

# A Reassessment of the Species Concept in *Eutypa lata*, the Causal Agent of Eutypa Dieback of Grapevine

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## ABSTRACT

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*Eutypa dieback* is a vascular disease of several cultivated crops and trees worldwide. The attribution of the name to the agent responsible for branch dieback is ambiguous. Pathogenicity of *Eutypa* sp. first was reported on apricot and the causal agent was named *E. armeniaca*. However, no morphological differences were reported with the previously described *E. lata*, and some authors considered both species synonymous. Others regarded them as distinct species on the basis of pathogenesis and molecular analysis. We further investigated the relatedness of both species by phylogenetic analyses of the internal transcribed spacer region

and  $\beta$ -tubulin gene. These analyses included several other taxa placed in the same family (*Diatrypaceae*), and yielded three groups. The isolates referred to as *E. lata* in previous work clustered with *Diatrype stigma* in one group. Isolates of *E. armeniaca* and *E. lata* clustered in a second group, supporting the synonymy of these species. The third group included other *Eutypa* spp. supporting the polyphyletic origin of this genus. Measurements of conidia length and secondary metabolite production of isolates supported the phylogenetic analyses. Secondary metabolites appeared to be a synapomorphic character shared by several taxa including *E. lata*, *E. armeniaca*, *E. laevata*, and *E. petrakii* var. *petrakii*.

*Additional keywords:* vegetative compatibility groups, Xylariales.

*Eutypa lata* (Pers.:Fr.) Tul. & C. Tul. (= *E. armeniaca* Hansf. & M. V. Carter) is a pathogen of woody plants worldwide (7) and is responsible for significant economic damage to the wine industry (37) by reducing vegetative growth and fruit yields (26). The anamorph and teleomorph of the fungus are produced on dead wood, but conidia do not play a role in the epidemiology of the disease. The pathogen is disseminated by ascospores (29,30), which infect wounds and slowly kill its hosts by producing an array of enzymes (11,36) and secondary metabolites (19–21,23,39,40).

The genus *Eutypa* (*Ascomycetes*, *Xylariales*, *Diatrypaceae*) was first established by Tulasne and Tulasne (44). However, *E. lata* originally was based on Persoon's (31) morphological description of *Sphaeria lata* fruiting bodies found on five woody plant species (*Acer* sp., *Prunus* sp., *Lonicera* sp., *Crataegus* sp., and *Tilia* sp.). However, these specimens deposited at Persoon's herbarium in Leiden are now lacking identifiable morphological characters and Rappaz (32) designated a neotype of *E. lata* from *Acer* sp., *Tilia* sp., *Crataegus* sp., and *Lonicera xylosteum*, with published descriptions of the anamorph and teleomorph (33), and deposited its collection at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). However, no information concerning the pathogenicity of these isolates was reported.

Pathogenicity of a *Eutypa* sp. first was observed on apricot (*Prunus armeniaca* L.) and the name *E. armeniaca* Hansf. & M. V. Carter was given to the agent responsible for branch dieback (4). No morphological features of the anamorph or teleomorph could differentiate *E. lata* from *E. armeniaca* (16,17,32,33), and the species later were regarded as synonymous (33). However,

some authors regarded *E. armeniaca* as an appropriate designation for pathogenic strains (22). DeScenzo et al. (9) separated *E. lata* and *E. armeniaca* based on sequence analysis of ribosomal DNA and amplified fragment length polymorphism (AFLP) patterns. They concluded that *E. armeniaca* was present on cultivated crops, whereas *E. lata* was present on the native Californian plant hosts valley oak (*Quercus lobata*) and madrone (*Arbutus menziesii*) and also on grapevine (*Vitis vinifera* L.), and that both species were pathogenic on their hosts. However, the molecular analysis of isolates of their collection did not include additional referable diatrypaceous specimens and, therefore, the grouping of their isolates could not be compared with related species classified in this family. Moreover, morphological identification of taxa in the family *Diatrypaceae* is often problematic (16,17,33,35), and misnaming of isolates is possible.

Molecular markers have provided a new tool for delimitation of fungal taxa that traditionally were identified based on morphological species concepts (3). Biochemical data also have proven to be useful in the circumscription of species in certain groups of fungi (2,25,27,45). In the present study, phylogenetic analysis of the internal transcribed spacer (ITS) region,  $\beta$ -tubulin gene, and combined data sets were used in combination with morphological data (conidia length) and biochemical data (secondary metabolite profiles) of several diatrypaceous fungi to determine whether or not *E. lata* and *E. armeniaca* are distinct species.

## MATERIALS AND METHODS

**Fungal collection.** A collection of 46 fungal isolates representing 15 species of the family *Diatrypaceae* (*Eutypella vitis* (Schwein.:Fr.) Ellis & Everth., *Diatrypella* sp., *Diatrype bullata* (Hoffm.:Fr.) Fr., *D. stigma* (Hoffm.:Fr.) Fr., *D. flavovirens* (Pers.:Fr.) Fr., *Eutypa lejoplaca* (Fr.:Fr.) Fuckel, *E. maura* (Fr.:Fr.) Fuckel, *E. astroidea* (Fr.:Fr.) Rappaz, *E. crustata* (Fr.:Fr.) Sacc.,

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*E. leptoplaca* Rappaz, *E. tetragona* (Duby) Sacc., *E. petrakii* Rappaz, *E. laevata* (Nitschke) Sacc., *E. armeniaca*, and *E. lata* was established at the University of California, Davis (UC Davis) (Table 1). Five isolates of *E. lata* were recovered from wood canker by classical isolation techniques. Seven isolates of *Diatrype bullata* sp. (one isolate), *Diatrype stigma* (one isolate), *D. bullata* (one isolate), and *E. lata* (four isolates) were cultured from ascospores obtained as described by Carter (5) and germinated on water-agar medium. A single hyphal tip was transferred to potato dextrose agar (PDA) plates in order to obtain a pure culture of each isolate. Isolates recovered from ascospores were identified based on morphological features of the teleomorph (17,33).

