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Research Papers

Species of *Diatrypaceae* associated with grapevine trunk diseases in Eastern Spain

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Summary. The presence and diversity of Diatrypaceae species occurring on grapevines in Eastern Spain were investigated. Several species were identified on the basis of morphological characters and phylogenetic analyses of the complete sequence of the internal transcribed spacers of the ribosomal DNA and part of the β-tubulin gene. Five species of Diatrypaceae isolated from the wood of diseased grapevines, pruning debris and/or perithecia were identified, including *Anthostoma decipiens*, *Cryptovalsa ampelina*, *Eutypa lata*, *Eutypella citricola* and *Eutypella microtheca*. Additionally, four taxa could not be identified to the species level but were closely related to *Eutypa tetragona* based on phylogenetic analyses. *Eutypa lata* was the most prevalent species and showed the greatest degree of genetic diversity. *Cryptovalsa ampelina* and *E. microtheca* ranked second in the frequency of isolations, while all the remaining species were less frequently isolated. *Eutypella citricola* and *E. microtheca* are reported for the first time as occurring on grapevine in Spain and this is the first report of *A. decipiens* occurring on grapevine.

Key words: *Anthostoma*, *Cryptovalsa*, *Eutypa*, *Eutypella*, *Vitis vinifera*, Bayesian inference.

Introduction

Several fungi in the Diatrypaceae are known to occur on grapevines (*Vitis vinifera* L.) in many grape-growing countries, including species in the genera *Cryptosphaeria* Ces. & De Not., *Cryptovalsa* Ces. & De Not. ex Fuckel, *Diatrype* Fr., *Diatrypella* (Ces. & De Not.) De Not., *Eutypa* Tul. & C. Tul. and *Eutypella* (Nitschke) Sacc. (Carter, 1988, 1991; Mostert *et al*., 2004; Trouillas and Gubler, 2004; Catal *et al*., 2007; Pitt *et al*., 2009; Úrbez-Torres *et al*., 2009; Trouillas and Gubler, 2010a, 2010b; Trouillas *et al*., 2010, 2011; Úrbez-Torres *et al*., 2011). To date, 14 known species of this family have been reported from grapevine, while about five taxa are still not assigned to any known species (Farr and Rossman, 2011). *Eutypa lata* (Pers.) Tul. & C. Tul., the causal agent of Eutypa dieback, is the most important grapevine pathogen known within this family (Carter, 1988, 1991). While identification, pathogenicity, epidemiology and control of *E. lata* have been thoroughly studied worldwide, the same subjects concerning other diatrypaceous fungi still require further investigation. However, recent reports on the identification and pathogenicity of other diatrypaceous taxa, including *Cryptovalsa ampelina* (Nitschke) Fuckel (Mostert *et al*., 2004; Luque *et al*., 2006; Martín *et al*., 2009; Trouillas and Gubler, 2010b), *Eutypa leptoplaca* (Mont.) Rappaz (Trouillas and Gubler, 2004, 2010b), *Eutypella vitis* (Schwein.) Ellis & Everh. (Catal *et al*., 2007; Jordan and Schilder, 2007; Úrbez-Torres *et al*., 2009, 2011), and *Cryptosphaeria pullmanensis* Glawe (Trouillas and Gubler,

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2010b) are contributing to a better knowledge about these fungi and, specifically, the role they play in the grapevine trunk diseases.

Recent studies in California and Australia have suggested a possible correlation between infection of grapevines by diatrypaceous species occurring on natural and ornamental host plants in the immediate proximity of the vineyards (Trouillas and Gubler, 2010b; Trouillas *et al*., 2010, 2011). Grapevine infection could be partly explained by a possible opportunistic lifestyle of these fungi, normally saprobic on their natural host(s) but occasionally pathogenic to other unusual hosts (e.g. grapevine) rendered susceptible due to predisposing factors (Trouillas *et al*., 2010, 2011). Pathogenicity experiments on *V. vinifera* vines using various species of Diatrypaceae have been conducted in California vineyards. Results showed that Diatrypaceae were capable of colonizing grapevine wood. However, although some species seemed to produce vascular discolorations, the disease caused by these fungi and their virulence remained unclear (Trouillas and Gubler, 2010b). As suggested by Trouillas and Gubler (2010b), extended incubation periods while conducting pathogenicity tests should be necessary to better characterize disease expression and aggressiveness of these fungi. In vineyards, symptoms caused by *E. lata* early in the season (*i.e.* stunted shoot growth with small, cupped and chlorotic leaves) are expressed 3 to 8 years after infection (Carter, 1988). However, in greenhouse assays, symptoms have been reported to occur within 4 weeks (Péros and Berger, 1994), 6 weeks (Jung *et al*., 2010), 8 months (Sosnowski *et al*., 2007), or even during the second growing season (Muruamendiaraz and Legorburu, personal communication).

