Host Range, Biological Variation, and Phylogenetic Diversity of *Eutypa lata* in California

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ABSTRACT

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The objectives of this study were to investigate the host range of *Eutypa lata* in the major grape-growing regions in California and to analyze the phenotypic variation and phylogenetic diversity of *E. lata* isolates. Perithecia of *E. lata* were found on grapevines, in apricot, almond, cherry, apple, and pear tree orchards, and on ornamentals (oleander) and native plant species (California buckeye, big leaf maple, and willow). Multigene phylogenies of ribosomal DNA internal transcribed spacer, β -tubulin, and *RPB2* genes confirmed the identity of *E.*

lata recovered from the various host plants but also revealed sequence differences among isolates. The intraspecific phylogenetic diversity of *E. lata* did not correspond to geography or source of isolation, and intraspecific groups were not consistent across the different DNA phylogenies. Significant phenotypic variation also was detected among *E. lata* isolates, including ascospore and conidium length as well as level of aggressiveness on grapevines. Pathogenicity studies proved that all isolates were infectious to grapevine, suggesting that the native vegetation surrounding vineyards can serve as inoculum sources that may constitute an important element in the epidemiology of *Eutypa* dieback in grapevines.

Eutypa lata (Pers.) Tul. & C. Tul. (syn: *E. armeniaca* Hansf. & M. V. Carter) (*Diatrypaceae*) is the pathogen responsible for a widespread and destructive fungal disease, mainly of grapevine (*Vitis vinifera*), apricot (*Prunus armeniaca*), and sweet cherry (*P. avium*), known as *Eutypa* dieback (2–4,8,22,26). In California, *Eutypa* dieback constitutes one of the most common fungal canker diseases of grapevine, particularly in the North and Central Coasts and in the Sacramento and Northern San Joaquin Valleys (52). Infection normally occurs during the pruning season when fresh pruning wounds become exposed to wind-dispersed ascospores, which are released following rainfall of ≈ 2 mm or greater (4,20). Typical symptoms of *Eutypa* dieback in grapevine include formation of wedge-shaped cankers in wood; death of spurs, cordons, and eventually the entire grapevine; leaf necrosis and chlorosis; and tattering as well as stunting of new shoots (2,4,22). *Eutypa* dieback also affects yield and longevity of vineyards and usually becomes more severe as vineyards become older (25). Recently, other pathogens in the families *Diatrypaceae* and *Botryosphaeriaceae* have been associated with grapevine trunk diseases in California (50–52). Overall, grapevine trunk diseases are responsible for significant economic losses to the wine industry estimated to be up to \$260 million annually (42).

Ascospores of *E. lata* are the primary form of inoculum and they infect grapevines through fresh pruning wounds during periods of rainfall (3,4,20). Ascospores are released from mature perithecia clustered in a black stroma embedded in decorticated wood or, sometimes, in the bark on dead trunks or branches of lignified angiosperms (3,4). *E. lata* perithecia are thought to develop in areas with at least 350 mm of annual rainfall (4). Perithecia have been observed on most continents (4) and have been found on at least 88 host plant species in 28 plant families (5). Until recently in California, only a few hosts of *E. lata* were

known (*Ceanothus* spp., *P. armeniaca*, *P. avium*, *P. dulcis*, *P. virginiana* var. *demissa*, and *V. vinifera*) (5,7,9,21,23,26). The inoculum that infects grapes in the Central Valley of California was thought to originate primarily from apricot trees in the Suisun Bay area on the east end of San Francisco (28,36), because perithecia had not been found on grapevines in the Central Valley. Thus, ascospores of *E. lata* were thought to travel long distances on air currents from coastal areas where perithecia were found (20,28,36) to the interior Central Valley of California and North Coast production areas, including Napa, Sonoma, and Mendocino Counties. Additional reports of the host range of *E. lata* in California have included *V. vinifera*, *P. avium*, and *P. dulcis* in Sacramento, Yolo, San Joaquin, and Napa Counties (26). However, despite the previous surveys, only a few individuals among the various host species were confirmed to support formation of perithecia; therefore, their importance as sources of inoculum as well as the current distribution of the perithecial stage of *E. lata* remained unclear. Identifying the inoculum sources for the disease constitutes critical knowledge of disease epidemiology and may ultimately help to reduce risks of infection if these sources can be eliminated.

Little effort has been made to analyze the population structure of *E. lata*, including isolates from a wide range of host plants, particularly isolates originating from both cultivated and indigenous plant species. The study of DeScenzo et al. (6) suggested the occurrence in California of two species of *Eutypa* (*E. armeniaca* and *E. lata*), both capable of infecting grape. They proposed *E. lata* sensu stricto to refer to strains found predominantly on native hosts and separated it from *E. armeniaca*, which they referred to strains associated with cultivated hosts. Subsequent studies of the strains used by DeScenzo et al. (6) revealed that strains from native host plants in California were, indeed, *Diatrype stigma* (Hoffm.) Fr. (40) and the occurrence of *E. lata* (syn: *E. armeniaca*) on native hosts remained unknown.

