Identification of *Eutypa lata* by PCR-RFLP

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ABSTRACT

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Eutypa lata is a vascular canker pathogen of woody plants commonly diagnosed by isolating the pathogen from infected tissue. Related fungi from the same family, the *Diatrypaceae*, also have been found in association with grapevine in Californian vineyards. An in situ polymerase chain reaction (PCR) method has been developed for detection of *E. lata* in infected wood tissue. However, our results indicate that this method also would amplify other *Diatrypaceae* fungi, which could potentially lead to an incorrect diagnosis. Therefore, we developed a PCR-restriction fragment length polymorphism (PCR-RFLP) assay. The internal transcribed spacer (ITS)1/5.8S/ITS2 ribosomal DNA region was amplified by PCR using universal primers, and RFLP patterns were determined after digestion with *AluI*. The restriction profiles obtained served to distinguish *E. lata* from wood trunk pathogens of grapevine (*Phomopsis viticola*, *Botryodiplodia* sp., *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora*), *Diatrypaceae* fungi (*Diatrype* sp., *Diatrypella* sp., *Eutypella vitis*, and *Eutypa leptoplaca*), and *Cryptovalsa* sp. found on dead wood of grapevine, and other *Eutypa* spp. (*E. petrakii* var. *hederiae*, *E. astroidea*, *E. crustata*, and *E. lejo-placa*), with the exception of *E. armeniacae*, which we regard as a synonym for *E. lata*, and *E. laevata*, which has not been found on grapevine.

Additional keywords: vegetative compatibility groups

Eutypa lata (Pers.) Tul. & C. Tul (= *E. armeniacae* Hansf. & M. V. Carter) is a major pathogen of cultivated crops such as apricot and grapevine and has been found all over the world (3). It is responsible for significant economic damage to the wine industry in California (22,25).

E. lata (Ascomycetes, *Diatrypaceae*) can be identified based on morphological features of the meiosporic stage on the dead wood of infected hosts (3,15,24). For disease diagnosis, positive identification of *E. lata* often is based on the mitospore stage isolated from infected wood on potato dextrose agar (PDA). On this medium, *E. lata* grows as a white cottony mycelium, which can produce conidia from conidiogenous cells aggregated into enclosed or open conidiomata (15).

Other fungi placed in members of *Diatrypaceae* are difficult to separate from each others based solely on morphological features of their mitospore stage. Also, the biology and taxonomy of this group of fungi is not well known. Glawe and Rogers (15) found other fungal species in

this family on dead or declining hosts, suggesting they may be somewhat pathogenic. *Cryptosphaeria populina* (Pers.) Sacc. (16) and *Eutypella parasitica* R. W. Davidson & Lorentz (8) are the only species other than *E. lata* reported to be pathogenic on aspen and maple, respectively. Trouillas et al. (26) reported the presence of several *Diatrypaceae* fungi in Californian vineyards on dead wood (*Eutypa lata*, *Diatrypella* sp., *Diatrype* sp., *Eutypella* sp., and *Cryptovalsa* sp.) including a second *Eutypa* sp., *Eutypa leptoplaca* Rappaz (27). These findings suggest that such fungi could be isolated from wood cankers instead of or in concert with *E. lata*. Therefore, incorrect identification of the target pathogen could occur.

Other techniques currently available to identify *E. lata* include serology (12,23), fatty acid analysis (11), and a polymerase chain reaction (PCR)-based method (19). However, neither fatty acid analysis nor serological methods are used extensively for disease diagnosis. Lecomte et al. (19) developed an in situ detection method by PCR using primers designed to specifically amplify 5.8S ribosomal DNA of *E. lata*. However, the three primer pairs (Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1) were not tested on other *Diatrypaceae* fungi. Given the potential for other fungi in this family to co-occur with *E. lata* in grape wood tissue (26,27), the possible lack of specificity of these primers may render this in situ detection procedure inadequate for accurate detection of *E. lata*.

After testing the specificity primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 designed by Lecomte et al. (19), our objective was to develop an alternative molecular method to quickly and reliably identify *E. lata* in culture and distinguish it from morphologically similar and taxonomically related fungi. For this purpose, we used a PCR-restriction fragment length polymorphism (PCR-RFLP) method, which has been used effectively for identification of other fungal pathogens (10,17,21).