The occurrence of Diatrypaceae species on grapevines in Spain is still poorly known. Studies published in the last decade confirmed the existence of only *E. lata* and *C. ampelina*. Eutypa dieback was reported for the first time in late 1970's in Extremadura, Southwestern Spain (Arias and Moral, 1981). Symptoms of this disease were later reported in the mid 1990's in the Rioja wine region, North Central Spain (Mateo, 1995). Several further studies have shown *E. lata* to be widely distributed in the grapevine growing regions in Spain (Armengol *et al*., 2001; Úrbez-Torres and Peláez, 2001; Péros and Berger, 2003; Santiago *et al*., 2005; Martín and Cobos, 2007; Luque *et al*., 2009; Muruamendiaraz *et al*., 2009). It is currently accepted that *E. lata* is the most common Diatrypaceae species found on grapevine in Spain. *Cryptovalsa ampelina* was reported for the first time in 2006 in Catalonia, Northeastern Spain, and was found mainly on pruning debris and rarely on standing vines showing symptoms of trunk diseases such as dieback and cankers (Luque *et al*., 2006). A moderate virulence has been suggested for this fungus (Mostert *et al*., 2004; Luque *et al*., 2006). Martín *et al*. (2009) also reported *C. ampelina* from the central region of Spain. To date, no other Diatrypaceae species have been reported from grapevines in Spain. Therefore, the present study aimed to determine the presence and diversity of other diatrypaceous fungi occurring on grapevines in this country. Morphological characters, such as colony morphology and conidial dimensions, were used in combination with phylogenetic analyses of the internal transcribed spacers of the rDNA and part of the β-tubulin gene to aid in the characterization and identification of the taxa.

Materials and methods

Fungal isolates and morphological characters studied

The isolates used in this study were obtained from infected shoots, cordons and trunks showing dieback and/or internal wood necroses, or directly from perithecia embedded in the bark of grapevines that were surveyed in Spain during the years 2002–2010 (Table 1). Wood chips obtained from the necrotic tissues were surface-sterilized (3–4 min in 70% ethanol), blotted on sterile filter paper to remove excessive ethanol, and plated onto Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA) amended with streptomycin sulphate (Sigma-Aldrich Co., St. Louis, MO, USA) at 100 units per ml (PDA-str) as described by Johnston and Booth (1983). Pure cultures of fungi were obtained by isolation of single hyphal tips. Monosporic isolations from perithecia were carried out as follows. Stromata were cut with a sterile blade to reveal the perithecial contents and a drop of sterile water (about 100 μL) was placed on the cut surface of the fruiting bodies. A water drop containing masses of ascospores was then collected with a pipette, plated onto PDA-str and spores were dispersed with a Digralsky spreader. Twenty four hours after plating, a single germinating spore was selected under the microscope and transferred to a fresh PDA Petri dish. Isolates were maintained at 4°C in sterile distilled water for long-term storage.

Table 1. Isolates of several Diatrypaceae species obtained from grapevine in different locations of Spain, used in this study.