The objectives of this study were to (i) establish the current distribution and host range of the perithecial stage of *E. lata* in California's main grape-growing regions, (ii) characterize the

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phenotypic and phylogenetic diversity of California population of *E. lata*, and (iii) test the pathogenicity in grapevine shoots of *E. lata* isolates collected from various host plants and regions in California.

MATERIALS AND METHODS

Surveys. During the years 2000–2002, we surveyed the main grape-producing regions of California for perithecia of *E. lata*. Surveys were conducted in vineyards and orchards and on native and ornamental woody plants surrounding vineyards in the counties of Napa, Sonoma, Solano, Sacramento, San Joaquin, Contra Costa, El Dorado, San Benito, Stanislaus, Merced, Fresno, Madera, Tulare, Kern, and Yolo. Plants with dead wood and dieback were checked for the presence of typical fruiting bodies of *E. lata*.

Isolate origin and cultivation. Isolates used in this study are presented in Table 1. *E. lata* isolates were obtained from perithecia on various woody host plants and from hyphae in infected wood of grapevines and apricot trees in California. Referenced isolates of *E. lata* and *Eutypa* spp. most closely

related to *E. lata* (1,37,40) were used in the phylogenetic study. These included *E. lata* var. *aceri*, *E. laevata*, and *E. petrakii* var. *petrakii*. Isolates obtained from perithecia were cultured using ascospores as described by Trouillas et al. (51). Colonies were grown on potato dextrose agar (PDA) amended with 100 ppm of tetracycline (PDA-tet). Hyphal tips were put on new PDA-tet plates to grow pure cultures. To recover fungi from cankers, the wood surface was treated with 0.5% sodium hypochlorite for 5 min, after which fragments of necrotic tissue were removed from leading edge of canker margins and placed on petri plates containing PDA-tet. Isolation plates were incubated in the laboratory for 1 week at the ambient laboratory light and temperature conditions.

Identification and morphological study. Identifications of the perithecia of *E. lata* were done using the keys and descriptions of Carter and Rappaz (4,37) and by comparison with type specimens of *E. lata* (L 0112789-0112795 under *Sphaeria lata*, Neotype) and *E. armeniaca* (WARI 4987, Holotype). Internal transcribed spacer (ITS) and β -tubulin sequences of *E. lata* isolates from California also were compared with GenBank sequences of *E. lata* isolate CBS208.87, which was referred to as *E. lata* sensu

TABLE 1. Isolates collected for this study and used either in the morphological, phylogenetic, or pathogenicity studies

