. Mycelial mats were collected by vacuum-filtration on Whatman No. 54 filter paper (Whatman Paper, Maidstone, England) and dried for 10 min by placing the mats between paper towels. Dried mats were ground to a fine powder in liquid nitrogen with a mortar and pestle.

DNA was extracted according to a modification of the method of Dellaporta et al. (7). Extraction buffer (100 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25% sodium dodecyl sulfate; 10 mM β -mercaptoethanol) at 65°C was added to mycelial mats at 2 ml/g, and the mixture was stored at –20°C until all isolates were ready for processing. Mixtures were incubated at 65°C for 30 min, followed by the addition of 40% (vol/vol) 5 M potassium acetate. Mixtures were stored at –20°C for 30 min followed by centrifugation at 25,000 $\times g$ for 20 min. Supernatant was filtered through Miracloth (CalBiochem-Behring Corp., La Jolla, CA), mixed with 0.58 volumes of ice-cold isopropanol, and placed at –20°C for 30 min. The resulting DNA precipitate was either spooled out on glass hooks or pelleted by centrifugation at 20,000 $\times g$ for 15 min. DNA was dried lightly prior to resuspension in 0.75 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and transfer to a 1.7-ml microcentrifuge tube. DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) followed by two extrac-

tions with chloroform-isoamyl alcohol (24:1, vol/vol). Polysaccharides were removed by adding an equal volume of 5 M NaCl, mixing well, and precipitating DNA with 2 volumes of 100% ice-cold ethanol. DNA was spooled out with a glass hook, dried, and resuspended in 500 μ l of ddH₂O. DNA was precipitated with 0.1 volumes of 7.5 M NH₄Ac and 1.1 ml of 100% ice-cold ethanol. DNA was quantified with a minifluorometer (TKO-100, Hoefer Scientific Instruments, San Francisco) following the manufacturer's instructions.

DNA sequencing. PCR was run on a PCR system (GeneAmp 9600, Perkin-Elmer Cetus Corp., Norwalk, CT). Target sequences were amplified from genomic DNA in 50- μ l reactions containing 5 μ l of GeneAmp 10 $\times$ PCR buffer, 8 μ l of 1.25 mM GeneAmp dNTPs, 0.25 μ l of AmpliTaq DNA polymerase (Perkin-Elmer, Applied Biosystems Division, Foster City, CA), 0.25 μ l each of ITS5 and ITS4 primers (58), and 36.25 μ l of dH₂O. Samples were processed through 30 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 2 min, followed by a final elongation step of 72°C for 10 min. PCR products were purified with PCR purification kits (QIAquick, Qiagen Ltd., Chatsworth, CA) to remove any excess primers, nucleotides, and polymerases. Purified PCR products (5 μ l) were run on a 1.2% agarose gel with 5 μ l of pGEM-3ZF(+) (Applied Biosystems) double-stranded DNA control template (0.2 g/liter) to estimate concentrations. All samples were sequenced directly from purified PCR products. Sequencing was performed on an automated DNA sequencer (ABI PRISM 377) using ABI PRISM dye terminator cycle-sequencing ready-reaction kits (Applied Biosystems). Cycle-sequencing products were purified over spin columns (Centri-Sep, Princeton Separations Inc., Adelphia, NJ) to remove excess primers, dye-labeled terminators, nucleotides, and polymerases. Samples were dried in a SpeedVac (Savant Instruments, Holbrook, NY) and run on the automated sequencer.

AFLP analysis. All reaction parameters and thermocycler profiles were performed following the manufacturers' instructions. PCR reactions were run on a PCR system (GeneAmp 9600). A single reaction was used to restrict DNA with *Eco*RI and *Mse*I and ligate adapters, followed by preselective amplification using the AFLP ligation kit (Applied Biosystems). Preselective amplification products were verified on agarose gels followed by selective amplification using the AFLP selective amplification kit (Applied Biosystems). Ten pairs of two selective-base primers (*Mse*I-*Eco*RI) were used in the analysis: CA-AT, CA-AC, CA-AA, CC-AC, CC-AA, CG-AC, CG-AG, CG-AA, CT-AC, and CT-AA. Approximately 0.5 μ l of selective amplification product was added to 1.5 μ l of loading buffer (1.0 μ l of formamide, 0.45 μ l of loading dye, 0.5 μ l of GS 500 ROX standard [Applied Biosystems]), denatured for 3 min at 90°C, and placed on ice.