*E. lata* isolates recovered from wood cankers were identified by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) patterns because of the paucity of identifiable morphological characters (35). The remaining 34 isolates were received from other culture collections (Table 1) and were named as identified by their source. *E. armeniaca* isolate 28120 from the American Type Culture Collection (ATCC; VA) was used by DeScenzo et al. (9) as reference for support of the *E. armeniaca* clade in their study. The collection received from E. & J. Gallo Winery Culture Collection (Modesto, CA) also included the group of eight isolates (E177.OK, E178.OK, E179.OK, E180.OK, E247.MD, E252.MD, E253.MD, and

TABLE 1. Species, identification number (ID), source, origin, and host of fungal taxa used for phylogenetic analyses, with GenBank accession numbers

Isolate ID <sup>a</sup>	Source <sup>b</sup>	Fungal species <sup>c</sup>	Host	Origin	GenBank accession number	
					ITS <sup>d</sup>	β-Tubulin
64171	ATCC	<i>Eutypella vitis</i>	<i>Vitis labrusca</i>	Illinois, United States	AJ302466	...
ELM13 <sup>myc</sup>	MSU	<i>Eutypella vitis</i>	<i>Vitis vinifera</i>	Michigan, United States	DQ006943	DQ006999
215.87	CBS	<i>Diatrype bullata</i>	<i>Salix</i> sp.	Switzerland	AJ302422	...
DCh400 <sup>per</sup>	UCD	<i>Diatrype bullata</i>	<i>Salix lasiolepis</i>	California, United States	DQ006946	DQ007002
205.87	CBS	<i>Diatrype disciformis</i>	<i>Fagus sylvatica</i>	Switzerland	AJ302437	...
211.87	CBS	<i>Diatrype stigma</i>	<i>Quercus</i> sp.	Ireland	AJ302438	...
DCash200 <sup>per</sup>	UCD	<i>Diatrype stigma</i>	<i>Quercus</i> sp.	California, United States	DQ006945	DQ007003
272.87 <sup>myc</sup>	CBS	<i>Diatrype flavovirens</i>	<i>Quercus ilex</i>	France	AJ302457	DQ006959
F-093,582	J. Checa	<i>Diatrype flavovirens</i>	<i>Eucalyptus globulus</i>	Spain	AJ302430	...
52484	ATCC	<i>Eutypella frostii</i>	<i>Acer</i> sp.	Unknown	AJ302441	...
DHb500 <sup>per</sup>	UCD	<i>Diatrypella</i> sp.	<i>Vitis vinifera</i>	California, United States	DQ006947	DQ007001
248.87 <sup>myc</sup>	CBS	<i>Eutypa lejoplaca</i>	<i>Acer pseudoplatanus</i>	Switzerland	DQ006922	DQ006974
219.87 <sup>myc</sup>	CBS	<i>Eutypa maura</i>	<i>Acer pseudoplatanus</i>	Switzerland	DQ006926	DQ006967
292.87 <sup>myc</sup>	CBS	<i>Eutypa astroidea</i>	<i>Fraxinus excelsior</i>	Switzerland	AJ302458	DQ006966
210.87 <sup>myc</sup>	CBS	<i>Eutypa crustata</i>	<i>Ulmus</i> sp.	France	AJ302448	DQ006968
287.87 <sup>myc</sup>	CBS	<i>Eutypa leptolaca</i>	<i>Frangula alnus</i>	Switzerland	DQ006924	DQ006961
286.87 <sup>myc</sup>	CBS	<i>Eutypa leptolaca</i>	<i>Arundo donax</i>	France	AJ302453	DQ006963
284.87 <sup>myc</sup>	CBS	<i>Eutypa tetragona</i>	<i>Sarothamnus scoparius</i>	France	DQ006923	DQ006960
244.87 <sup>myc</sup>	CBS	<i>Eutypa petrakii</i> var. <i>petrakii</i>	<i>Prunus spinosa</i>	Switzerland	AJ302455	DQ006958
245.87 <sup>myc</sup>	CBS	<i>Eutypa petrakii</i> var. <i>petrakii</i>	<i>Salix borealis</i>	Normay	AJ302456	DQ006970
291.87 <sup>myc</sup>	CBS	<i>Eutypa laevata</i>	<i>Salix</i> sp.	Switzerland	AJ302449	DQ006962
622.84 <sup>myc</sup>	CBS	<i>Eutypa armeniaca</i>	<i>Vitis vinifera</i>	Italy	AJ302446	DQ006964
28120 <sup>myc</sup>	ATCC	<i>Eutypa armeniaca</i>	<i>Prunus armeniaca</i>	Australia	DQ006948	DQ006975
290.87 <sup>myc</sup>	CBS	<i>Eutypa lata</i> var. <i>aceri</i>	<i>Acer pseudoplatanus</i>	Switzerland	DQ006925	DQ006965
217.87 <sup>myc</sup>	CBS	<i>Eutypa lata</i> var. <i>aceri</i>	<i>Acer campestre</i>	France	AJ302451	DQ006970
208.87 <sup>myc</sup>	CBS	<i>Eutypa lata</i>	<i>Tilia</i> sp.	Switzerland	DQ006927	DQ006969
289.87 <sup>myc</sup>	CBS	<i>Eutypa lata</i>	<i>Crataegus</i> sp.	France	DQ006928	DQ006973
247.87 <sup>myc</sup>	CBS	<i>Eutypa lata</i>	<i>Lonicera xylosteum</i>	Switzerland	AJ302459	DQ006972
8D <sup>myc</sup>	INRA	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	France	DQ006930	DQ006986
ESm <sup>per</sup>	Maurice Carter	<i>Eutypa lata</i>	<i>Shinus molle</i>	Australia	DQ006942	DQ006998
EA1 <sup>per</sup>	Maurice Carter	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Australia	DQ006937	DQ006994
ESw <sup>per</sup>	Adrian Bolay	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Switzerland	DQ006941	DQ006987
MD1 <sup>myc</sup>	IPV	<i>Eutypa lata</i>	Unknown	Italy	DQ006929	DQ006985
1776 <sup>myc</sup>	WRP	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Australia	DQ006936	DQ006993
E201 <sup>myc</sup>	WRP	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Australia	DQ006940	DQ006997
D001 <sup>myc</sup>	WRP	<i>Eutypa lata</i>	<i>Prunus armeniaca</i>	Australia	DQ006938	DQ006995
G004 <sup>myc</sup>	WRP	<i>Eutypa lata</i>	<i>Prunus armeniaca</i>	Australia	DQ006939	DQ006996
E117.NY <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	New York, United States	DQ006949	DQ006976
E177.OK <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Quercus</i> sp.	California, United States	DQ006950	DQ006977
E178.OK <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Quercus</i> sp.	California, United States	DQ006951	DQ006978
E179.OK <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Quercus</i> sp.	California, United States	DQ006952	DQ006979
E180.OK <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Quercus</i> sp.	California, United States	DQ006953	DQ006980
E247.MD <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Arbutus menziesii</i>	California, United States	DQ006954	DQ006981
E252.MD <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Arbutus menziesii</i>	California, United States	DQ006955	DQ006982
E253.MD <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Arbutus menziesii</i>	California, United States	DQ006956	DQ006983
E256.GR <sup>myc</sup>	E. & J. Gallo	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, United States	DQ006957	DQ006984
E30 <sup>can</sup>	UCD	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, United States	DQ006934	DQ006991
E7 <sup>per</sup>	UCD	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, United States	DQ006944	DQ007000
E38 <sup>can</sup>	UCD	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, United States	DQ006935	DQ006992
E31 <sup>can</sup>	UCD	<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, United States	DQ006933	DQ006990
E454 <sup>can</sup>	UCD	<i>Eutypa lata</i>	<i>Prunus armeniaca</i>	California, United States	DQ006931	DQ006988
E456 <sup>can</sup>	UCD	<i>Eutypa lata</i>	<i>Prunus armeniaca</i>	California, United States	DQ006932	DQ006989

<sup>a</sup> Isolate received as pure mycelium (myc), obtained from perithecia (per), or recovered from canker (can).