MATERIAL AND METHODS

Isolates of *E. lata*. Over 30 *E. lata* isolates were collected over the years at the University of California, Davis (UC Davis) from several plant hosts, including tree hosts and cultivated crops, and from several geographic locations worldwide. A subsample of 11 *E. lata* isolates was presented for this study (Table 1). Isolates

Table 1. Designation, geographic origin, plant host, and source of 11 *Eutypa lata* isolates used in this study^a

<i>E. lata</i> isolate	Geographic origin	Plant host	Isolate source
E30	United States—California	<i>Vitis vinifera</i>	Canker
E31	United States—California	<i>V. vinifera</i>	Canker
E38	United States—California	<i>V. vinifera</i>	Canker
E454	United States—California	<i>Prunus armeniaca</i>	Canker
E455	United States—California	<i>P. armeniaca</i>	Canker
ESW	Switzerland	<i>V. vinifera</i>	Ascospore
CBS 208.87	Switzerland	<i>Tilia</i> sp.	Culture
E201	Australia	<i>V. vinifera</i>	Culture
D001	Australia	<i>P. armeniaca</i>	Culture
MD1	Italy	Unknown	Culture
8D	France	Unknown	Culture

^a All 11 *E. lata* isolates were tested with the polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) method. *E. lata* isolate CBS (Centraalbureau voor Schimmelcultures, Netherlands) 208.87 was used as the reference isolate for evaluating PCR primers Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 (19), and for comparison of RFLP banding patterns to other fungal specimens listed in Table 2.

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were cultured from cankers in wood (five isolates) or from fruiting bodies (one isolate) as described by Carter (4). Each isolate was cultured on water agar before transferring a single hyphal tip to PDA in order to obtain a pure culture. The remaining five isolates were received as pure cultures from France (Institut National de la Recherche Agronomique, Villenave d'Ornon), Italy (Istituto di Pathologia Vegetale, Milano), Australia (Waite Research Precinct, Adelaide), and the Netherlands Centraalbureau voor Schimmelcultures (CBS). All isolates were stored at 4°C in sterile water.

Other fungal isolates. *Phomopsis viticola* (Sacc.) Sacc., *Botryodiplodia* sp., *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai, and *Phaeomoniella chlamydospora* W. Gams, Crous, M. J. Wingf. & L. Mugnai (Table 2) were isolated on PDA medium from wood necrosis of diseased grapevines in California, and were identified by their mitospore stages based on the keys of Barnett and Hunter (2) and Crous et al. (7). Meiosporic stages of *Diatrypella* spp., *Cryptovalsa* spp., and *Diatrype* spp. were collected from grapevine in different regions in California and were identified based on the keys of Rappaz (24) and Glawe and Rogers (15). A culture of *Eutypella vitis* was obtained from Michigan State University and identified by a BLAST search in the GenBank database (1) using the internal transcribed spacer (ITS)1/5.8S/ITS2 region (*E. vitis* accession number AJ302466), because only the mitospore stage was available for identification. Pure cultures of *Eutypa armeniacae* Hansf. & M. V. Carter, *E. laevata* (Nitschke) Sacc., *E. petrakii* var. *hederae* Rappaz, *E. astroidea* (Fries:Fries) Rappaz, *E. lejoiplaca* (Fries:Fries) Fuckel, *E. crustata* (Fries:Fries) Saccardo, and *E. leptoplaca* were received from the CBS collection.

DNA extraction. Mycelium for DNA extraction was obtained by cutting an agar plug drawn from the edge of a growing culture and placing it on a sterile cellophane strip (3 by 3 cm) overlaid on PDA. Petri plates were inoculated at room temperature for 5 days in the dark. Mycelium was scraped off the cellophane, and DNA was extracted as described by Cenis (6).

Evaluation of specific PCR primers. Primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 (19) were tested independently on crude DNA of *Phomopsis viticola*, *Botryodiplodia* sp., *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Cryptovalsa* sp., *Diatrype* sp., *Diatrypella* sp., *Eutypella vitis*, *Eutypa lata* (CBS 208.87), *E. armeniacae*, *E. laevata*, *E. petrakii* var. *hederae*, *E. astroidea*, *E. leptoplaca*, *E. crustata*, and *E. lejoiplaca* (Table 2). PCR assays were set up in the same conditions as described by Lecomte et al. (19) and were performed at least two times for each fungal isolate. PCR products were run on a 1× Tris-borate EDTA (TBE) 1% agarose gel (BMA, Rockland, ME), stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Vegetative compatibility test. Isolates of *E. lata* (Table 1) were tested for vegetative compatibility in order to make sure they were not clones. Isolates were paired in all possible combinations on PDA for 2 weeks at room temperature in the dark. If formation of a barrage was observed, the isolates were placed in different vegetative compatibility groups (VCGs); whereas, if no barrage was observed, isolates were placed in the same VCG (13). Isolates also were paired with themselves as a positive control (i.e., no barrage).