Gel electrophoresis. Samples were analyzed on an ABI PRISM 377 automated fluorescent sequencer. Approximately 1.5 μ l of denatured sample was loaded onto a 36-cm well-to-read gel containing 5% LongRanger (FMC BioProducts, Rockland, ME) and 7 M urea in 1 $\times$ TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.45). AFLP gels used 36-lane square-tooth combs and were run at 2,650 V for 3.5 h with 1 $\times$ TBE buffer. Scans were collected from 1,000 to 8,500 data points. Sequencing gels used 36-lane square-tooth combs and were run at 1,680 V for 9 h with 1 $\times$ TBE buffer.

AFLP data analysis. AFLP analysis is a powerful tool for detecting DNA polymorphisms in genetically similar organisms. Fluorescent AFLP analysis is highly sensitive to genetic variation due to computer-generated gel files and data analysis. Low copy number DNA fragments in the genome are detectable by fluorescent AFLP and are reproducible and informative. However, the ABI PRISM 377 system for fluorescent AFLP analysis is very sensitive to artifactual errors due to sample preparation, gel electrophoresis conditions, and data analysis methods (R. DeScenzo, unpublished data). To maximize the information extracted from each primer pair, it is imperative to accurately detect the presence or absence of all fragments. To assure accuracy in AFLP data analysis, it is critical to

normalize lanes within gels to each other, as well as to lanes from the other gels used in the study. Identifying a target number of bands (TNB) for each primer pair and adjusting analysis parameters so a similar number of bands is detected for each sample effectively normalizes all samples. In our experience, this process eliminates the majority of artifactual variations initially observed with fluorescent AFLP.

Data were acquired using GeneScan version 2.1 (Applied Biosystems). Lanes were tracked manually and extracted. Initial analysis parameters were set as: analysis range of 1,400 to 8,000 scans

based on migration of 50- and 500-base GS 500 ROX standards; data processing, baseline and multicomponent options, and smooth options light checked; size call range from 50 to 500; local southern method for size calling method; split peak correction at none; and peak detection at 100 for red and the two primer colors not used, at 15 for the primer color used on the gel, and minimum peak half width at 5 points. After initial analysis, sample files were imported into GenoTyper version 2.0 (Applied Biosystems), and scale factors were calculated for each sample file. Scale factors were used to assign dye color-specific peak amplitude thresholds (PAT) for each

TABLE 1. Fungal isolates used in amplified fragment length polymorphism and rDNA internal transcribed spacer (ITS) sequence analysis