<sup>b</sup> ATCC = American Type Culture Collection, MSU = Michigan State University, UCD = University of California, Davis, CBS = Centraalbureau voor Schimmelfcultures, Utrecht, The Netherlands, WRP = Waite Research Precinct, Adelaide, Australia, INRA = Institut National de la Recherche Agronomique, Bordeaux, France, IPV = Instituto di Pathologia Vegetale, Milan, Italy, and E. & J. Gallo, Modesto, CA.

<sup>c</sup> Named as identified by their source.

<sup>d</sup> ITS = internal transcribed spacer.

E256.GR) found on madrone, oak, and grape in California and referred to as *E. lata* by DeScenzo et al. (9), and one isolate from grape (E117.NY) that grouped with neither *E. lata* nor *E. armeniaca* in their molecular analysis. All isolates were stored on PDA plugs at 4°C in sterile water.

**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). If no barrage was observed, isolates were placed in the same VCG (15). Isolates also were paired with themselves as a positive control.

**DNA extraction and sequencing.** Mycelium for DNA extraction was obtained by cutting an agar plug from the outer margin of a growing culture and placing it on a sterile cellophane strip (3 by 3 cm) overlaid on PDA. Inoculated plates were incubated at room temperature for 5 days in the dark. Mycelium was scraped off the cellophane, and DNA was extracted as described by Ceniz (8). The ITS1/5.8S/ITS2 region and partial coding sequence of the  $\beta$ -tubulin gene were PCR amplified in both directions in a 50- $\mu$ l reaction as described by Rolshausen et al. (35), using primer pairs ITS1/ITS4 (47) and  $\beta$ t2a/ $\beta$ t2b (14), respectively. PCR products were checked on a 1 $\times$  Tris-borate EDTA 1% agarose gel, stained with ethidium bromide, and visualized under UV light. PCR products were cleaned using a QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and were sequenced with the amplification primers at the DNA sequencing facility on the UC Davis campus.

**Phylogenetic analysis.** Sequence data were imported into Sequencher (version 4.1; Gene Codes Corporation, MI), in which forward and reverse sequences were aligned and spliced in a consensus sequence. Sequence data of fungal taxa were imported and aligned in ClustalX (41). Sequences were imported into MacClade (version 4.0; Sinauer Associates Inc., Sunderland, MA). Phylogenetic trees were constructed by the maximum parsimony method using the heuristic search algorithm of the Phylogeny Analysis Using Parsimony (PAUP) program (version 4.0b8; Sinauer Associates). Heuristic search was performed with simple addition of sequences and tree bisection-reconnection (TBR) branch swapping. Trees were rooted with *Eutypella vitis* as the out-group taxon prior to the analysis. All alignments and subse-

quent analyses were deposited in TreeBase (accession number S1385). Regions of the ITS1/5.8S/ITS2 sequences that could not be aligned with certainty and generated a high number of parsimonious trees were excluded from the analysis to facilitate tree construction (characters 25–26, 35, 181–182, 426–433, 487–488, 500, 518–519, 548, and 559). Both complete and truncated matrices of the ITS1/5.8S/ITS2 region are available in TreeBase (matrix accession numbers M2470 and M2472, respectively). The complete sequences of the  $\beta$ -tubulin gene were used for tree construction (matrix accession number M2471). In all the analyses performed, gaps were treated as missing data and characters had equal weight. Consistency index (CI), homoplasy index (HI), retention index (RI), and tree length were calculated for all trees. Support for internal nodes was assessed by bootstrapping with 1,000 replicates. Sequence identity between taxa was calculated by pairwise comparison. The GenBank taxa *Eutypella vitis* 64171, *Diatrypella frostii* 52484, *Diatrype bullata* 215.87, *D. disciformis* 205.87, *D. stigma* 211.87, and *D. flavovirens* F-093,582 were included only in the phylogenetic analysis of the ITS region because the nucleotide sequences of the  $\beta$ -tubulin region were not available in the GenBank database. Congruence of the combined ITS and  $\beta$ -tubulin data set was tested using the partition homogeneity test as implemented in PAUP (13) with 1,000 replicates.

**Determination of secondary metabolites produced by fungal isolates.** Fungal isolates listed in Table 2 were cultured on PDA medium and a 1-cm plug of agar was drawn from the edge of the growing culture and introduced into a 50-ml grape-based media (19) supplemented with 1% sucrose. Secondary metabolite production was assessed after 1 month in static culture at room temperature (20 to 22°C). The fungal mass was removed from the medium by filtration (Whatman No. 1) and the filtrate partitioned with an equal volume of ethyl ether. The ether was concentrated under reduced pressure and the dried extract dissolved in methanol (1 ml) and filtered through a 0.45- $\mu$ m nylon syringe filter. Aliquots (20  $\mu$ l) were analyzed quantitatively for fungal metabolites by reverse-phase high-performance liquid chromatography (Varian Microsorb 100-5 C18, 250 by 4.6 mm i.d.) using a mobile phase gradient of 100% water containing 0.5% acetic acid to 100% acetonitrile over 30 min at a flow of 1 ml/min, with detection at 254 nm using a photodiode array detector (Agilent 1100) (19). The experiment was repeated twice and taxa were considered as secondary metabolite producers if production of one of

TABLE 2. Secondary metabolites production by fungal taxa used in this study after 1 month of growth in static liquid culture of grape cane extract