Species	Isolate	Location	Province	Isolation year	Grapevine variety	Source details
Anthostoma decipiens	JL567	Falset	Tarragona	2004	Tempranillo	Wood necrosis, arm
Cryptovalsa ampelina	JL413	Vimbodí	Tarragona	2003	Red Grenache	Perithecia, on pruning debris
Cryptovalsa ampelina	JL424	Capçanes	Tarragona	2003	Carignane	Perithecia, on pruning debris
Cryptovalsa ampelina	JL476	Bot	Tarragona	2003	Macabeo	Canker, arm
Cryptovalsa ampelina	JL717	El Pla del Penedès	Barcelona	2009	Cabernet Sauvignon	Wood necrosis, living shoot
Eutypa lata	JL355	Caldes de Montbui	Barcelona	2002	Chardonnay	V-shaped necrosis, arm
Eutypa lata	JL399	Pacs del Penedès	Barcelona	2003	Cabernet Sauvignon	Canker, arm
Eutypa lata	JL407	Mediona	Barcelona	2003	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL411	Vimbodí	Tarragona	2003	Red Grenache	V-shaped necrosis, arm
Eutypa lata	JL427	La Vilella Baixa	Tarragona	2003	Cabernet Sauvignon	Canker, arm
Eutypa lata	JL431	Capçanes	Tarragona	2003	Carignane	V-shaped necrosis, arm
Eutypa lata	JL432	Òdena	Barcelona	2003	Macabeo	V-shaped necrosis, arm
Eutypa lata	JL479	Batea	Tarragona	2003	Red Grenache	Canker, arm
Eutypa lata	JL600	Vilajuïga	Girona	2005	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL677	Barbastro	Huesca	2007	Cabernet Sauvignon	Canker, arm
Eutypa lata	JL720	Barriobusto	Álava	2006	Tempranillo	Wood necrosis, arm
Eutypa lata	JL721	Lanciego	Álava	2006	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL723	Laguardia	Álava	2008	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL725	Bargota	Navarra	2008	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL726	Labastida	Álava	2008	Tempranillo	V-shaped necrosis, arm
Eutypa lata	JL727	Navaridas	Álava	2007	Tempranillo	Perithecia, on arm
Eutypa lata	JL731	La Morra	Burgos	2002	Tempranillo	Wood necrosis, unknown part
Eutypa lata	JL732	Malagón	Ciudad Real	Unknown	Red Grenache	Wood necrosis, unknown part
Eutypa lata	JL739	Albacete	Albacete	2010	Unknown	Wood necrosis, unknown part
Eutypa lata	JL740	Yecla	Murcia	2009	Unknown	Wood necrosis, unknown part
Eutypa lata	JL741	Villena	Alicante	2009	Unknown	Wood necrosis, unknown part
Eutypa lata	JL743	La Cañada	La Rioja	2010	Tempranillo	Wood necrosis, unknown part
Eutypa lata	JL744	Azofra	La Rioja	2010	Tempranillo	Wood necrosis, unknown part

(*Continued*)

Table 1. *Continues.*

Species	Isolate	Location	Province	Isolation year	Grapevine variety	Source details
Eutypa sp.	JL488	Batea	Tarragona	2003	Red Grenache	V-shaped necrosis, arm
Eutypa sp.	JL688	Barbastro	Huesca	2007	Merlot	V-shaped necrosis, arm
Eutypa sp.	JL690	Barbastro	Huesca	2007	Chardonnay	V-shaped necrosis, arm
Eutypa sp.	JL742	Albacete	Albacete	2010	Unknown	Wood necrosis, unknown part
Eutypella citricola	JL583	Olèrdola	Barcelona	2004	Chenin Blanc	Wood necrosis, trunk
Eutypella citricola	JL734	Murcia	Murcia	2009	Unknown	Wood necrosis, unknown part
Eutypella microtheca	JL609	Vilajuïga	Girona	2005	Tempranillo	Perithecia, on pruning debris
Eutypella microtheca	JL625	Peralada	Girona	2005	Don Mariano	Wood necrosis, living shoot
Eutypella microtheca	JL735	Villanueva de Alcolea	Castellón	2009	Unknown	Wood necrosis, unknown part
Eutypella microtheca	JL738	Novelda	Alicante	2009	Unknown	Wood necrosis, unknown part

All isolates available through IRTA (J. Luque). All isolates obtained from infected wood except for *Cryptovalsa ampelina* JL413 (monosporic), *C. ampelina* JL424 (monosporic), *Eutypa lata* JL727 (multisporic), and *Eutypella microtheca* JL609 (monosporic), which were all obtained from perithecia. Other accession number for selected isolates deposited at Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands are: JL411, CBS 121487; JL413, CBS 117484; JL424,CBS 117485; JL476, CBS 117486.

Sporulation was enhanced by culturing isolates on PDA at 25°C with a 12/12 hour photoperiod, under near UV (Philips TLD 18W/08; Philips Electronics N.V., Amsterdam, Netherlands) and white fluorescent light (Osram L 18W/840; Osram GmbH, Munich, Germany). Isolates that did not sporulate within 3 months of incubation in the above conditions were not used for the measurements of conidia. The mean, standard deviation, 95% confidence intervals and minimum and maximum values were calculated from the measurements made with the ×100 microscope objective of 50 conidia mounted in water. Colony characters of isolates were recorded after growing the fungi on PDA at the above cited conditions for 4 weeks. When possible, fungi were tentatively identified from their colony and conidial morphology by comparing with previous studies (Trouillas and Gubler, 2010b; Trouillas *et al*., 2010; Trouillas *et al*., 2011).