ID ^a	Origin ^b	Fungal species ^c	Host	Source	Origin ^d
E001*	Australia	<i>Eutypa armeniacae</i>	Apricot	ATCC 28120	Unknown
E002*	South Africa	<i>Eutypa armeniacae</i>	Apricot	ATCC 28900	Unknown
E050*	Illinois	<i>Eutypella vitis</i>	Grape	ATCC 64171	Unknown
E100*	Yolo Co.	<i>Eutypa lata</i>	Almond	G. Munkvold	Stromata
E101*	Sonoma Co.	<i>E. lata</i>	Blueberry	D. Gubler	Wood
E102*	Solano Co.	<i>E. lata</i>	Kiwi	D. Gubler	Wood
E103*	San Joaquin Co.	<i>E. lata</i>	Bing cherry	G. Munkvold	Stromata
E104*	Yolo Co.	<i>E. lata</i>	Bing cherry	G. Munkvold	Wood
E105*	Yolo Co.	<i>E. lata</i>	Apricot	G. Munkvold	Stromata
E106*	Solano Co.	<i>E. lata</i>	Apricot	G. Munkvold	Stromata
E107*	Merced Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood 1
E108*	Merced Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E109*	Merced Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E110*	Sacramento Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E111*	Sacramento Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E112*	Sacramento Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E113*	Napa Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E114*	Napa Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E116*	Sonoma Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E117*	New York	<i>E. lata</i>	Grape	R. Pearson	Unknown
E118*	Yolo Co.	<i>E. lata</i>	Grape	G. Munkvold	Stromata
E119*	San Joaquin Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood
E120*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 2
E121*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 2
E122*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 2
E123*	Michigan	<i>E. lata</i>	Grape	D. Ramsdell	Unknown
E124*	Michigan	<i>E. lata</i>	Grape	D. Ramsdell	Unknown
E125*	Italy	<i>E. lata</i>	Grape	S. Di Marco	Wood
E126*	Merced Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood 1
E127*	Merced Co.	<i>E. lata</i>	Grape	G. Munkvold	Wood 1
E128*	Lake Co.	<i>E. lata</i>	Grape	D. Rizzo	Wood
E129	Lake Co.	<i>E. lata</i>	Grape	D. Rizzo	Wood
E134	Suisun Co.	<i>E. lata</i>	Kiwi	D. Rizzo	Wood 3
E135	Suisun Co.	<i>E. lata</i>	Kiwi	D. Rizzo	Wood 3
E176	Stanislaus Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E177*	Sonoma Co.	<i>E. lata</i>	Valley oak ^e	E. & J. Gallo Winery	Wood 4a
E178*	Sonoma Co.	<i>E. lata</i>	Valley oak	E. & J. Gallo Winery	Wood 4a
E179*	Sonoma Co.	<i>E. lata</i>	Valley oak	E. & J. Gallo Winery	Wood 4a
E180*	Sonoma Co.	<i>E. lata</i>	Valley oak	E. & J. Gallo Winery	Wood 4a
E182	Napa Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E183	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 5a
E185	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 5a
E186	Mendocino Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 6a
E187	Mendocino Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 6a
E188*	Stanislaus Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E189*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7a
E190*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7a
E191	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7
E192*	Mendocino Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E193	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7b
E194	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7b
E195	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7c

(continued on next page)

^a * indicates isolate was used in rDNA ITS region DNA sequence analysis.

^b Counties are in California.

^c As originally identified.

^d Wood followed by the same number indicates same vineyard or orchard; wood followed by the same letter indicates same vine or tree.

^e *Quercus lobata*.

^f *Prunus* sp.

^g *Arbutus menziesii*.

^h *Ceanothus* sp.

lane in a gel. Analysis parameters were set (as above) for each lane based on the adjusted PAT. Lanes were reanalyzed, and the number of bands detected per lane was determined. The lane with the lowest assigned PAT was used to determine the TNB for each primer pair. PAT was adjusted, when necessary, in the other 35 lanes to detect TNB plus or minus three bands. The results from the gel analysis were exported into a text file containing all band information for each lane. Text files were reformatted for use with the AFLP program (W. Lamboy, Cornell University, Geneva, NY), which converted the text data into a binary format for further analysis. A

band in one lane was scored as 1 for presence or 0 for absence. Analysis parameters for AFLP were set as: minimum peak size = 15; minimum fragment size (base pairs) = 50; maximum fragment size (base pairs) = 500; interval length (base pairs) in which there can only be one fragment in a lane = 0.8; and maximum difference in length between two fragments considered the same size = 0.4.

The binary data files from 10 primer pairs were combined into a single file and formatted for NTSYS-pc version 2.02f (Exeter Biological Software, Setauket NY). Similarity was determined using the SIMQUAL module and DICE coefficient. A dendrogram was

TABLE 1. (continued from preceding page)