Fungal specimen <sup>a</sup>	Secondary metabolites ( $\mu$ g/ml) <sup>b</sup>					
	Eutypinol	Eulatachromene	Methoxyeutypinol	Eutypine	Siccayne	Eulatinol
<i>Eutypa lata</i> 208.87	0.3	0.1	–	–	–	–
<i>E. lata</i> 289.87	6.5	0.2	0.2	–	–	–
<i>E. lata</i> E7	1	0.1	0.1	–	0.2	0.2
<i>E. lata</i> E38	0.6	0.1	0.1	–	–	–
<i>E. lata</i> E31	12.7	1.2	1.9	tr	0.1	0.1
<i>E. lata</i> E30	5.7	0.1	0.2	–	–	–
<i>E. lata</i> E454	1.5	0.1	0.3	–	tr	tr
<i>E. lata</i> E456	6.7	0.4	0.4	0.2	–	–
<i>E. lata</i> EA1	2.1	0.2	0.1	tr	–	–
<i>E. lata</i> ESm	0.3	–	–	–	–	–
<i>E. lata</i> D001	7.9	0.1	1.6	–	–	–
<i>E. lata</i> 8D	5.8	3.2	2.3	–	–	–
<i>E. lata</i> ESw	0.9	–	–	–	–	–
<i>E. lata</i> MD1	30	1.6	3.7	0.2	–	–
<i>E. lata</i> var. <i>aceri</i> 290.87	5.6	0.3	tr.	tr	tr	–
<i>E. armeniaca</i> 622.84	11.9	0.3	0.3	tr	–	tr
<i>E. laevata</i> 291.87	13.5	0.9	–	–	–	–
<i>E. petrakii</i> var. <i>petrakii</i> 244.87	40.2	1.1	2.7	–	–	–
E117.NY	0.2	0.2	2.5	–	–	–

<sup>a</sup> *Eutypa lata* isolates 247.87, G004, and 1776; *E. petrakii* var. *petrakii* 245.87; *E. tetragona* 284.87; *E. maura* 219.87; *E. astroidea* 292.87; *E. leptoplaca* isolates 286.87 and 287.87; *Diatrype flavovirens* 272.87; *D. bullata* DCh400; *Diatrypella* sp. DHB500; and Gallo isolates E177.OK, E178.OK, E179.OK, E180.OK, E247.MD, E252.MD, E253.MD, and E256.MD did not produce detectable quantities of the secondary metabolite listed in this table.

<sup>b</sup> Secondary metabolite production was determined based on the average of two replicates; tr = traces (below 0.1  $\mu$ g/ml) and – = no detectable quantities produced.

the acetylenic phenol compounds (eutypine, eulatachromene, eulatinol, eutypinol, methyleutypinol, or siccayne) was observed in at least one replication.

**Conidia measurement.** Fungal isolates listed in Table 3 were cultured on PDA medium. Isolates were placed in the dark at room temperature until conidiomata were produced. Conidiomata were removed from the culture medium and prepared by staining in cotton blue before taking measurements of 30 conidia per isolate under microscope using the software SPOT (version 3.5.2; Technical Instrument San Francisco, Burlingame, CA). Conidia measurements reflected the curved length of the spore.

## RESULTS

**Phylogenetic analyses.** A heuristic maximum parsimony analysis of the ITS1/5.8S/ITS2 region and partial coding sequence of the  $\beta$ -tubulin gene yielded a total of 535 (Fig. 1) and 4 (Fig. 2) equally most-parsimonious trees of 307 steps (CI = 0.603, RI = 0.861, HI = 0.397) and 404 steps (CI = 0.624, RI = 0.852, HI = 0.376), respectively. In all, 540 characters of the ITS region

TABLE 3. Conidia length ( $\mu\text{m}$ ) measured on PDA medium and secondary metabolites produced in liquid culture of grape cane extract of fungal specimens used in this study

Fungal specimen	Conidia ( $\mu\text{m}$ )		Prod <sup>c</sup>
	Measured <sup>a</sup>	Reported <sup>b</sup>	
<i>Eutypa lata</i> 208.87	26.4 $\pm$ 2.3	18–39	+
<i>E. lata</i> 289.87	29.8 $\pm$ 2.6	18–39	+
<i>E. lata</i> 247.87	nd	32–41	–
<i>E. lata</i> var. <i>aceri</i> 290.87	24.8 $\pm$ 2	15–28	+
<i>E. lata</i> var. <i>aceri</i> 217.87	24.3 $\pm$ 2	15–28	nd
<i>E. armeniaca</i> e 28120	nd	...	+
<i>E. armeniaca</i> e 622.84	25.0 $\pm$ 1.9	...	+
<i>E. lata</i> ESm	30.9 $\pm$ 2.2	...	+
<i>E. lata</i> ESsw	29.1 $\pm$ 4.8	...	+
<i>E. lata</i> E7	28.4 $\pm$ 2.3	...	+
<i>E. lata</i> E454	34.0 $\pm$ 2.8	...	+
<i>E. lata</i> E456	27.5 $\pm$ 3.2	...	+
<i>E. lata</i> E31	29.5 $\pm$ 3.4	...	+
<i>E. lata</i> MD1	26.3 $\pm$ 2.8	...	+
<i>E. lata</i> 1776	24.8 $\pm$ 2	...	–
<i>E. lata</i> G004	nd	...	–
<i>E. lata</i> D001	nd	...	+
<i>E. lata</i> EA1	nd	...	+
<i>E. lata</i> 8D	nd	...	+
<i>E. lata</i> E38	nd	...	+
<i>E. lata</i> E30	nd	...	+
E117.NY	19.4 $\pm$ 3	...	+
<i>E. laevata</i> 291.87	nd	34–55	+
<i>E. petrakii</i> var. <i>petrakii</i> 244.87	nd	24–45	+
<i>E. petrakii</i> var. <i>petrakii</i> 245.87	nd	24–45	+
<i>E. tetragona</i> 284.87	nd	20–37	–
<i>E. leptoplaca</i> 287.87	nd	17–39	–
<i>E. astroidea</i> 292.87	nd	26–43	–
<i>E. maura</i> 219.87	nd	11–20	–
<i>Diatrype flavovirens</i> 272.87	nd	20–31	–
<i>D. stigma</i> 211.87	nd	4.5–7.5	nd
Gallo E178.OK	6.6 $\pm$ 0.7	...	–
Gallo E179.OK	7.5 $\pm$ 0.5	...	–
Gallo E180.OK	7.3 $\pm$ 0.6	...	–
Gallo E247.MD	5.9 $\pm$ 0.8	...	–
Gallo E252.MD	6.6 $\pm$ 0.7	...	–
Gallo E253.MD	6.8 $\pm$ 0.7	...	–
Gallo E256.GR	6.2 $\pm$ 0.6	...	–
<i>D. bullata</i> DCh400	nd	15–25	–
<i>Diatrypella</i> sp. DHb500	nd	...	–

<sup>a</sup> nd = not determined.

<sup>b</sup> As reported by Rappaz (33).