Evaluation of PCR-RFLP method. The ITS1/5.8S/ITS4 region was amplified in a 50-µl reaction using a Taq PCR Core Kit (Qiagen, Valencia, CA) containing 36 µl of distilled water (GIBCO, Invitrogen

Corporation, Scotland, UK), 5 µl of 10× PCR buffer, 4 µl of MgCl₂, 2 µl of dNTPs, 1 µl of ITS1 and ITS4 primers (28), 0.25 µl of Taq DNA polymerase, and 1 µl of DNA extract per reaction. A negative control (no DNA) was included in the PCR. Samples were processed through 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min 30 s. PCR products were run on a 1× TBE 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The ITS1/5.8S/ITS2 region was further digested in a 30-µl reaction containing 2 µl of restriction enzyme *AluI* (New England Biolabs, Beverly, MA), 25 µl of PCR product, and 3 µl of 10× enzyme buffer. The restriction digestion profile was run on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The PCR-RFLP method was performed at least two times for each fungal isolate listed in Tables 1 and 2.

RESULTS

No PCR products were obtained with primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 designed by Lecomte et al. (19) when tested with *Phomopsis viticola*, *Botryodiplodia* sp., *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora*. However, amplicons of the expected size were obtained with all three primer pairs when tested with a *Cryptovalsa* sp., *Diatrype* sp., *Diatrypella* sp., *Eutypella vitis*, *Eutypa lata*, and *E. leptoplaca* (Fig. 1). Moreover, PCR products also were obtained with all three primer pairs when tested on *E. armeniacae*, *E. laevata*, *E. petrakii* var. *hederae*, *E. astroidea*, *E. crustata*, and *E. lejoiplaca* (data not shown).

Eleven isolates of *E. lata* were tested in all combinations and found to be all vegetatively incompatible, indicating that no clone was represented more than once in our sample. Digestion of the PCR-amplified ITS1/5.8S/ITS2 region with *AluI* yielded three bands of approximately 0.3, 0.2, and 0.1 kb for all isolates of *E. lata* (Fig. 2). To confirm our findings with the initial group of isolates, both vegetative compatibility tests and PCR-RFLP assays also were applied to the entire collection of *E. lata* isolates established at UC Davis. Isolates always were placed in different VCGs and intraspecific polymorphism was never detected among them (data not shown).

The PCR-RFLP method also was tested with other fungal pathogens found in association with grapevine (18,26,27). Restriction profiles of *Phomopsis viticola*, *Botryodiplodia* sp., *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* clearly were different from that of *E. lata* (Fig. 3). Different banding patterns also could separate *E. lata* from a *Diatrype* sp., *Diatrypella* sp., *Eutypella vitis*, and *Cryptovalsa* sp. (Fig. 4). However, *Diatrype* sp. and *Diatrypella* sp. shared the

Table 2. Designation, geographic origin, and plant host of fungal organisms used in this study^a

Fungal organism	Plant host	Geographic origin	Isolate number ^b
<i>Phomopsis viticola</i>	<i>Vitis vinifera</i>	United States—California	P470
<i>Phaeoacremonium aleophilum</i>	<i>V. vinifera</i>	United States—California	Pa61
<i>Phaeomoniella chlamydospora</i>	<i>V. vinifera</i>	United States—California	Pc44
<i>Botryodiplodia</i> sp.	<i>V. vinifera</i>	United States—California	B422
<i>Cryptovalsa</i> sp.	<i>V. vinifera</i>	United States—California	A001
<i>Diatrypella</i> sp. ^c	<i>V. vinifera</i>	United States—California	DHb500
<i>Eutypella vitis</i> ^c	<i>V. vinifera</i>	United States—Michigan	ELM13
<i>Diatrype</i> sp. ^c	<i>V. vinifera</i>	United States—California	DHb200
<i>Eutypa armeniacae</i> ^c	<i>V. vinifera</i>	Italy	CBS 622.84
<i>E. laevata</i> ^c	<i>Salix</i> sp.	Switzerland	CBS 291.87
<i>E. petrakii</i> var. <i>hederae</i> ^c	<i>Hedera helix</i>	Switzerland	CBS 285.87
<i>E. astroidea</i> ^c	<i>Fraxinus excelsior</i>	Switzerland	CBS 292.87
<i>E. leptoplaca</i> ^c	<i>Arundo donax</i>	France	CBS 286.87
<i>E. crustata</i> ^c	<i>Ulmus</i> sp.	France	CBS 210.87
<i>E. lejoiplaca</i> ^c	<i>Acer pseudoplatanus</i>	Switzerland	CBS 248.87

^a DNA of each fungal organism was used for evaluating polymerase chain reaction (PCR) primers Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 (19), and PCR-restriction fragment length polymorphism method.