DNA extraction and sequencing

DNA was extracted from the fungal mycelium as described by Alves *et al*. (2004). Amplification of the ITS1 and ITS2 regions flanking the 5.8S ribosomal RNA gene was carried out using the universal primers ITS1 and ITS4 (White *et al*., 1990). Part of the β-tubulin gene was amplified by using the primers Bt2a and Bt2b (Mostert *et al*., 2006). All reactions were performed on a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, Foster City, CA, USA), following the temperature profiles described in Luque *et al*. (2005) for the ITS region, and Mostert *et al*. (2006) for the β-tubulin gene. Purification of PCR products was according to the methods described in Luque *et al*. (2005). The purified amplicons were sequenced in both directions using the aforementioned primers and the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The resulting fragments were analyzed on an ABI Prism 377 automated DNA sequencer (Perkin Elmer, Norwalk, CT, USA). Sequences were read and edited with BioEdit Sequence Alignment Editor Version 7.0.8 (Hall, 1999). All sequences were checked manually, and nucleotide arrangements at ambiguous positions were clarified using sequences from both strands. Nucleotide sequences obtained in this study were deposited in GenBank (Table 2). Identification of isolates was confirmed by comparing the DNA sequences of the above mentioned regions

with those deposited at GenBank, and by combining these sequences in the phylogenetic analyses.

Phylogenetic analyses

Thirty seven isolates obtained in this study were used in the phylogenetic analyses. Additional sequences corresponding to other Diatrypaceae (26 for the ITS and 25 for the β-tubulin datasets) were obtained from GenBank (Figures 1 and 2) to be included in the analyses. *Daldinia concentrica* (Bolton) Ces. & De Not. (Xylariaceae) was used as the outgroup (with GenBank accession numbers FJ185300 for ITS and FJ185285 for β-tubulin). Taxa from GenBank were selected to include diatrypaceous species previously reported from grapevine as well as species showing high similarity with our query sequences. DNA sequences of both ITS and β-tubulin datasets were aligned using ClustalW (Thompson *et al*., 1994). Alignments were verified and adjusted manually using BioEdit. ITS and b-tubulin datasets were combined into a single dataset, which did not include *Anthostoma decipiens* (DC.) Nitschke because no β-tubulin sequence was available for this species in GenBank. The incongruence-length difference test (ILD) (Farris *et al*., 1995) or partition homogeneity test (HomPart) was performed in PAUP* ver. 4.0b10 (Swofford, 2002) to determine whether the ITS and b-tubulin datasets could be combined. Prior to the ILD, uninformative characters were removed and the ILD test was run using a heuristic search and simple addition of taxa for 1,000 random partitions of the data.

Prior to Bayesian inference (BI) analyses, the most appropriate nucleotide substitution models were chosen using jModeltest version 0.1.1 (Posada, 2008). BI analyses were carried out using Mr. Bayes version 3.2 (Ronquist and Huelsenbeck, 2003). Four Markov chains were run simultaneously for 1×10^6 generations, and these were sampled every 100 generations. Data from the first 1,000 generations were discarded as the burn-in period, and after confirming that likelihood values were stabilized prior to the 1,000th generation. The 50% majority rule consensus tree and posterior probability of the tree nodes were calculated from the pooled samples.

Results

A total of 38 isolates of putative Diatrypaceae species from Spain were used in this study (Table 1). Most of these isolates were obtained from infected wood of diseased vines, whereas two isolates of *C. ampelina* and one isolate of *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler were obtained from perithecia occurring on grapevine pruning debris. One isolate of *E. lata* (JL727) was also obtained from perithecia, but found on old dead wood. Isolates were obtained from typical V-shaped cankers as well as from irregular-shaped necroses. Shapes of cankers associated with the various isolates collected are summarized in Table 1. While 37 isolates were used in the phylogenetic analyses, conidial measurements were obtained for 21 isolates only. These included one isolate of *A. decipiens*, three of *C. ampelina*, 12 of *E. lata*, two of *Eutypella citricola* Speg., and three of *E. microtheca*. Production of conidia was not observed for *Eutypa* sp. isolates JL488, JL688, JL690 and JL742.

PCR amplification of the ITS region gave products of approximately 0.6 kb while those of the β-tubulin were about 0.4 kb. The ITS and β-tubulin dataset contained 64 and 63 sequences, respectively, including the outgroup. The ITS matrix consisted of 604 aligned characters and the β-tubulin matrix consisted of 403 characters, including gaps. Results of the ILD test ($P<0.05$) showed that the ITS and β-tubulin data were incongruent and thus could not be combined. For this reason, two separated BI analyses were carried out and the resulting phylogenetic trees are presented in Figures 1 and 2. The Akaike Information Criterion (AIC) implemented in jModeltest was used to determine the best fitting models for the ITS and β-tubulin datasets, and resulted as GTR+G for the ITS dataset and HKI+I+G for the β-tubulin dataset.