<sup>c</sup> Secondary metabolite production based on results as shown in Table 2: + = production of at least one of the acetylenic phenol compounds (eutypine, eulatachromene, eulatinol, eutypinol, methyleutypinol, or siccayne), – = no detectable quantities of acetylenic phenol compounds produced, and nd = not determined.

were used for tree reconstruction, of which 396 were constant, 36 were parsimony uninformative, and 108 were parsimony informative. Of 418 characters derived from the partial coding sequence of the  $\beta$ -tubulin gene, 249 were constant, 46 were parsimony-uninformative, and 123 were parsimony informative. The combined analysis of both nucleotide sequences yielded 260 most parsimonious trees (Fig. 3) of 723 steps (CI = 0.609, RI = 0.833, HI = 0.391). In all, 1,000 characters of the combined regions were used for tree reconstruction, of which 681 were constant, 112 were parsimony uninformative, and 207 were parsimony informative.

*D. stigma* DCash200 and *D. bullata* DCh400 showed 100% nucleotide sequence homology with sequences posted in GenBank (Table 1) of *D. bullata* 215.87 and *D. stigma* 211.87, respectively. *Diatrypella* sp. DHb500 showed 97.7% sequence homology and 100% bootstrap support with GenBank isolate of *Diatrypella frostii* 52484. Thus, phylogenetic analyses of the  $\beta$ -tubulin and combined data sets of ITS and  $\beta$ -tubulin could be performed with confidence in order to establish taxonomic affiliation and relationships of fungal taxa classified within the family *Diatrypaceae*.

Phylogenetic reconstruction of the combined sequences yielded tree topology similar to that for individual ITS and  $\beta$ -tubulin sequences analyses, with separation of three distinct groups of taxa (Figs. 1, 2, and 3). Partition homogeneity tests demonstrated congruence between the analysis of the two individual data sets ( $P = 0.068$ ). The analysis of the combined data sets yielded strong support for the three groups, all of which were accorded bootstrap values greater than 95% (Fig. 3). In all three analyses, *Diatrypella* sp. and *Diatrype flavovirens* were excluded from the three groups.

The first group included *Diatrype bullata*, *D. stigma*, and the eight Gallo isolates previously referred to as *E. lata* (9). Within group 1, the clustering of the eight Gallo isolates with *D. stigma* was strongly supported at a 99% bootstrap value in the combined data set analysis (Fig. 3).

Group 2 included *Eutypa lejoplaca*, *E. crustata*, *E. astroidea*, *E. maura*, *E. tetragona*, and *E. leptoplaca* and was strongly supported by bootstrap in all three data set analyses (Figs. 1, 2, and 3).

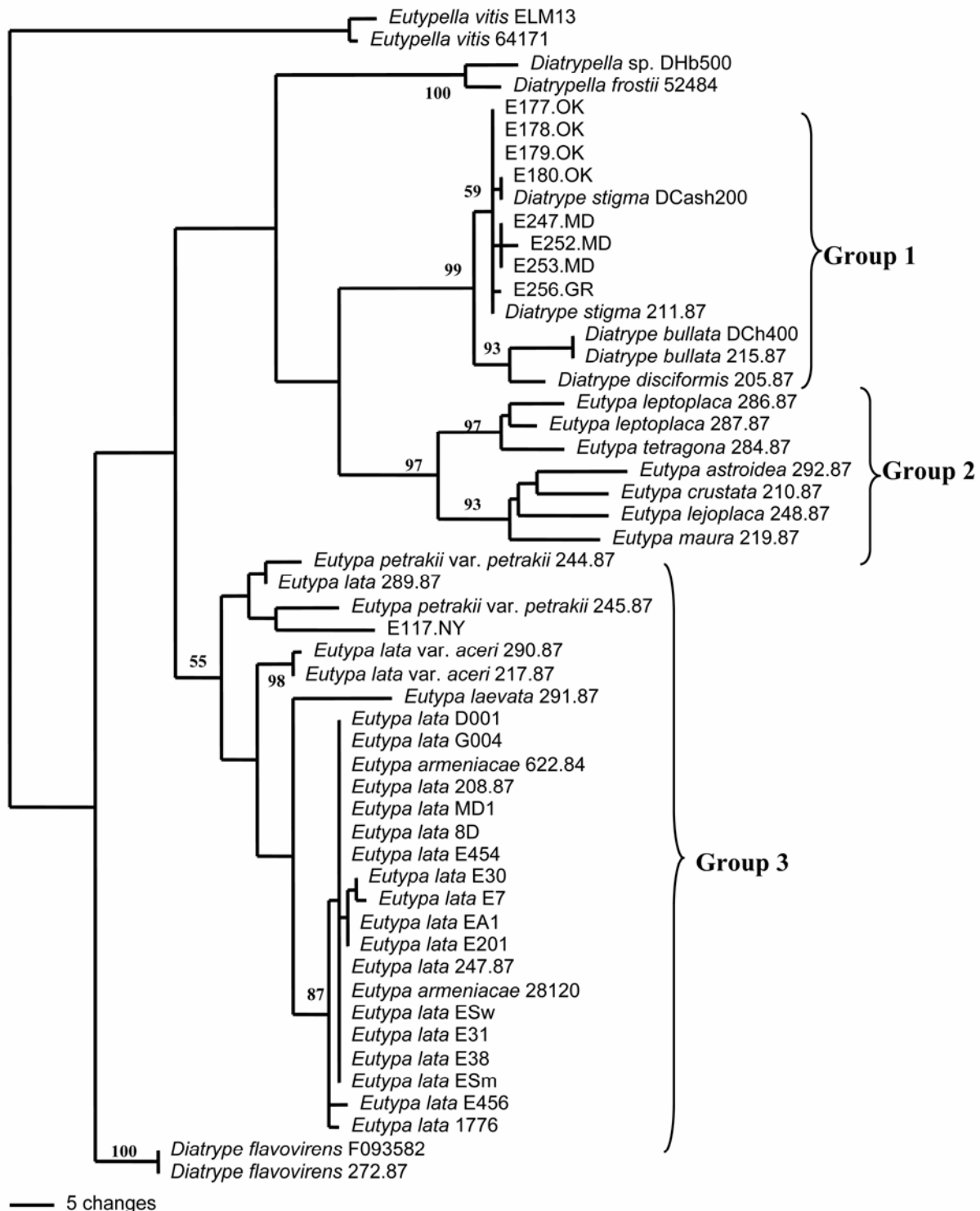
Bootstrap support of group three, that included *E. armeniaca*e, *E. laevata*, *E. petrakii* var. *petrakii*, *E. lata*, and isolate E117.NY, was 95% in the combined analysis (Fig. 3), whereas much lower values were obtained in individual analyses (Figs. 1 and 2). Within this group, monophyly of a clade that included the two *E. armeniaca*e isolates and most *E. lata* isolates was supported with a 99% bootstrap value. The isolates of this clade showed over 99% sequence homology in the combined data set. All *E. lata* isolates of this clade were placed in different VCGs, indicating that they were not clonally related. However, this clade excluded CBS isolates *E. lata* 289.87 (97.8% sequence homology) and *E. lata* var. *aceri* 217.87 and 290.87 (95.7% sequence homology). The two *E. lata* var. *aceri* isolates grouped together with a 100% bootstrap support and also shared a common ancestor with *E. laevata* (96% bootstrap support). The two isolates of *E. petrakii* var. *petrakii* showed 5.3% heterogeneity between their nucleotide sequences of the combined data set. *E. petrakii* var. *petrakii* isolate 245.87 clustered with E117NY with a 56% bootstrap support, whereas *E. petrakii* var. *petrakii* isolate 244.87 did not cluster with any taxon.