Isolate ID ^v	Fungal species	Host	Origin ^w	Collector ^x	GenBank accession no.		
					ITS rDNA ^y	β -Tubulin	RPB2 ^z
CAB5A c	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Napa Co.	F. P. Trouillas			
DCA700 b	<i>E. lata</i>	<i>V. vinifera</i>	Napa Co.	F. P. Trouillas	HM164714	HM164748	HM164782
DCA900b	<i>E. lata</i>	<i>V. vinifera</i>	Sonoma Co.	F. P. Trouillas	HM164715	HM164749	HM164783
DHB100 b	<i>E. lata</i>	<i>V. vinifera</i>	Sonoma Co.	F. P. Trouillas			
EAMS100 b	<i>E. lata</i>	<i>V. vinifera</i>	Yolo Co.	F. P. Trouillas	HM164730	HM164764	HM164798
UCD379St c	<i>E. lata</i>	<i>V. vinifera</i>	Stanislaus Co.	J. R. Úrbez-Torres	HM164705	HM164739	HM164773
UCD401Mo c	<i>E. lata</i>	<i>V. vinifera</i>	Monterey Co.	J. R. Úrbez-Torres	HM164706	HM164740	HM164774
UCD715SJ c	<i>E. lata</i>	<i>V. vinifera</i>	San Joaquin Co.	J. R. Úrbez-Torres	HM164707	HM164741	HM164775
UCD732SJ c	<i>E. lata</i>	<i>V. vinifera</i>	San Joaquin Co.	J. R. Úrbez-Torres	HM164708	HM164742	HM164776
UCD777St c	<i>E. lata</i>	<i>V. vinifera</i>	Stanislaus Co.	J. R. Úrbez-Torres	HM164709	HM164743	HM164777
UCD795St c	<i>E. lata</i>	<i>V. vinifera</i>	Stanislaus Co.	J. R. Úrbez-Torres	HM164710	HM164744	HM164778
DDES200 b	<i>E. lata</i>	<i>Acer macrophyllum</i>	El Dorado Co.	F. P. Trouillas			
DCA200 b	<i>E. lata</i>	<i>A. macrophyllum</i>	Napa Co.	F. P. Trouillas	HM164718	HM164752	HM164786
DAP100 b	<i>E. lata</i>	<i>Prunus armeniaca</i>	Contra Costa Co.	F. P. Trouillas	HM164713	HM164747	HM164781
DRO100 b	<i>E. lata</i>	<i>P. armeniaca</i>	Stanislaus Co.	F. P. Trouillas	HM164724	HM164758	HM164792
DJAM100 b	<i>E. lata</i>	<i>P. avium</i>	San Joaquin Co.	F. P. Trouillas	HM164719	HM164753	HM164787
DMUNK100 b	<i>E. lata</i>	<i>P. avium</i>	San Joaquin Co.	F. P. Trouillas	HM164720	HM164754	HM164788
USC100 b	<i>E. lata</i>	<i>P. avium</i>	Santa Clara Co.	F. P. Trouillas	HM164729	HM164763	HM164797
DCE624 c	<i>E. lata</i>	<i>Malus domestica</i>	Mendocino Co.	F. P. Trouillas			
DNA100 b	<i>E. lata</i>	<i>Malus</i> sp.	Napa Co.	F. P. Trouillas	HM164721	HM164755	HM164789
DPEAR100 b	<i>E. lata</i>	<i>Pyrus communis</i>	El Dorado Co.	F. P. Trouillas	HM164722	HM164756	HM164790
DPEAR200 b	<i>E. lata</i>	<i>P. communis</i>	El Dorado Co.	F. P. Trouillas	HM164723	HM164757	HM164791
DRUSS100 b	<i>E. lata</i>	<i>Prunus dulcis</i>	Yolo Co.	F. P. Trouillas	HM164725	HM164759	HM164793
DCHR100 b	<i>E. lata</i>	<i>Salix cf lasiolepis</i>	Napa Co.	F. P. Trouillas	HM164716	HM164750	HM164784
DCHR200 b	<i>E. lata</i>	<i>S. cf lasiolepis</i>	Napa Co.	F. P. Trouillas	HM164717	HM164751	HM164785
DSA200 b	<i>E. lata</i>	<i>Salix</i> sp.	San Joaquin Co.	F. P. Trouillas	HM164726	HM164760	HM164794
DSA300 b	<i>E. lata</i>	<i>Salix</i> sp.	Contra Costa Co.	F. P. Trouillas			
DSA400 b	<i>E. lata</i>	<i>Salix</i> sp.	Santa Clara Co.	F. P. Trouillas	HM164727	HM164761	HM164795
DSA500 b	<i>E. lata</i>	<i>Salix</i> sp.	Napa Co.	F. P. Trouillas	HM164728	HM164762	HM164796
WILLOWC c	<i>E. lata</i>	<i>Salix</i> sp.	Napa Co.	F. P. Trouillas	HM164731	HM164765	HM164799
DCHES200 b	<i>E. lata</i>	<i>Aesculus californica</i>	Sonoma Co.	F. P. Trouillas	HM164732	HM164766	HM164800
OLEA2 b	<i>E. lata</i>	<i>Nerium oleander</i>	Yolo Co.	F. P. Trouillas			
OLEA3 b	<i>E. lata</i>	<i>N. oleander</i>	Yolo Co.	F. P. Trouillas	HM164733	HM164767	HM164801
3802 b	<i>E. lata</i>	<i>Frangula alnus</i>	Roches, Switzerland	F. Rappaz	HM164711	HM164745	HM164779
1908021 b	<i>E. lata</i>	<i>Hedera helix</i>	Roches, Switzerland	F. Rappaz	HM164712	HM164746	HM164780
CBS217.87 a	<i>E. lata</i> var. <i>aceri</i>	<i>Acer campestre</i>	France	F. Rappaz	HM164734	HM164768	HM164802
CBS290.87 a	<i>E. lata</i> var. <i>aceri</i>	<i>A. pseudoplatanus</i>	Switzerland	F. Rappaz	HM164736	HM164770	HM164804
CBS291.87 a	<i>E. laevata</i>	<i>Salix</i> sp.	Switzerland	F. Rappaz	HM164737	HM164771	HM164805
DSA600 b	<i>E. cf laevata</i>	<i>Salix lasiolepis</i>	San Joaquin Co.	F. P. Trouillas	HM164738	HM164772	HM164806
DRSN100 b	<i>E. leptoplaca</i>	<i>V. vinifera</i>	Sonoma Co.	F. P. Trouillas	AY684236	AY684211	HM164807
CBS244.87 a	<i>E. petrakii</i> var. <i>petrakii</i>	<i>P. spinosa</i>	Switzerland	F. Rappaz	HM164734	HM164769	HM164803
UCD730SJ c	<i>Cryptosphaeria pullmanensis</i>	<i>V. vinifera</i>	San Joaquin Co.	J. R. Úrbez-Torres	GQ293929	GQ294013	HM164808

^v Isolate received as pure culture (a), obtained from perithecia (b), or isolated from canker (c).

^w Co. = county in California, United States.

^x Collector or isolator.

^y Internal transcribed spacer (ITS) ribosomal (r)DNA.