^b CBS = Centraalbureau voor Schimmelcultures, Netherlands.

^c Fungal organism taxonomically classified in the family *Diatrypaceae* (*Ascomycetes*).

same banding patterns, as did *Eutypella vitis* and *Cryptovalsa* sp. Within the genus *Eutypa*, no separation could be made between *Eutypa lata*, *E. armeniaca*, and *E. laevata*. Likewise, *E. astroidea*, *E. leptoplaca*, and *E. crustata* shared a common banding pattern (Fig. 5). The restriction profiles of these two groups were different from each other and also from *E. lejoplaca* and *E. petrakii* var. *hederae*.

DISCUSSION

The taxonomic disposition of fungi within the family *Diatrypaceae* remains unclear and somewhat artificial. Also, the biology of these organisms is poorly understood. Many species in this family are found on dead hosts (15). Trouillas et al. (26) identified several fungi (*Diatrype* sp., *Diatrypella* sp., *Cryptovalsa* sp., and *Eutypella vitis*) on dead wood of grapevine in addition to the pathogen *Eutypa lata*, including another *Eutypa* sp., *E. leptoplaca* (27). *E. lata* is being recognized as the main pathogen responsible for grapevine dieback, but the frequency of occurrence of other *Diatrypaceous* fungi in woody tissue of grapevine and their pathogenic abilities have not been determined yet.

Lecomte et al. (19) developed an identification method of *E. lata* by PCR directly from infected wood tissue. The design of their primer pairs from the ITS region was based on one *E. lata* isolate. The BLAST search for specificity of their designed primers showed no match with other *Diatrypaceous* fungi, because this group was not represented in the GenBank database at the time. Nucleotide sequences of several species of *diatrypaceous* fungi have been deposited in the GenBank database since, and evidence of the lack of specificity of primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 now can be demonstrated by BLAST search (1). We further demonstrated the lack of specificity of all three primer pairs by performing PCR on several fungal isolates. No PCR products were obtained from fungi that are found in dead grapevine wood but are taxonomically distinct from *E. lata*, such as *Phomopsis viticola*, *Botryodiplodia* sp., *Phaeoacremonium aleophilum*, and *Phaeoconiella chlamydospora* (18), which confirmed the results obtained by Lecomte et al. (19). However, a lack of specificity of all three primer pairs was demonstrated with taxonomically related *Diatrypaceous* fungi and a *Cryptovalsa* sp. Given the potential for these fungi to inhabit grape wood tissue, the in situ PCR detection method may be inadequate for positive detection of *E. lata*. Lecomte et al. (19) also developed three other primer pairs (SCA 10A/SCA 10B, SCB 02A/SCB 02B, and SCD 18A/SCD 18B) from random amplified polymorphic (RAPD) DNA fragments that were not tested in the present study. These primers may be more suitable for detection and diagnosis of

Fig. 1. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS)1/5.8S/ITS2 region using primer pair **A**, Lata 1/Lata 2-1, **B**, Lata 1/Lata 2-2, and **C**, Lata 3/Lata 2-1 from DNA of five *Diatrypaceous* fungi and a *Cryptovalsa* sp. found in their meiosporic stages on dead wood of grapevine in California. The PCR products were run on a 1× Tris-borate EDTA 1% agarose gel, stained with ethidium bromide, and checked under UV light. Lane 1, 1-kb ladder; lane 2, negative control (no DNA); lane 3, *Eutypa lata* (CBS 208.87); lane 4, *E. leptoplaca* (CBS 210.87); lane 5, *Diatrype* sp. (DHb200); lane 6, *Diatrypella* sp. (DHb500); lane 7, *Eutypella vitis* (ELM13); lane 8, *Cryptovalsa* sp. (A001).

Fig. 2. Restriction fragment length polymorphism patterns of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 region using universal primer pair ITS1/ITS4 digested with restriction enzyme *AluI*, from DNA of 11 *Eutypa lata* isolates. Digested PCR products were run on a 1× Tris-borate EDTA 1.5 % agarose gel, stained with ethidium bromide, and checked under UV light. Lane 1, 1-kb ladder; lane 2, negative control (no DNA); lane 3, E30; lane 4, E31; lane 5, E38; lane 6, MD1; lane 7, 8D; lane 8, ESW; lane 9, CBS 208.87; lane 10, E201; lane 11, D001; lane 12, E454; lane 13, E455.