The phylogenetic tree of ITS showed five main clades that were supported by posterior probabilities over 95%. These groups included fungi in *Cryptovalsa* (group 1), *Diatrype* and *Diatrypella* (group 2), *Eutypella* and *Anthostoma* (group 3), and *Eutypa* (groups 4 and 5) (Figure 1). On the other hand, the phylogeny of the β-tubulin gene showed four large clades, coinciding with the genera *Cryptovalsa* (group 1), *Diatrype* and *Diatrypella* (group 2), *Eutypella* (group 3), and *Eutypa* (group 4), although *Eutypa* (group 4) only received a posterior probability value of 0.81 (Figure 2). *Anthostoma decipiens* JL567 was also included in this clade.

In addition to *C. ampelina* and *E. lata*, already known from grapevine in Spain, phylogenetic analyses also detected *E. citricola*, *E. microtheca* and *A. decipiens.* Species identification of these five taxa was

Table 2. Nucleotide sequences of the Spanish isolates of Diatrypaceae deposited in GenBank.

Species	Isolate	ITS	β-tubulin
Anthostoma decipiens	JL567	JN975370	JN975407
Cryptovalsa ampelina	JL413	JN975335	JN975371
Cryptovalsa ampelina	JL424	AY920391	JN975372
Cryptovalsa ampelina	JL476	JN975336	JN975373
Cryptovalsa ampelina	JL717	JN975337	JN975374
Eutypa lata	JL355	JN975338	JN975375
Eutypa lata	JL399	JN975339	JN975376
Eutypa lata	JL407	JN975340	JN975377
Eutypa lata	JL411	JN975341	JN975378
Eutypa lata	JL427	JN975342	JN975379
Eutypa lata	JL432	JN975343	JN975380
Eutypa lata	JL479	JN975344	JN975381
Eutypa lata	JL600	JN975345	JN975382
Eutypa lata	JL677	JN975346	JN975383
Eutypa lata	JL720	JN975347	JN975384
Eutypa lata	JL721	JN975348	JN975385
Eutypa lata	JL723	JN975349	JN975386
Eutypa lata	JL725	JN975350	JN975387
Eutypa lata	JL726	JN975351	JN975388
Eutypa lata	JL727	JN975352	JN975389
Eutypa lata	JL731	JN975353	JN975390
Eutypa lata	JL732	JN975354	JN975391
Eutypa lata	JL739	JN975355	JN975392
Eutypa lata	JL740	JN975356	JN975393
Eutypa lata	JL741	JN975357	JN975394
Eutypa lata	JL743	JN975358	JN975395
Eutypa lata	JL744	JN975359	JN975396
Eutypa sp.	JL488	JN975360	JN975397
Eutypa sp.	JL688	JN975361	JN975398
Eutypa sp.	JL690	JN975362	JN975399
Eutypa sp.	JL742	JN975363	JN975400
Eutypella citricola	JL583	JN975364	JN975401
Eutypella citricola	JL734	JN975365	JN975402
Eutypella microtheca	JL609	JN975366	JN975403
Eutypella microtheca	JL625	JN975367	JN975404
Eutypella microtheca	JL735	JN975368	JN975405
Eutypella microtheca	JL738	JN975369	JN975406

strongly supported by the high sequence similarities with reference sequences from GenBank (99 to 100%) and by the posterior probabilities obtained in the BI analyses (0.97 to 1). The ITS and β-tubulin analyses also distinguished a separate clade of another putative *Eutypa* sp. that remained unidentified but showed a close relatedness to *Eutypa tetragona* (Duby) Sacc. (Figures 1 and 2).

Several isolates of each species were obtained from different geographic regions (Table 1). *Eutypa lata* was the diatrypaceous species mostly isolated (23 isolates) and showed the greatest phylogenetic diversity in both genes (Figures 1 and 2). However, the intraspecific diversity of *E. lata* did not correspond to a relationship of geographical proximity, and intraspecific groups were not consistent across the two DNA phylogenies. ITS and β-tubulin sequences of *C. ampelina* (4), *E. citricola* (2), and *E. microtheca* (4) showed no genetic variation among isolates of each species despite their different geographical origins.