^z RPB2 = DNA-dependant RNA polymerase II.

stricto by Rolshausen et al. (40). Perithecia and stromata characteristics were examined in the laboratory using a stereoscopic microscope (Leica MZ95). Perithecial contents were mounted in water and examined by light microscopy (Leica DM LB). Ascospores and conidia of the various isolates collected were measured in water with a bright-field compound microscope at $\times 1,000$ magnification. Measurements were taken on 90 ascospores/isolate. Thirty conidia per isolate were measured as conidial masses became visible from colonies maintained under fluorescent lighting (Underwriters Laboratories Inc., Georgia) (12 h/day) at 24°C. Conidia measurements reflected the straight-line distance between the endpoints of the spore (chord length). Means of ascospore and conidium sizes were compared statistically using analysis of variance (ANOVA) and Tukey's highly significant difference test in SAS (version 9.1; SAS Institute, Cary, NC).

DNA extraction, sequence alignment, and phylogenetic analyses. Phylogenetic analyses were carried out to confirm results of morphological identification and further analyze the intraspecific variation of *E. lata* isolates from California. Phylogenetic analyses were based on the sequence comparison of the ITS region of the ribosomal (r)DNA, portions of the β -tubulin and DNA-dependant RNA polymerase II (*RPB2*) genes. Multigene phylogeny of the combined datasets of the ITS, β -tubulin, and *RPB2* genes were also carried out.

DNA was extracted from fresh mycelium of pure cultures using a commercial DNA extraction kit (Bio-Rad Laboratories, Hercules, CA). The 5.8S nuclear rDNA region and ITS1 and ITS2 were amplified by polymerase chain reaction (PCR) using primers ITS1 and ITS4 (54). The β -tubulin gene was amplified using primers T1 (27) and Bt2b (11). DNA-dependant RNA polymerase II gene was amplified using primers *RPB2*-7F and *RPB2*-11aR (17). Samples were then processed through 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min and 30 s for amplification in a programmable thermal controller machine. PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Both strands of the three DNA regions were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CN) at the Division of Biological Sciences sequencing facility at the University of California, Davis. Sequencing results were edited and assembled using Sequencher version 3.1.1. Sequences were aligned using ClustalW multiple alignment program (49) and sequence alignments were improved manually. Phylogenetic analyses were performed with PAUP version 4.0b10 (47) using maximum parsimony (MP) with a heuristic search and 1,000 random addition sequence replicates and tree bisection-reconnection (TBR) as the branch swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Measures including tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. All characters were unordered and equally weighted. Alignment gaps were treated as missing data. Bootstrap support (BS) was evaluated using 1,000 replicates to test branch strengths. Branches with bootstrap value $<50\%$ are not represented. Phylogenetic analyses neighbor-joining (NJ) analyses were also performed to test results obtained from the MP analyses. Trees were rooted with *Cryptosphaeria pullmanensis* and *E. leptoplaca* as outgroups.

Pathogenicity tests. Two pathogenicity tests were conducted on *V. vinifera* grapevines using green shoots. A field experiment was conducted on mature Sauvignon Blanc grapevines planted at the Viticulture department experimental station of the University of California, Davis. Shoots were inoculated in June 2007 using 19 isolates of *E. lata* obtained from various host plants (Table 1). Inoculations were made at internodes 4 to 6 using a 4-mm cork borer and placing an agar plug with fresh mycelium obtained from the margin of 10-day-old colonies. One cane on each of six grapevine plants per fungal treatment was inoculated in a completely randomized design. Control shoots were inoculated in the

same way using sterile agar plugs. Inoculations were then covered with petroleum jelly and wrapped with Parafilm (Pechiney, Chicago). Shoots were collected after 8 months for measurement of the total length of vascular discoloration extending upward and downward from the point of inoculation. Canes were surface disinfected using 0.5% sodium hypochlorite for 5 min and air dried. Fragments of necrotic tissue developing outside the area of inoculation were cultured on PDA-tet from the leading edge of canker, and *E. lata* isolates obtained from reisolation were identified based on colony shape, color, and growth rate.

A second experiment was conducted in green shoots of 3-year-old potted Chardonnay grapevine plants maintained in a lath house at the field station of the Plant Pathology department of the University of California, Davis. Inoculations were done as described above using 18 of the 19 isolates of *E. lata* used in the first experiment. One shoot on each of five plants per isolate and control were inoculated in a completely randomized design. Observations of the length of vascular discolorations and reisolations were done 10 months following inoculations.

Statistical analyses were performed using ANOVA in SAS (version 9.1). Log transformations were performed to reduce heteroscedasticity and stabilize variance. Fisher's least significance difference (LSD) test was used to assess significant differences in the extent of vascular discoloration between the different treatment group means.

RESULTS

Surveys. The host range and geographic distribution of the perithecia of *E. lata* in California are summarized in Figure 1. Agricultural crop hosts in this study on which perithecia of *E. lata* were identified included crabapple (*Malus* sp.) in Napa County; pear (*Pyrus communis*) in Mendocino, El Dorado, and Sacramento Counties; sweet cherry (*Prunus avium*) in San Joaquin and San Benito Counties; apricot (*P. armeniaca*) in San Joaquin, Contra Costa, Stanislaus, and San Benito Counties; and grapevine (*V. vinifera*) in San Joaquin, San Benito, Napa, Sonoma, Yolo, and El Dorado Counties. Stromata of *E. lata* on almond (*P. dulcis*) were rare and only one tree with a poorly developed stroma was found in an orchard in Yolo County, despite extensive surveys of almond orchards in Central and Northern California. Perithecia in vineyards and apricot and cherry orchards were found mostly in ≈ 20 -year and older plantings.