Fig. 3. Restriction fragment length polymorphism patterns of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 region using universal primer pair ITS1/ITS4 digested with restriction enzyme *AluI*, from DNA of fungi isolated from wood necrosis of grapevine. Digested PCR products were run on a 1× Tris-borate EDTA 1.5% agarose gel, stained with ethidium bromide, and checked under UV light. Lane 1, 1-kb ladder; lane 2, negative control (no DNA); lane 3, *Eutypa lata* (CBS 208.87); lane 4, *Phaeopsis viticola* (P470); lane 5, *Phaeoacremonium aleophilum* (Pa61); lane 6, *Phaeoniella chlamydospora* (Pc44); lane 7, *Botryodiplodia* sp. (B422).

Fig. 4. Restriction fragment length polymorphism patterns of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 region using universal primer pair ITS1/ITS4 digested with restriction enzyme *AluI*, from DNA of five fungal genera found in their meiotic stage on dead wood of grapevines. Digested PCR products were run on a 1× Tris-borate EDTA 1.5% agarose gel, stained with ethidium bromide, and checked under UV light. Lane 1, 1-kb Ladder; lane 2, negative control (no DNA); lane 3, *Eutypella vitis* (ELM13); lane 4, *Cryptovalsa* sp. (A001); lane 5, *Eutypa lata* (CBS 208.87); lane 6, *Diatrype* sp. (DHB200); lane 7, *Diatrypella* sp. (DHB500).

E. lata because of the increased specificity they have to offer versus primers designed from the ribosomal DNA region. However, Lecomte et al. (19) found the RAPD primers to be less sensitive than primers designed from the ITS region for detection of *E. lata* directly from infected wood because of the high copy number of ribosomal DNA targeted by the primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1 in comparison to the RAPD primer pairs.

The PCR-RFLP method developed in this study was specific enough to identify

Fig. 5. Restriction fragment length polymorphism patterns of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS)1/5.8S/ITS2 region using universal primer pair ITS1/ITS4 digested with restriction enzyme *AluI*, from DNA of several *Eutypa* spp. Digested PCR products were run on a 1× Tris-borate EDTA 1.5% agarose gel, stained with ethidium bromide, and checked under UV light. Lane 1, 1-kb ladder; lane 2, negative control (no DNA); lane 3, *Eutypa lata* (CBS 208.87); lane 4, *E. lata* (E31); lane 5, *E. armeniaca* (CBS 622.84); lane 6, *E. laevata* (CBS 291.87); lane 7, *E. petrakii* var. *hederiae* (CBS 285.87); lane 8, *E. astroidea* (CBS 292.87); lane 9, *E. leptoplaca* (CBS 286.87); lane 10, *E. crustata* (CBS 201.87); lane 11, *E. lejoplaca* (CBS 248.87).

E. lata from wood trunk pathogenic fungi found in association with grapevine dieback (18), and from other genera in the family *Diatrypaceae* found on dead wood of grapevine (26,27) as well as several other *Eutypa* spp. However, no polymorphisms distinguished *E. lata* from *E. armeniaca* or *E. laevata*. *E. laevata* was identified only on *Salix* sp. (24) and, therefore, is not likely to be confused with *E. lata* during isolation from grapevine. *E. armeniaca* was described originally on *Prunus armeniaca* (3) and is responsible for branch dieback. *E. armeniaca* was reported to be morphologically identical to *E. lata* (14). These species are regarded as synonymous by some authors (24) but are separated by others (9,20). The PCR-RFLP method was not tested directly in situ for identification of *E. lata*, because the use of universal primers ITS1/ITS4 for PCR (1) would potentially amplify the ITS1/5.8S/ITS2 region of several fungi inhabiting wood tissue, resulting in multiple PCR products. Therefore, recognition of *E. lata* by RFLP pattern could not be achieved after digestion by *AluI*. Thus, the PCR-RFLP method requires fungi to be cultured for identification purposes.

In summary, the PCR-RFLP assay we have developed appears to be a reliable method for identification of the causal agent of *Eutypa* dieback. The utility of this method is based on the assumption that *E. laevata* is not found on grapevine and that *E. armeniaca* is truly synonymous with *E. lata*. This method proved to be more specific than the PCR detection method developed by Lecomte et al. (19) when using primer pairs Lata 1/Lata 2-1, Lata 1/Lata 2-2, and Lata 3/Lata 2-1, although it cannot be applied directly in situ. The PCR-RFLP method potentially could be used for diagnosis on other cultivated crops on which *E. lata* has been reported to be pathogenic (3,5). Considerable research is yet needed to clarify the taxonomy and the

biology of the family *Diatrypaceae* in order to achieve more effective management of the diseases caused by this group of fungi.

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