**Secondary metabolites.** Analysis of secondary metabolites indicated that *E. lata* isolates 208.87, 289.87, ESm, ESsw, E7, E454, E456, E31, MD1, D001, EA1, 8D, E38, and E30; *E. lata* var. *aceri* 290.87; *E. armeniaca*e 622.84; *E. laevata* 291.87; *E. petrakii* var. *petrakii* isolates 244.87 and 245.87; and one Gallo isolate, E117.NY, produced at least one of the following compounds: eutypine, eulatachromene, eulatinol, eutypinol, methyleutypinol, or siccayne (Table 2). Eutypinol, methyleutypinol, and eulatachromene were the most widely distributed and abundant metabolites among strains, whereas eutypine, siccayne, and eulati-

nol occasionally were produced in small amounts by fungal isolates. The eight Gallo isolates E177.OK, E178.OK, E179.OK, E180.OK, E247.MD, E252.MD, E253.MD, and E256.GR; *E. lata* isolates 247.87, 1776, and G004; *E. tetragona* 284.87; *E. leptoplaca* 287.87; *E. astroidea* 292.87; *E. maura* 219.87; *D. flavovirens* 272.87; *D. bullata* DCh400; and *Diatrypella* sp. DHb500 did not produce detectable quantities of these secondary metabolites.

**Conidia length.** The lengths of conidia of *E. lata* isolates 208.87 and 289.87 were measured at  $26.4 \pm 2.3$  and  $29.8 \pm 2.6$   $\mu\text{m}$ ,

respectively, and were in accordance with the measurements of the same isolates made by Rappaz (33) and presented in Table 3. Likewise, *E. lata* isolates ESm, ESw, E7, E454, E456, E31, MD1, and 1776 ranged from  $24.8 \pm 2$  to  $34 \pm 2.8$   $\mu\text{m}$  (Table 3). *E. lata* var. *aceri* isolates 290.87 and 217.87 were measured at  $24.8 \pm 2$  and  $24.3 \pm 2$   $\mu\text{m}$ , respectively, which was in accordance with the measurements made by F. Rappaz for the same isolates (33). The conidia length of *E. armeniaca* 622.84 was measured at  $25 \pm 1.9$   $\mu\text{m}$ . The eight Gallo isolates (E177.OK, E178.OK, E179.OK, E180.OK, E247.MD, E252.MD, E253.MD, and E256.GR) had

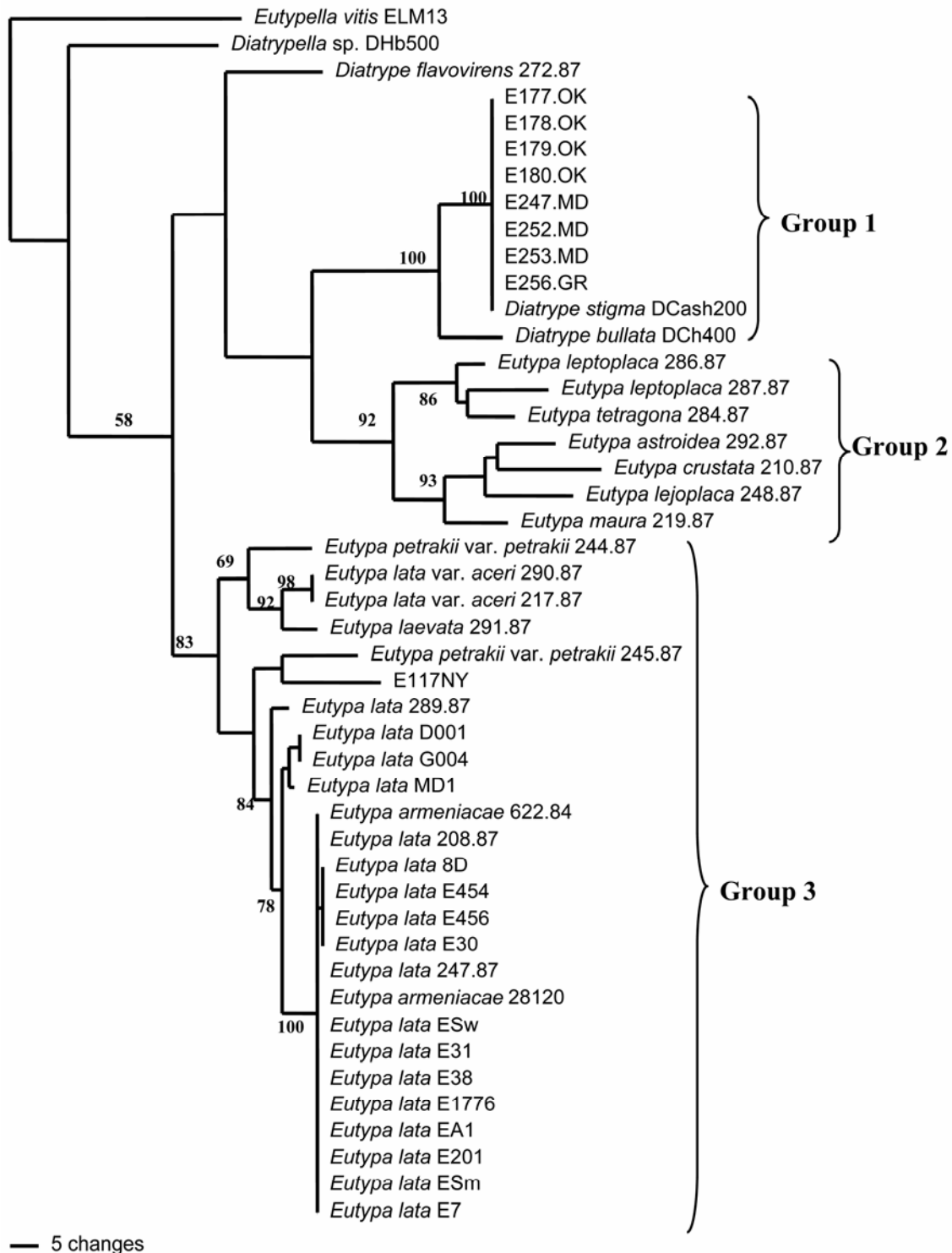


**Fig. 1.** One of the 535 most parsimonious phylogenetic trees based on sequence differences in the internal transcribed spacer (ITS)1/5.8S/ITS2 region. Clades that are supported by bootstrap analysis (1,000 replications) are indicated by numbers next to the clade (when more than 50%). Tree length = 307, consistency index = 0.603, retention index = 0.861, and homoplasy index = 0.397.