ID ^a	Origin ^b	Fungal species ^c	Host	Source	Origin ^d
E196*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7c
E197	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7c
E198*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7c
E199*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7d
E200	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7d
E201*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8
E202*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8
E203*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8a
E204	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8b
E205*	Fresno Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E206*	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E207	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9a
E208*	Solano Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 10
E209*	Solano Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 10
E210	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8a
E211	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8a
E212	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8a
E213	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8a
E214*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8c
E215*	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8c
E216	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8b
E217*	Merced Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E218	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 11a
E219	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 11a
E220	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E221	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9a
E222	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9a
E224	Solano Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 10a
E225	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9b
E226	Stanislaus Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E227*	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7d
E228	Madera Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 7d
E229	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8c
E230*	Sonoma Co.	<i>E. lata</i>	Cherry ^f	E. & J. Gallo Winery	Wood 8
E231	Merced Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E232	Sonoma Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 8c
E233	Fresno Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood
E234	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9b
E235	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9a
E236	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 12a
E237	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 12a
E238	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 12a
E239	San Joaquin Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 12
E240	Yolo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 9a
E241	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13a
E242	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13a
E243*	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13b
E244	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13b
E245	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13c
E246	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13c
E247*	El Dorado Co.	<i>E. lata</i>	Madrone ^g	E. & J. Gallo Winery	Wood 13d
E248	El Dorado Co.	<i>E. lata</i>	Buckthorn ^h	E. & J. Gallo Winery	Wood 13e
E249*	El Dorado Co.	<i>E. lata</i>	Buckthorn	E. & J. Gallo Winery	Wood 13e
E250*	El Dorado Co.	<i>E. lata</i>	Buckthorn	E. & J. Gallo Winery	Wood 13e
E251*	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13f
E252*	El Dorado Co.	<i>E. lata</i>	Madrone	E. & J. Gallo Winery	Wood 13d
E253*	El Dorado Co.	<i>E. lata</i>	Madrone	E. & J. Gallo Winery	Wood 13d
E254*	El Dorado Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 13f
E255*	San Luis Obispo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 14
E256*	San Luis Obispo Co.	<i>E. lata</i>	Grape	E. & J. Gallo Winery	Wood 14
L001*	Italy	<i>Libertella viticola</i>	Grape	S. DiMarco	Wood
L002*	Italy	<i>L. viticola</i>	Grape	S. DiMarco	Wood
L003*	Italy	<i>L. viticola</i>	Grape	S. DiMarco	Wood

constructed using the unweighted pair-group method with arithmetic averages (UPGMA) in the SAHN module. Additional analysis was performed on a subset of 67 isolates with the combined data from 10 primer pairs for comparison with ITS sequence data. In addition, DICE similarity was calculated for both the entire data set, as well as the subset, and for each primer pair. Pearson product moment correlation coefficient (r) was calculated between individual DICE-based distance matrices for each primer pair to compare the ability of different AFLP primer pairs to detect genetic diversity. In addition, the r value can be used as an indicator of genome coverage. A high r value among a set of primers suggests the selected primers amplify similar areas of the genome.

DNA sequence analysis. Sequence data were imported into Sequence Navigator version 1.0.1 (Applied Biosystems), in which forward and reverse reads were aligned. CLUSTAL W (53) was used for multiple alignments, sequences were transferred into a PAUP version 4.0b1 (Sinauer Associates Inc., Sunderland, MA) file, and alignment was verified visually.

Tamura-Nei (52) distances for each spacer sequence were compared to determine whether spacers diverged at different rates. The spacer sequences were not analyzed separately because they diverged at the same rate (discussed below) and the individual DNA fragments were too short to recover a meaningful phylogeny (2,48).

PAUP software was used for all sequence analyses. Heuristic (tree-bisection and reconnection) search algorithms were used because the large number of isolates precluded exhaustive searches. Each nucleotide was treated as an unordered character with four alternative states, and gaps were treated as missing characters. Phylogenetic hypotheses were generated for the DNA sequence data under the principle of maximum parsimony with 10 random addition sequences with equally weighted characters. Support for internal nodes was assessed by bootstrapping (11) with 500 replicates. To evaluate the sensitivity of tree topology to the algorithm used, trees also were generated for the DNA sequence data by the neighbor-joining algorithm (49) with both Kimura-2 parameter (26) and Tamura-Nei (52) distance measures.