In addition, the sexual stage of *E. lata* was found on trees in riparian areas and natural forests. Perithecia were particularly common on dead branches of willows (*Salix lasiolepis*) in the counties of Napa, San Benito, Stanislaus, Sacramento, Yolo, Merced, Solano, and San Joaquin. Perithecia also were collected commonly from the dead wood of big leaf maple (*Acer macrophyllum*) in the Napa and El Dorado Counties and on California buckeye (*Aesculus californica*) in some mixed evergreen forest of Napa and Sonoma Counties. *E. lata* perithecia also were found on oleander (*Nerium oleander*) in Yolo County.

Morphological study. Mean length of ascospores for all putative isolates of *E. lata* ranged between 8.3 ± 0.6 μm (isolate DCHES200) and 9.7 ± 1.1 μm (DCHR100) and were significantly different among isolates ($P < 0.0001$) (Table 2). ANOVA revealed significant differences in conidia size among isolates of *E. lata* ($P < 0.0001$). Conidia sizes varied between 23.6 ± 2.4 μm (DCHR200) and 35.0 ± 4.0 μm (isolate DAP100). Mean length of ascospores and conidia of isolate DSA600 (7.6 ± 0.9 and 54.6 ± 7.2 μm , respectively) significantly differed from all other isolates (Table 2), which separated from *E. lata* also in phylogenetic analyses.

Phylogeny. PCR amplifications using primers ITS1 and ITS4 gave products of ≈ 0.56 kb. The 5.8S rDNA region was conserved among all the strains. Parsimony analysis of the molecular data set generated nine MP trees. One of the nine MP trees derived

from the analysis of the rDNA-ITS region is shown in Figure 2A. A complete alignment was achieved for 566 bp, with 480 constant characters, 46 parsimony-informative characters, and 40 parsimony-uninformative characters. The length of the tree was 111 steps, with CI = 0.8559, RI = 0.8416, and RC = 0.7203. Although the relationship of isolates within the *E. lata* clade varied between the nine MP trees, all MP trees generated identical lineages.

The β -tubulin dataset consisted of 605 characters after alignment; 392 were constant, 143 were parsimony-uninformative, and 70 were parsimony-informative. The analysis retained one MP tree of 283 steps after the heuristic search (CI = 0.9152, RI = 0.8983, and RC = 0.8221) (Fig. 2B).

The parsimony heuristic analysis of the *RPB2* gene yielded one MP tree with length of 195 steps (CI = 0.9077, RI = 0.8741, and RC = 0.7934) (Fig. 2C). The aligned *RPB2* dataset contained 824 characters, of which 666 were constant, 89 were parsimony-uninformative, and 69 were parsimony-informative.

The combined data set of the ITS-rDNA, β -tubulin, and *RPB2* contained 1,995 characters, with 1,538 constant characters, 272 parsimony-uninformative characters, and 185 parsimony-informative characters. The heuristic search resulted in 6,879 equally MP trees 611 steps long, with CI = 0.7377, RI = 0.8333, and RC = 0.7242. All 6,879 MP trees shared similar lineage topology (Fig. 2D).

Fig. 1. Host range and geographical distribution of the perithecial stage of *Eutypa lata* in the main grape-growing regions of California. Areas with gray background correspond to areas that were surveyed.

NJ analyses generated tree topologies identical to those obtained using MP analyses for all data sets. Therefore, only results of MP analyses were used to illustrate tree topologies.

All sequence analyses using the three nuclear regions were concordant and distinguished four closely related and previously recognized morphospecies, including *E. lata*, *E. lata* var. *aceri*, *E. laevata*, and *E. petrakii* var. *petrakii*. Isolates of *E. lata* from California were similar to *E. lata* reference isolates CBS208.87 (40) as well as *E. lata* isolates 3802 and 1908021 (ITS, β -tubulin, and *RPB2* sequence comparison) obtained from Rappaz, thus confirming morphological identifications. All analyses also separated isolate DSA600 from the main clade of *E. lata* isolates and suggested that it is related to *E. laevata* isolate CBS 291.87. All other isolates from California formed a monophyletic clade recognized as *E. lata* and including collections made from fruit trees and wine grapes, as well as natural and ornamental host plants. Several isolates of *E. lata* from California were unevenly separated into subclades; however, the low bootstrap values associated with these clades suggested that they do not constitute distinct species but, rather, illustrate genetic variation for *E. lata*. On the other hand, a major clade with strong bootstrap support, including all isolates collected (except for isolate DSA600), was identified in all analyses. These major clades are regarded as *E. lata* sensu stricto in the present study.