conidia lengths ranging from  $5.9 \pm 0.8$  to  $7.5 \pm 0.5$   $\mu\text{m}$ . Finally, the conidia length of isolate E117.NY was measured at  $19.4 \pm 3$   $\mu\text{m}$ . The conidia lengths of other diatrypaceous fungi already were published by Rappaz (33) and also are represented as a reference in Table 3. The conidia length of isolates in Table 3 was combined with the absence or presence of secondary metabolite production as determined in Table 2 in order to improve clarity in the results.

## DISCUSSION

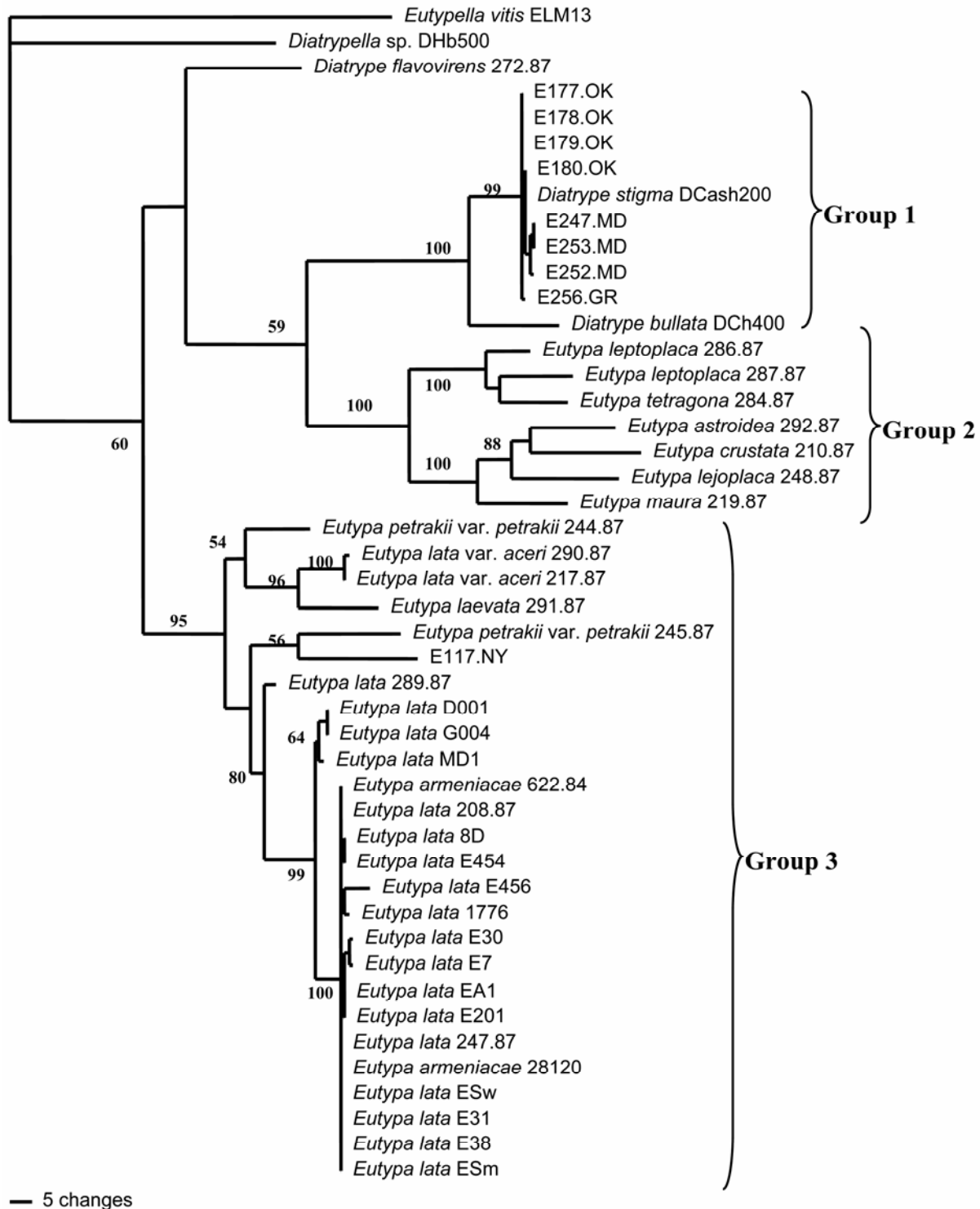
The phylogenetic reconstruction of individual and combined data sets support the results obtained by Acero et al. (1). The tree topology of the ITS region of several taxa placed in the family *Diatrypaceae* obtained by Acero et al. (1) distinguished nine groups within this family, from which groups 8, 3, and 6 correspond to groups 1, 2, and 3, respectively, as presented in our analysis.



**Fig. 2.** One of the four most parsimonious phylogenetic trees based on sequence differences in the  $\beta$ -tubulin gene. Clades that are supported by bootstrap analysis (1,000 replications) are indicated by numbers next to the clade (when more than 50%). Tree length = 404, consistency index = 0.624, retention index = 0.852, and homoplasy index = 0.376.

Our data confirmed the polyphyletic origin of the genus *Eutypa*, but did not support recognition of *E. lata* and *E. armeniaca* as two distinct species as suggested by DeScenzo et al. (9). The group of eight isolates found on madrone, oak, and grape in California and referred to as *E. lata* in DeScenzo's work was shown to be related to *D. stigma* in our analysis (group 1). The attribution of names to Gallo's isolates collected in California was based on the morphology of the anamorph, and teleomorph when available, using the key of Glawe and Rogers (16,17). Identification of fungi placed in the family *Diatrypaceae* is often problem-

atic because of interspecific resemblance and intraspecific polymorphism of morphological characters. Phylogenetic analysis established by Acero et al. (1) did not support the morphological schemes used within the family *Diatrypaceae* for species delimitation. Moreover, the molecular analyses conducted by DeScenzo et al. (9) did not include other diatrypaceous fungi; therefore, they could not compare the grouping of their isolates with related taxa. Phylogenetic reconstruction using a combination of two genomic regions of several taxa placed in the family *Diatrypaceae* indicates that the eight Gallo isolates are referable to *D. stigma*.