Somatic compatibility analysis. Isolates of *E. lata* were grown in 100 × 15-mm petri plates on potato dextrose agar (PDA) (Difco Laboratories, Detroit). Agar plugs (5-mm diameter) containing mycelia from the colony margins were paired on fresh PDA plates for somatic compatibility testing. plates were incubated at 25°C in the

dark for 6 weeks. Multiple isolates from the same vine were tested against each other and self-paired. In addition, selected isolates from different locations were paired against each other. Plates were scored for compatibility reactions: strong = definite barrage reaction with an easily visible, dark, depressed region; weak = clear area between two isolates devoid of mycelia; and no reaction. All pairings were repeated at least three times.

RESULTS

AFLP analysis. Analysis of fungal DNA was reproducible when selective amplification was repeated on the same set of preselective amplification products. Artifactual variation in reproducibility was observed most often when DNA from different isolations was examined. Additional artifacts also can be introduced, to a lesser extent, during the restriction-ligation reaction step (R. DeScenzo, unpublished data).

Initial data collection and formatting were performed using GeneScan version 2.1. Individual analysis parameter files were configured for each primer-lane combination to achieve the predetermined TNB. The TNB for the 10 primer combinations varied from 35 to 76 bands per lane (Table 2). Conversion of GeneScan data to a binary format using the AFLP program yielded a different number of band classes for each primer pair (Table 2). A total of 3,153 band positions was observed for the 10 primer pairs. There were 137 monomorphic band positions, 790 autapomorphic band positions, and 2,226 polymorphic band positions. Similarity matrices generated from individual primer pairs were compared using the Pearson product moment correlation. Correlation coefficients (r) ranged from 0.58 to 0.94 (Table 3). Primer pair CT-AC had the lowest r value when paired with other primers. A low correlation coefficient indicated the two primer pairs amplified markers from genetically different or specific regions of the genome. A high level of correlation between primer pairs indicated the AFLP markers either were from genetically similar regions of the genome or widely distributed throughout the genome. The overall high r values observed between most of the primer pairs used in this study suggest the AFLP markers are probably widely distributed throughout the genome.

The DICE similarity coefficient was calculated from a total of 365,748 data points. A dendrogram was generated using the genetic similarity matrix for the 1 *Eutypella vitis* strain and 115 *Eutypa* strains. The matrix was computed using the combined AFLP data from 10 primer-pair data sets. The dendrogram consisted of one large cluster (Fig. 1, group A1) and one small cluster (Fig. 1, group B). Group B consists of four strains from valley oak (*Quercus lobata*), three strains from madrone (*Arbutus menziesii*), and one strain from grape. The strains were separated from the remaining 107 isolates by a genetic distance of 0.73. Group A1 consisted primarily of one cluster containing 104 strains at an overall genetic distance of ≈0.45. The genetic distance of the remaining three *Eutypa* isolates (Fig. 1, group A2), from California, Michigan, and New York, and the *Eutypella vitis* isolate ranged from 0.68 to 0.62. The three A2 *Eutypa* strains may represent an intermediate genotype between the isolates from groups A1 and B. Within group A1, individual isolates from the same vineyard in California often clustered together. However, there was no evidence of clustering based on

TABLE 2. Target number of bands and number of band classes observed in *Eutypa lata* for individual amplified fragment length polymorphism primer pairs

Primer pair (<i>MseI-EcoRI</i>)	Target no. of bands	No. of band classes
CA-AA	71	319
CA-AC	45	303
CA-AT	70	339
CC-AA	76	323
CC-AC	52	324
CG-AA	60	305
CG-AC	35	300
CG-AG	55	313
CT-AA	70	299
CT-AC	50	328

TABLE 3. Correlation values (r) for DICE similarity matrices between individual primer pairs (*MseI-EcoRI*) used to analyze *Eutypa lata*