Pathogenicity tests. Green shoot inoculations of Sauvignon Blanc grapevines with *E. lata* isolates collected in the survey showed that most isolates were capable of causing disease. After 8 months, the extent of vascular discoloration induced by *E. lata* isolates varied almost continuously from 10.8 to 32.2 mm (DRUSS100) (Fig. 3A). ANOVA revealed highly significant differences (at $\alpha = 0.05$) among the isolates ($P < 0.0001$). Fisher's LSD test showed significant differences between some isolates, indicating a gradient of virulence among the various isolates (Fig. 3A). Percentage of inoculations from which the pathogen was recovered varied from 0% for plants inoculated with isolates DMUNK100 and DSA300 from cherry and willow trees, respectively, to low to moderate for plants inoculated with isolates UCD401 (*V. vinifera*), DRUSS100 (*P. dulcis*), and DCA900 (*Acer macrophyllum*), which were recovered at 16.7, 33.4, and 57.4%, respectively. Percentage of recovery for all other isolates was 66.7 to 100%.

TABLE 2. Mean length of ascospore and conidium of putative *Eutypa lata* isolates collected for this study^z

Isolate ID	Mean length of ascospores (μm) $\pm$ SD	Mean length (chord length) of conidia (μm) $\pm$ SD
DCHR100	9.7 $\pm$ 1.1 a	29.3 $\pm$ 2.8 defg
DMUNK100	9.5 $\pm$ 1.1 ab	30.2 $\pm$ 1.9 def
EAMS100	9.4 $\pm$ 1.1 abc	nd
DSC100	9.4 $\pm$ 1.1 abc	31.3 $\pm$ 3.4 cd
DCHR200	9.4 $\pm$ 1.2 abcd	23.6 $\pm$ 2.4 h
DJAM100	9.3 $\pm$ 1.2 abcde	26.8 $\pm$ 3.4 gh
DSA200	9.3 $\pm$ 1.1 abcde	27.5 $\pm$ 4.2 fg
DPEAR200	9.2 $\pm$ 0.1 abcde	30.6 $\pm$ 2.5 def
DNA100	9.2 $\pm$ 1.0 abcde	28.0 $\pm$ 4.7 efg
DHB100	9.1 $\pm$ 1.0 bcdef	nd
DCA900	9.1 $\pm$ 1.0 bcdef	29.3 $\pm$ 2.3 defg
DAP100	9.1 $\pm$ 1.1 bcdefg	35.0 $\pm$ 4.0 b
DCA700	9.0 $\pm$ 0.8 bcdefg	30.7 $\pm$ 3.9 cde
DCA200	8.9 $\pm$ 1.2 cdefg	nd
DRO100	8.9 $\pm$ 1.0 cdefgh	34.6 $\pm$ 2.4 b
DSA500	8.9 $\pm$ 0.8 defgh	33.8 $\pm$ 3.4 bc
DSA300	8.8 $\pm$ 1.1 efgh	nd
DSA400	8.6 $\pm$ 0.1 fgh	27.8 $\pm$ 1.8 efg
OLEA3	8.5 $\pm$ 0.7 gh	nd
DCHES200	8.3 $\pm$ 0.6 h	26.2 $\pm$ 2.5 gh
DSA600	7.6 $\pm$ 0.9 i	54.6 $\pm$ 7.2 a

^z Means followed by different letters differ significantly ($\alpha = 0.05$) according to Tukey's highly significant difference test. SD = standard deviation and nd = not determined.

Green shoot inoculation of Chardonnay grapevines with the wide host range collection of *E. lata* showed that all isolates tested were capable of infecting grapevine shoots. After 10 months, the extent of vascular discoloration varied from 7.6 mm (isolate DSA200) to 16.8 mm (DSC100). Mean of vascular discoloration observed in the control was 5.6 mm (Fig. 3B). ANOVA showed significant differences (at $\alpha = 0.05$) among the treatments ($P = 0.0216$). Fisher's LSD test revealed significant differences of virulence among the various isolates tested (Fig. 3B). Percentage of recovery was only 20% for isolate DPEAR100; all other percentages of recovery were high across the experiment and varied from 60 to 100% for the isolates tested.

DISCUSSION

In California, damage due to *Eutypa* dieback has a severe economic impact on the wine industry (42), yet no eradication control methods are available, except for severe pruning. Most control methods rely on preventing infection using pruning-wound protectants, as well as appropriate cultural practices, sanitation methods, and remedial surgery (39,43–45,53). Identifying the inoculum sources for the disease constitutes critical knowledge of disease epidemiology and may ultimately help to reduce risks of infection if these sources can be eliminated.