**Fig. 3.** One of the 260 most parsimonious phylogenetic trees based on sequence differences in the combined internal transcribed spacer (ITS)1/5.8S/ITS2 region and  $\beta$ -tubulin gene. Clades that are supported by bootstrap analysis (1,000 replications) are indicated by numbers next to the clade (when more than 50%). Tree length = 723, consistency index = 0.609, retention index = 0.833, and homoplasy index = 0.391.

Glawe and Rogers (17) noticed that the specimens placed in the collection referable to *D. stigma* presented morphological polymorphisms and suggested that it could be separated into five morphological groups. Rappaz (34) also attributed two different species names (*D. decortica* and *D. undulata*) to the specimens that were morphologically distant from *D. stigma* sensu stricto. Based on our results, the conidia lengths of the eight Gallo isolates fit well with *D. stigma* group 3 as described by Glawe and Rogers (17) and *D. stigma* sensu stricto as described by Rappaz (34).

Four *Eutypa* spp. clustered in group 3 (*E. lata*, *E. armeniaca*, *E. petrakii* var. *petrakii*, and *E. laevata*). However, species delimitation within this group as established by Rappaz (33) using morphological characters was not totally supported by phylogenetic reconstruction. The grouping of the two *E. petrakii* var. *petrakii* isolates and the monophyly of *E. lata* were not demonstrated. A strong monophyletic clade that included most *E. lata* isolates as well as the two *E. armeniaca* isolates segregated from the rest of the taxa placed in group 3. *E. lata* and *E. armeniaca* were described in the literature as morphologically identical (16,17, 32,33), and our phylogenetic analyzes supported the synonymy of these two species. However, *E. lata* var. *aceri* (isolates 217.87 and 290.87) showed genetic divergence compared with the *E. lata* and *E. armeniaca* clade. Rappaz (33) also noted morphological differences between *E. lata* var. *aceri* and *E. lata*, such as pronounced aerial conidiogenesis and shorter conidia, but did not consider these specimens to be different species. Interestingly, the two *E. lata* var. *aceri* isolates are host specific to *Acer* spp. and were closely related to *E. laevata*, host specific to *Salix* spp. We propose that *E. lata* var. *aceri* should be renamed on the basis of the morphological and molecular features. The two taxa also differ in host range, with *E. lata* being a pathogen of many woody plants. The position of *E. lata* isolate 289.87 from *Cratageus* spp. is ambiguous, because it showed more nucleotide divergence than the other *E. lata* isolates and was placed outside the *E. lata* clade, but was the most closely related taxon. Rappaz noted distinctive morphological features of this isolate, such as formation of large pycnidia and production of aerial mycelium in rays. Based on these indications of morphological and molecular divergence, *E. lata* 289.87 also should not be considered as *E. lata* sensu stricto. Thus, only *E. lata* isolates 208.87 and 247.87 deposited at CBS should be regarded as authentic. Application of the biological species concept could clarify species delimitation within the *Diatrypaceae* family; however, to our knowledge, sexual reproduction of compatible isolates in vitro has not yet been possible.

The production of secondary metabolites by taxa clustered in group 3 suggested the synapomorphy of this character. None of the other taxa produced detectable quantities of the secondary metabolites analyzed, although *E. leptoplaca* (group 2) was found to produce different secondary metabolites in culture medium (N. Mahoney, unpublished data). To date, secondary metabolite production by specimens classified in the family *Diatrypaceae* has not been extensively investigated. However, research conducted in *Xylariaceae*, a sister family of *Diatrypaceae* (38), showed the production of a wide range of novel metabolites, including antibiotics and phytotoxic compounds (46). Whalley and Edwards (45) also determined that production of secondary metabolites in the family *Xylariaceae* was a constant and reliable feature, and that it could be used as an additional taxonomic character for species delimitation and to predict intra- and intergeneric associations. Biochemical data also have proven to be useful to reinforce species separation in other groups of fungi (2,25,27). However, more research is yet needed to determine whether these chemotaxonomic schemes hold on within the family *Diatrypaceae*.

In our analysis, differences in qualitative and quantitative secondary metabolite production was observed among *Eutypa* spp. and *E. lata* isolates in group 3, ranging from high (10 to 40 µg/ml) to no detectable levels. Several of these secondary metabolites

have phytotoxic effect (19) and are considered virulence factors. Eutypine, for example, was reported to disrupt the functioning of mitochondria in *Vitis vinifera* cells (10,12), but the sites targeted in planta by the other secondary metabolites have not yet been identified. However, our results indicated that eutypine was not the major secondary metabolite produced. These results also were supported by Mahoney et al. (20). Thus, the tolerance of grapevine cultivars to *E. lata* cannot be explained only by the detoxification of a single compound (i.e., eutypine) as previously supported (12,18). The disparity in qualitative or quantitative production of secondary metabolites among *E. lata* isolates could partly explain their variation in virulence as previously observed on apricot (6) and grapevines (28,29), although such correlation has yet to be strongly established. *E. lata* is an outcrossing species presenting high genetic diversity (29,30). Therefore, phenotypic differences, such as virulence, are expected to vary within *E. lata* populations. Consequently, delimitation of species based on pathogenic properties may not be appropriate within this group of fungi.

The results of this study also confirmed the occurrence of several diatrypaceous species on grapevines. As discussed above, *D. stigma* (E256GR) and Gallo isolate E117NY were recovered from grapevines. Trouillas et al. (43) also found the teleomorph of a *Diatrype* sp. on dead wood of grapes. A *Diatrypella* sp. also was recovered from wood cankers of grapevines (F. Trouillas, personal communication). Moreover, *E. leptoplaca* (42) and *Cryptovalsa ampelina* (24) were reported to be pathogenic on grapes. Ongoing studies are establishing the occurrence of these taxa within and in the vicinity of Californian vineyards. The presence of several related taxa on grapevines also suggests that precautions have to be taken for positive identification of the causal agent responsible for disease. Disease diagnosis commonly is done by isolating the organism from wood cankers on PDA medium; however, because of the morphological resemblance of species in this group of fungi in culture, misidentification can occur (35). Considerable work is still needed to establish proper species delimitations within the family *Diatrypaceae*, which will allow for more accurate identification of pathogens and development of more appropriate control strategies.

In conclusion, these results confirmed the polyphyletic origin of the genus *Eutypa*. Secondary metabolite production of isolates in the family *Diatrypaceae* showed good correlation with phylogenetic reconstruction. These data have proven to be useful to establish species recognition within this family of fungi, and to clearly confirm that *E. lata* and *E. armeniaca* do not represent different species but should continue to be regarded as synonymous.

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