	CA-AC	CA-AT	CC-AA	CC-AT	CG-AA	CG-AC	CG-AG	CT-AA	CT-AC
CA-AA	0.83	0.92	0.91	0.71	0.91	0.92	0.87	0.79	0.60
CA-AC		0.80	0.81	0.69	0.83	0.82	0.82	0.73	0.58
CA-AT			0.94	0.76	0.93	0.90	0.86	0.81	0.64
CC-AA				0.72	0.93	0.89	0.87	0.81	0.64
CC-AC					0.73	0.71	0.70	0.77	0.77
CG-AA						0.89	0.88	0.82	0.63
CG-AC							0.85	0.77	0.58
CG-AG								0.75	0.59
CT-AA									0.65

Fig. 1. Dendrogram generated by unweighted pair-group method with arithmetic average cluster analysis of amplified fragment length polymorphism (AFLP) data from 115 isolates of *Eutypa lata* and 1 isolate of *Eutypella vitis*. Genetic similarities were calculated using the DICE coefficient in the SIMQUAL module of NTSYS-pc (version 2.02f), based on 3,153 band positions observed for 10 AFLP primer pairs. Clusters are indicated by brackets.

geographic area, i.e., North Coast, Central Valley, etc., with AFLP data. A tree produced by parsimony analysis of AFLP data (not shown) maintained topological congruency with the DICE-UPGMA analysis.

Strains from four native host species were examined. The three strains from madrone and four strains from valley oak were genetically distinct from the majority of *Eutypa* strains. Strains from native buckthorn (*Ceanothus* sp.) and cherry (*Prunus* sp.) were not

Fig. 2. Strict consensus of the six most parsimonious trees from analysis of nucleotide sequences spanning ITS1 and ITS2 for a subset of 66 *Eutypa lata* isolates and 1 *Eutypella vitis* isolate, showing support for internal nodes from bootstrap resampling (≥ 50 shown) (length = 232, consistency index = 0.8190, retention index = 0.7730). Clusters are indicated by brackets.

genetically distinct from the majority of strains. Strains obtained from buckthorn were genetically closest to strains from grape in an adjacent vineyard. The isolate of *Eutypella vitis* was genetically distinct from group A1. However, it was more similar to groups A1 and A2 than to *Eutypa* isolates from group B.

ITS DNA sequence. The 5.8 S rDNA sequences were identical in all isolates and, therefore, were excluded from the analysis. The final DNA sequence data set included 380 bp that spanned all of the ITS1 and ITS2 for the 1 *Eutypella vitis* isolate and 66 *Eutypa* isolates (Table 1). Many isolates had identical sequences. Therefore, the 66 *Eutypa* isolates exhibited only 30 sequence haplotypes; the most prevalent haplotype was present in 11 isolates from various geographic regions. The number of nucleotide differences among isolates of *Eutypa* ranged from 0 to 46, whereas the number of differences among *Eutypella vitis* and *Eutypa* isolates ranged from 50 to 67. No difficulties were encountered in aligning the sequences. Variation in length was observed in both spacer sequences. ITS1 varied from 166 to 199 bp (excluding gaps) in *Eutypa* compared with 169 bp in *Eutypella vitis*, and ITS2 varied from 157 to 161 bp (excluding gaps) in *Eutypa* compared with 167 bp in *Eutypella vitis*. Much of the variation in length in *E. lata* ITS1 was due to an 11-bp minisatellite tandemly repeated from one to four times from positions 66 to 109. The vast majority of isolates possessed three repeats. One isolate each exhibited one (E251), two (E117), and four (E189) repeats. Of the 380 total characters, 207 were constant, 94 were parsimony-uninformative, and 79 were parsimony-informative.

Pairwise comparisons of Tamura-Nei (52) genetic distances across all taxa for the two spacer sequences produced a strong correlation ($r = 0.964$), verifying that they diverged at essentially the same rate. However, in *Eutypa*, ITS1 is slightly more variable than ITS2, as has been found in other filamentous fungi (34). A parsimony analysis in which all nucleotide substitutions were weighted equally produced six equiparsimonious trees, the consensus of which is shown in Figure 2. Neighbor-joining analyses with both Kimura-2 parameter and Tamura-Nei (52) distance measures produced a topology with an overall arrangement of taxa congruent with that shown in Figure 2. The use of different distance measures with different underlying assumptions did not affect topology. There was strong support for a clade containing the *Eutypa* isolates from madrone and valley oak trees, as well as an anomalous isolate from grape, which agrees with the AFLP data (group A2). There was only weak to moderate support for the internal branches in the remainder of the tree, although there was strong support for several branches leading to terminal polytomies. Therefore, it may be more accurate to state that a basal polytomy exists between isolates that are more derived than the New York-Michigan clade.