In this study, surveys revealed that perithecia of *E. lata* occur in the main wine regions of the North and Central Coasts and in the Sacramento and Northern San Joaquin Valleys in California. Perithecia of *E. lata* were found to be prevalent in the counties of Napa, Sonoma, Yolo, Sacramento, Contra Costa, San Benito, El Dorado, Mendocino, San Joaquin, and Stanislaus. In fruit crops, stromata of *E. lata* were common in many of the older cherry and apricot orchards and vineyards that were surveyed. Several trees in a cherry orchard in San Joaquin County previously reported to have perithecia (26) were still bearing viable ascomata. Trees and vines with stromata generally were associated with large pruning wounds on the scaffold or main trunk. Stromata spread widely along the trunk surface and often shared alternate zones of living and dead perithecia. New hosts for the sexual stage of *E. lata* in California on woody crops included crabapple (*Malus* sp., not *Malus domestica*) and pear (*Pyrus communis*). Preliminary surveys for pathogens associated with dieback in commercial apple trees (*M. domestica*) in California have revealed an abundance of *E. lata* in cankers collected from diseased trees in Mendocino County (10). Other studies in the United States have shown the association of *E. lata* with a canker disease of *M. domestica* (12).

In the present study, we report the occurrence of perithecia on common native Californian trees including *A. macrophyllum*, *Aesculus californica*, and *S. lasiolepis* and on ornamentals such as *N. oleander*. Perithecia of *E. lata* were found to be particularly well established on dead branches of willow species occurring along natural creeks and irrigation waterways. Stromata were common and usually well developed on the dead wood of willow, which suggested that this host plant might represent an important reservoir of inoculum. This work introduces the role of ornamental plants and native vegetation as potential inoculum sources for *Eutypa* dieback in California and illustrates the increased importance of grapevine as an overwintering host. In many wine regions of California, vineyards have been planted near riparian areas, forests, and managed landscapes. Pathogenicity tests have shown that *E. lata* isolates collected from native, ornamental, and agricultural hosts could cause *Eutypa* dieback of grapevine. The role of native vegetation in the epidemiology of *Eutypa* dieback remains to be established but removal of the dead wood of potential hosts of *E. lata* in proximity to vineyards may be advisable. Removal and burning of infected wood, which can usually be identified by the distinctive black stroma of *E. lata*, should reduce the number of spores released in winter.

No perithecia were detected from surveys conducted in Tulare, Fresno, Madera, Kings, and Kern Counties. Yearly average rainfall is 266 mm in Tulare County, 145 mm in Kern County, and 304 mm in Fresno and Madera Counties. These findings are

consistent with previous reports suggesting an influence of rainfall on the distribution of *E. lata* perithecia (4). Also, Urbez-Torres et al. (52) showed a low or zero incidence of *E. lata* in grapevine wood cankers collected from grapevines grown in these

Fig. 2. **A**, One of the nine most-parsimonious trees obtained from the internal transcribed spacer (ITS) sequence data; tree length (TL) = 111 steps, consistency index (CI) = 0.8559, retention index (RI) = 0.8416, and rescaled consistency index (RC) = 0.7203. Bootstrap support values from 1,000 replicates >50% are reported at the nodes. **B**, Most-parsimonious trees obtained from the β -tubulin sequence data; TL = 283 steps, CI = 0.9152, RI = 0.8983, and RC = 0.8221. Bootstrap support values from 1,000 replicates >50% are reported at the nodes. **C**, Most-parsimonious trees obtained from the *RPB2* sequence data; TL = 195 steps, CI = 0.9077, RI = 0.8741, and RC = 0.7934. Bootstrap support values from 1,000 replicates >50% are reported at the nodes. **D**, One of the 6,879 most-parsimonious trees obtained from the combined ITS, β -tubulin, and *RPB2* sequence data; TL = 611 steps, CI = 0.8691, RI = 0.8333, and RC = 0.7242. Bootstrap support values from 1,000 replicates >50% are reported at the nodes.

southern regions and further showed the prevalence of *Botryosphaeriaceae* fungi as the cause of grapevine cankers in the southern San Joaquin Valley. Moreover, the distribution of *E. lata* perithecia in California appears to coincide with the distribution of the disease. The identification of many new hosts on which *E. lata* produces perithecia calls into question the importance of long-distance dispersal of ascospores in causing infections on grapevines in the San Joaquin Valley (28,36).

The occurrence of the sexual stage of *E. lata* on cultivated grapevine and fruit trees as well as on native plant species in forests and riparian areas raises questions about the origin of the pathogen. Carter and Peros have discussed the possibilities that *E. lata* originally acquired its pathogenicity in grapevine in Asia Minor and, subsequently, expanded its range of susceptible hosts (4,31). Another possibility suggested by the same authors (4,31) is that *E. lata* was present as a weak pathogen on different host

Fig. 3. A, Mean length of vascular discolorations 8 months after inoculation of various isolates of *Eutypa lata* in Sauvignon Blanc grapevine green shoots under field conditions using mycelium plugs. Isolates with mean lengths of vascular discoloration followed by different letters were significantly different from each other according to Fisher's least significant difference (LSD) ($\alpha = 0.005$). **B,** Mean length of vascular discoloration 10 months after inoculation of various isolates of *E. lata* in Chardonnay grapevine green shoots inoculated using mycelium plugs and maintained in a lath house. Isolates with mean lengths of vascular discoloration followed by different letters were significantly different from each other according to Fisher's LSD ($\alpha = 0.005$).