Colonies on PDA of the various fungal species were overall quite similar in morphology. However, slight differences could be observed. Colonies of *C. ampelina* on PDA were white to cream-white, woolly, with diffuse margins, and rapid growth (9 cm in diam. in 4 days at 25°C). The reverse of colonies was first pale-yellow, later (>20 days) developing irregular, mostly central, dark areas. The sporodochium-like conidiomata consisted of conidiophores aggregated on blackened mycelial crusts, and produced cream coloured conidial masses within 4 weeks. Cultures of *E. lata* on PDA were variable in morphology: they were white when young (<1 week at 25°C), sometimes turning cream-white with age (4 weeks), and with diffuse margins. Occasionally, production of brownish exudates was detected in some isolates

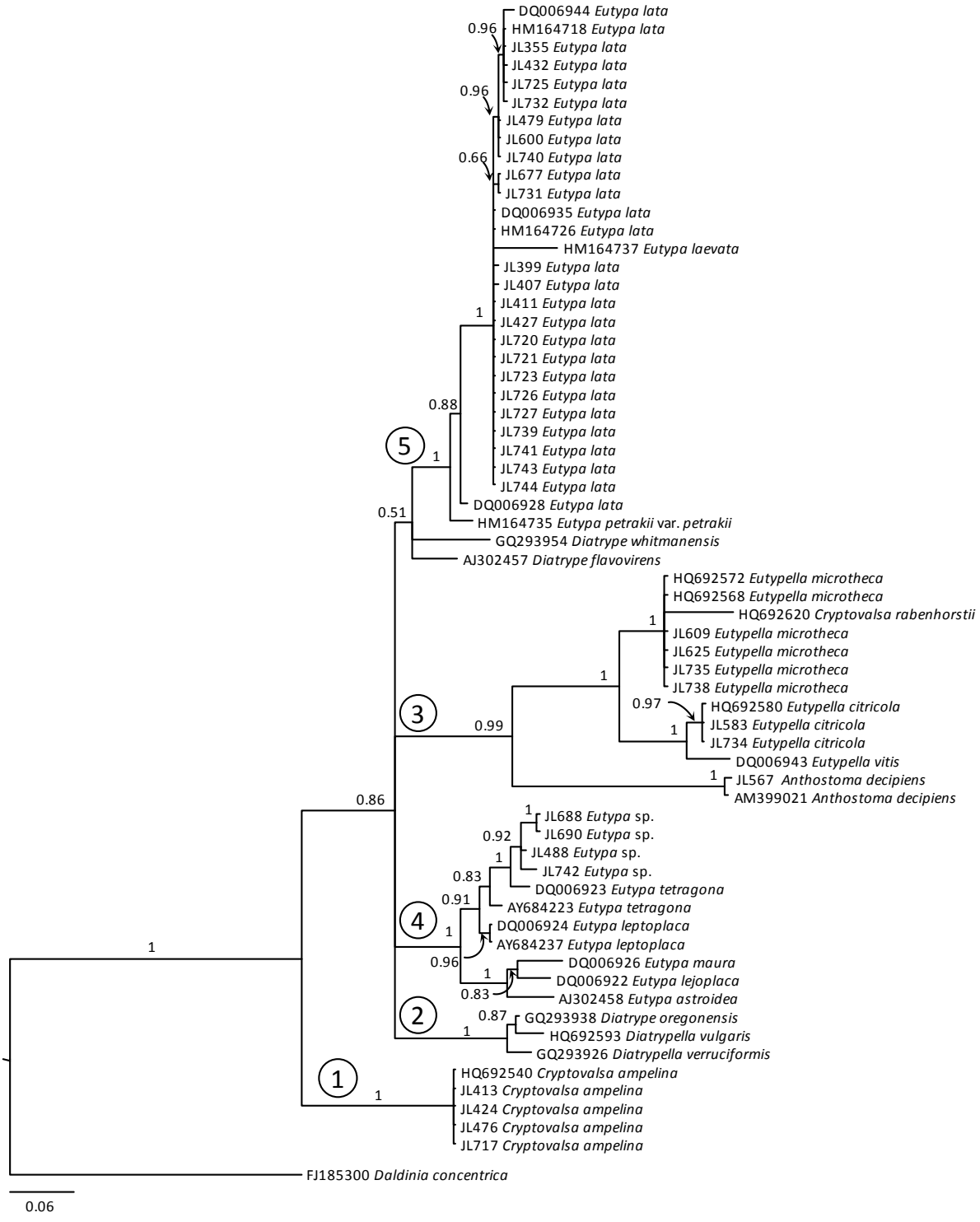

Figure 1. Majority rule consensus tree resulting from the Bayesian analysis of the ITS sequence data, with posterior probabilities reported at the nodes. Sequences obtained from GenBank are indicated by their accession numbers while isolates obtained in this study are indicated by their code numbers. Clades are numbered in circles. Bars represent expected changes per site.