Somatic compatibility. Isolates obtained from the same or adjacent vines were tested for somatic compatibility. There were no incompatible reactions observed in any of the self-pairings. All self-pairings resulted in mycelia that grew together with no visible barrage zone (Fig. 3). In all nonself-pairings, isolates exhibited somatic incompatibility and produced barrage zones ranging from weak to strong. A faint, yet definite, zone with sparse mycelia was observed in weak reactions. In contrast, barrage zones devoid of mycelia and with a visibly depressed, darkened region were observed in strong reactions (Fig. 3). In all pairings in which the genetic distance was less than 0.15, a weak reaction was observed. In pairings in which the genetic distance was greater than 0.15, a strong reaction was observed. In addition, pairings of any of the other selected strains with isolate E252 resulted in extensive mycelial lysis of the paired strain (Fig. 3D).

DISCUSSION

In this study, we observed a genetically distinct group of eight *Eutypa* isolates, group B, that consisted of three isolates from madrone, four isolates from valley oak, and one isolate from grape. The results support the concept of two species of *Eutypa* (*E. lata*

sensu stricto and *E. armeniaca*), both capable of infecting grape and native hosts. Prior to 1987, *E. armeniaca* and other taxa were not synonymous with *E. lata* (30). McKemy et al. (30) initially suggested that *E. armeniaca* be retained as the name for pathogenic strains from diseased, cultivated hosts. Previous studies, based on morphology and pathogenicity (14–16), have suggested that the species predominantly found on grape and cultivated hosts (group A1) is *E. armeniaca* and is distinct from *E. lata*. The presence in group A1 of two strains obtained from the American Type Culture Collection (Manassas, VA) and originally identified as *E. armeniaca* supports this classification. We suggest that strains found predominantly on native hosts (group B) are probably *E. lata sensu stricto*.

Group B (*E. lata sensu stricto*) was more distant from group A1 (*E. armeniaca*, associated with cultivated hosts) than was *Eutypella vitis*, another member of the family Diatrypeaceae. Group B was linked at a genetic distance of ≈ 0.73 to the remaining 107 *Eutypa* strains. Group A2, consisting of three strains from grape, had a genetic distance ranging from 0.62 to 0.68. The AFLP data, obtained using two selective-base primers, indicate that the *Eutypella vitis* isolate is more similar to group A1 than to group B, which suggests the strains from group B and A2 may represent different taxa than isolates from group A1.

Data from the 95 California strains collected from nonnative species indicate an overall high level of variability, with an average genetic distance of 0.34. Based on RAPD analysis, Péros et al. (44) reported a high level of genetic variation (Jaccard distance of ≈ 0.3) in a sample population of 18 *E. lata* isolates representing 10 geographic regions in France. They reported polymorphisms between isolates from the same stroma and observed that isolates from different stromata generally were separated in cluster analysis. In a later study, Péros et al. (43) analyzed 32 polymorphic RAPD markers in a population of 55 *E. lata* isolates from a single vineyard in France. They detected the same high level of genetic variation within the vineyard, with an average Jaccard distance of 0.36. These levels of genetic diversity are expected for a pathogen that is primarily dispersed by sexual spores. Péros et al. (43) were unable to correlate pathogenicity with a particular RAPD profile, and UPGMA analysis did not indicate any population substructure. They did observe one genetically distinct isolate in their study but were unable to positively identify it as *E. lata* based solely on RAPD and iso-

Fig. 3. Somatic compatibility interactions of *Eutypa* isolates from the same or adjacent vines. **A**, Self-pairing; **B**, strong reaction; **C**, weak reaction; and **D**, isolate E252 (bottom) caused mycelial lysis when paired with other isolates.

zyme analyses. They suggested this isolate could be a variant of *E. lata* or a different species of *Eutypa*.