plants around the world and only recently has developed the ability to kill agricultural crops such as apricot and grape. Recently, a study of members of *Diatrypaceae* associated with grapevine canker diseases has suggested that these fungi may constitute opportunistic pathogens occurring naturally on the indigenous flora but capable of infecting newly introduced agricultural host plants (51). Surveys in Central Europe have shown that *E. lata* apparently occurs on many native plant species in this region, seemingly coexisting in a state of equilibrium without causing disease to the hosts, which suggests that host and fungus have long been associated (4). The coevolution event in a host–pathogen combination has been known to favor a high level of resistance of a particular host and, reciprocally, a low degree of virulence of a pathogen (46). The evolutionary history of *E. lata* and trend (direction) of host-jumping events between indigenous and domesticated flora should be investigated next to differentiate the founder populations of the pathogen and elucidate the origin (emergence) of diseases caused by the fungus *E. lata* in California.

The dominant operational species concept in *E. lata* has been the morphological species concept (48), and no biological species concept has yet been established for *E. lata* despite previous attempts to obtain sexual fruiting bodies in vitro from sexual reproduction of compatible isolates (P. E. Rolshausen, *personal communication*). Moreover, identification of the teleomorph of *E. lata* has been based generally on the examination of ascospore shape and size, perithecial organization within stroma (37), and shape of perithecial ostioles (37,50). In this study, we detected morphological plasticity and provided an expected range of ascospore and conidium size. Moreover, disease diagnosis for Eutypa dieback has been done mostly by isolating the pathogen from cankered tissues and observing typical white colonies and filiform conidia on PDA medium. However, because of the difficulty in separating *E. lata* from closely related *Eutypa* spp. using morphology alone (13), and the occurrence on grapevine of related species (41,50,51), molecular- and metabolite-based techniques have been recently developed to accurately identify *E. lata* (14,16,19,41).

Identification of *E. lata* from the various hosts was confirmed using multigene phylogenies of rDNA ITS, β -tubulin, and *RPB2* genes. The analyses also revealed sequence differences among isolates, indicating some intraspecific genetic diversity for *E. lata* populations from California. However, the degree of phylogenetic diversity and the intraspecific groups were not consistent across the various DNA phylogenies. Moreover, subgroups did not correlate with the sampling site or source of isolation. One probable explanation is that random mating and gene flow through dispersal of ascospore are sufficient to preclude differentiation of regional populations (30,32). Moreover, the proximity of native hosts for *E. lata* to cultivated hosts may play a major role in defining the overall population structure of the pathogen and unrestricted gene flow (31).

Under the conditions of our tests, all isolates of *E. lata* from California were infectious to grapevines but differences in virulence were significant. These results are in agreement with previous reports of great diversity in aggressiveness among strains of *E. lata* from the same country, within a single vineyard, and even from the same stroma (29–33). Similar indications of diversity are apparent from reports of differential production of secondary metabolites across strains of *E. lata* (18,24,38,40). Isolates DMUNK100 and DSA300 from cherry and willow trees, respectively, could not be reisolated from the previously inoculated Sauvignon Blanc shoots. This indicates that these isolates were probably not pathogenic to the Sauvignon Blanc cultivar under the condition of our experiment.

Overall, differences in ascospore and conidium length as well as differences in virulence on grapevine shoots did not correlate with differences in DNA sequences or phylogenetic groups. Our

results illustrate the intraspecific phenotypic and genetic variation within the species *E. lata* inferred by examination of numerous isolates from California. Morphology and, most particularly, ascospore and conidium sizes generally provide important taxonomy features useful for diagnostic. Here, we characterized the intraspecific morphological variation of *E. lata* using multiple strain measurements of ascospores and conidia. Because of the many strains examined for this study, our results provide confident size ranges for these morphological characters. The morphological and phylogenetic analyses excluded the willow isolate DSA600 (initially considered as *E. lata* based on perithecia and colony morphologies) from the main clade of *E. lata* isolates. These analyses also suggested that it relates to *E. laevata*.

Previous studies have analyzed the population structure of *E. lata* at various geographical scales, varying from a single vineyard population to continental populations. The studies have shown high levels of genetic diversity among populations of *E. lata* in Europe, Australia, and North America using randomly amplified polymorphic DNA, amplified fragment length polymorphism, isozyme, and vegetative compatibility data (6,15,30–35).

The wide geographical distribution and host range of the pathogen *E. lata* have raised questions about the true boundaries of this species. In this study, we have concluded, on the basis of phylogenetic analyses of the ITS region of the rDNA and portions of the β -tubulin and DNA-dependant RNA polymerase II genes, that the wide host range and phenotypic variations observed among isolates corresponded to the intraspecific variability of California populations of *E. lata* sensu stricto. Therefore, the occurrence of putative cryptic species of *Eutypa* among isolates in our collection of *E. lata* from California is doubtful but future research should further investigate the population structure and genetic diversity of *E. lata* for possible subdivisions, as well as levels of gene flow occurring between agroecosystems and native environment populations of the pathogen.

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