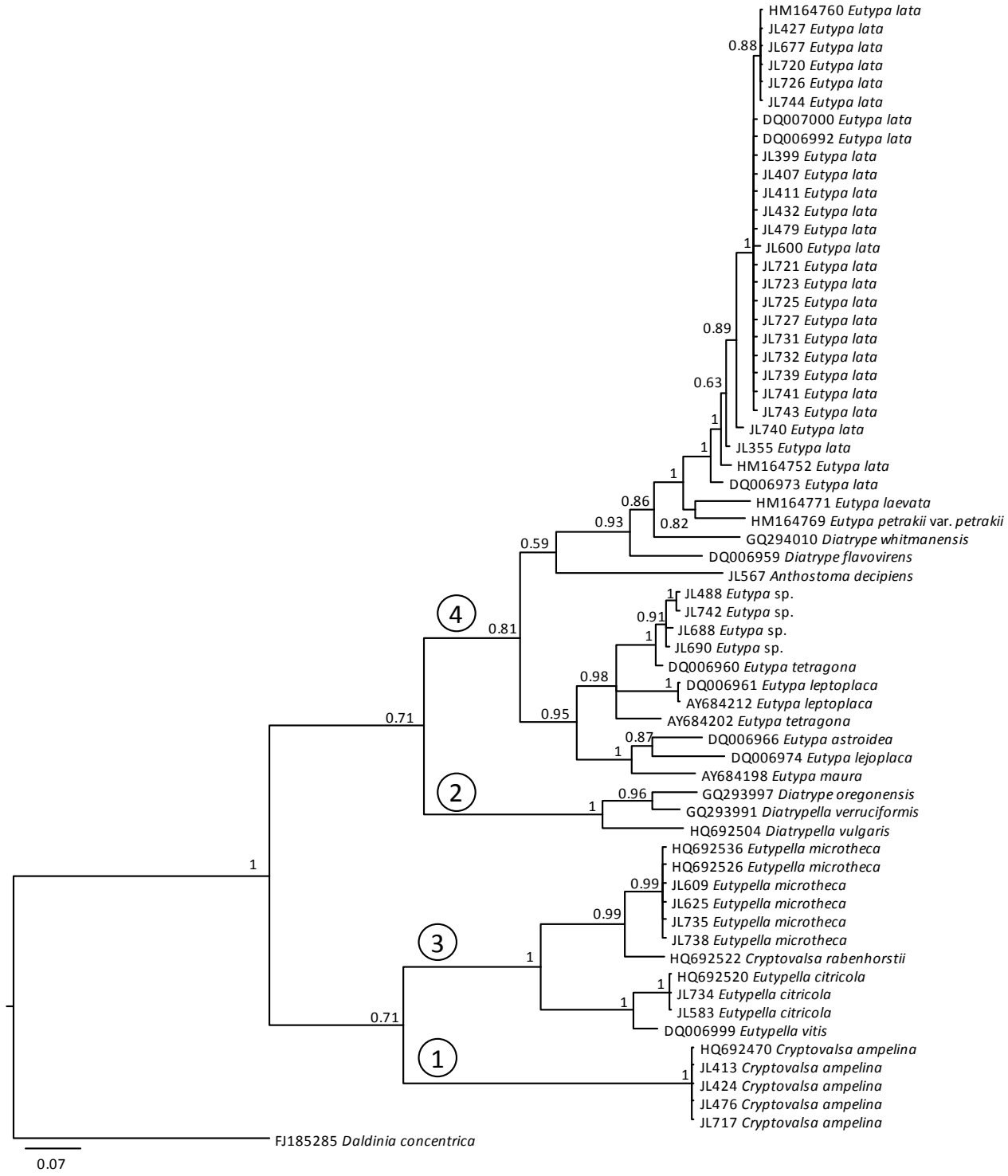

Figure 2. Majority rule consensus tree resulting from the Bayesian analysis of the β-tubulin sequence data, with posterior probabilities reported at the nodes. Sequences obtained from GenBank are indicated by their accession numbers while isolates obtained in this study are indicated by their code numbers. Clades are numbered in circles. Bars represents expected changes per site.