DNA sequence comparisons can provide a more direct assessment of genetic diversity and relationships and, therefore, can be extremely informative if the correct DNA region is analyzed. The rDNA ITS region is not highly conserved, and a number of studies have demonstrated its usefulness for resolving species and subspecies differences (20,40,47). The ITS sequence data in our study strongly support the distinction, made also based on AFLP data, of the group B cluster (Fig. 2). The data also moderately support the separation of group A2 isolates from the other isolates. The three A2 isolates are more similar to group A1 than to group B based on ITS sequence data. The average transition to transversion ratio between groups A1 and A2 is 3.2, whereas the ratio between groups A1 and B is 0.89. The balance of the tree, however, with the exception of branches leading to terminal polytomies, remains poorly resolved. Similar levels of ITS sequence identity have been observed in *Acremonium* and *Epichloe* (50), *Armillaria* (1), *Sclerotium* (3), *Phomopsis* (47), and *Ganoderma* (21). Therefore, the evolutionary rate of the ITS region, coupled with its short length in certain genera of fungi, appears to greatly impair its phylogenetic utility at the subspecific level, which reduces its effectiveness for ascertaining intraspecific relationships, while enhancing its usefulness for determining species boundaries.

Isolates from individual California counties in specific geographic regions, i.e., North Coast, Central Coast, and Central Valley, did not display greater similarity to each other than to isolates from different regions. However, genetic analyses indicated that isolates from a single vineyard or vine were more genetically similar to each other than to those from different locations. Somatic compatibility assays indicated the absence of clonal lineages among *Eutypa* isolates tested and, therefore, agreed with the genetic differences observed between isolates. Fungal somatic compatibility is regulated by several polymorphic loci, and only strains with identical alleles at each locus are compatible. Péros et al. (43) observed somatic incompatibility between all *Eutypa* isolates tested. Interestingly, they also observed one isolate that caused extensive mycelial lysis when paired with other isolates, similar to isolate E252 in our study.

The absence of somatic compatibility between isolates obtained from the same vine and their distinct AFLP fingerprints and ITS sequences can help verify multiple infections in a single vine. The similarity of same-vine isolates suggests a common source of inoculum. It is highly probable that ascospores from a single perithecialium or stroma could produce multiple infections on a single vine. The AFLP and ITS sequence data corroborate this theory, because many of the isolates from single or adjacent vines formed discrete groups in cluster analysis.

The data presented here demonstrate the efficiency and applicability of AFLP and ITS sequence analyses for studying genetic similarity in *Eutypa*. In addition, our study represents the first step in using molecular tools to understand the genetic structure of *Eutypa* populations in California. The data suggest the presence of two species of *Eutypa* isolated from native and cultivated hosts. Three strains obtained from buckthorn (*Ceanothus* sp.) were not genetically distinct from *E. armeniaca*. The buckthorn bordered a vineyard, and strains from the buckthorn were genetically closest to *E. armeniaca* isolates from the adjacent vineyard, suggesting the potential for cultivated species to be sources of *E. armeniaca* inoculum for native species and vice versa. We were unable to determine whether the one isolate from grape in the *E. lata* sensu stricto group was pathogenic or saprophytic on grape. However, its isolation from a canker margin on a symptomatic plant suggests the isolate was pathogenic. This finding opens the possibility of native hosts acting as reservoirs for *E. lata* sensu stricto inoculum on grape.

The ability of either species to infect both grape and native hosts warrants additional investigations into native hosts as reservoirs for a genetically diverse source of *Eutypa* inoculum. The presence of two species that are both capable of infecting grape and the

potential of unlimited inoculum from native hosts would complicate efforts to control this disease. A comprehensive understanding of the genetics of these pathogens would contribute to the development of a successful disease management strategy.

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