(JL720, JL726, JL741). The reverse of colonies was first white, later (>20 days) pale-yellow to creamy in most isolates, and cultures producing exudates each exhibited a brown reverse after 4 weeks. Most isolates developed blackened, circular areas, which coalesced with time (over 4 weeks) to each show a blackish colony reverse for some isolates. Production of conidiomata and conidia were only observed in half of the studied isolates. Cultures of *E. citricola* on PDA were white, with low dense aerial mycelium and woolly mycelium aggregates, sometimes turning to pale cream (3 weeks). Blackened, circular areas bearing sporodochia were seen occasionally on the colony surfaces. Cultures of *E. microtheca* on PDA were similar to those of *E. citricola* but developing a light pinkish-orange colour in the central parts of the colonies in some isolates (JL625, JL735), as reported by Trouillas *et al*. (2011). The culture of *A. decipiens* JL567 on PDA was white to cream-white and woolly, with a moderate growth (9 cm in diam. in 7 days at 25°C). The reverse of the colony was first pale yellow, later (>2 weeks) creamy, with irregular blackened areas. Old colonies (4 weeks) turned to dark grey. Colonies of isolates JL488, JL688, JL690 and JL742 on PDA were overall similar, however slight differences of morphology could be observed. All colonies appeared white initially, with little aerial mycelium, but rapidly (about 3 days) turned into a pale-cream colour. After one week, isolates JL688 and JL690 developed dense, cottony mycelium with moderate aerial growth, while mycelium in JL488 and JL742 remained almost appressed to the culture medium. Reverse colour of the colonies were first white (<7 d), turning pale yellow in isolates JL488 and JL742 after 10 d while isolates JL688 and JL690 remained white. Nevertheless, 4-week-old cultures were similar in colour for all isolates, mostly pale-cream in both sides. Isolates JL688 and 690 (approx. 9 cm in diam. after 7 d) grew a little faster than JL488 and JL742 (approx. 9 cm in diam. after 12 d). Production of conidiomata and conidia were not observed in any of these *Eutypa* sp. isolates in two independent assays.

Conidial dimensions of representative isolates of all species, except for the unknown *Eutypa* sp., which did not sporulate, are shown in Table 3. Conidia of *A. decipiens* were lunate, (7)–9– (11) × (1) –1.05– (1.1) μm, and were the smallest conidia of the studied isolates. Conidia of the remaining species were similar in form (curved at the obtuse end and straight at the truncate base) and with overlapping lengths, which did not allow for species differentiation based on conidial form. Mean length of conidia for *C. ampelina* ranged from 21.5 μm to 24.2 μm, while a great phenotypic variation of mean conidium length was detected among *E. lata* isolates, which ranged from 21.9 μm (isolate JL723) to greater than 34 μm (JL427 and JL 431) (Table 3). Mean length of conidia for *E. citricola* ranged from 14.7 μm to 15.6 μm, which overlapped with the corresponding range in *E. microtheca* (15.2 μm to 15.9 μm). Mean widths of conidia for nearly all isolates ranged from 1.0 μm to 1.5 μm (Table 3).

Discussion

This study has confirmed the occurrence of five species of Diatrypaceae associated with grapevines in Eastern Spain, namely *Anthostoma decipiens*, *Cryptovalsa ampelina*, *Eutypa lata*, *Eutypella citricola* and *Eutypella microtheca*. Additionally, four unidentified isolates of a *Eutypa* closely related to *E. tetragona* were detected on the basis of the phylogenetic analyses. However, we did not consider introducing new species names for these isolates as none of them (JL488, JL688, JL690 and JL742) produced either conidia *in vitro* or sexual reproductive structures required for the description of new fungal species. Furthermore, the ITS and β-tubulin phylogenetic analyses showed incongruent branch topologies for these isolates, which did not allow us to definitely conclude if multiple *Eutypa* spp. occurred within this clade.

Despite the incongruence found between the ITS and β-tubulin phylogenies, both trees inferred through Bayesian methods showed that species identification of our isolates was consistent among the estimated phylogenies. However, the position of *A. decipiens* still remains unclear. While this taxon was a sister clade to *Eutypella* in the ITS tree, it was included in the large clade of *Eutypa* in the β-tubulin tree. Regarding the new putative *Eutypa* sp. isolates, morphological observations were in accordance with the groupings observed in the ITS and the β-tubulin trees; thus, JL688 and JL690 showed similar characteristics (i.e. colony colour and growth) to JL488 and JL742. In addition, these isolates that were closely related to *E. tetragona* could in turn be distant relatives of *E. leptoplaca*.

While *C. ampelina* and *E. lata* were already known in Spain from previous studies (Arias and Moral, 1981; Armengol *et al*., 2001; Santiago *et al*., 2005; Luque *et al*., 2006; Martín and Cobos, 2007; Martín *et al*., 2009; Muruamendiaraz *et al*., 2009), *A. decipi-*

Table 3. Measurements of conidia from Diatrypaceae species isolated from grapevine in Spain.

Species	Isolate	(Min) 95% CI (Max)	Mean ± SD	Ratio L/W
Anthostoma decipiens	JL567	(7)-8.9-9.4-(11) × (1)-1.05-(1.1)	9.2 ± 0.1 × 1.0 ± 0.1	9.1 ± 0.1
Cryptovalsa ampelina	JL424	(18)-20.8-22.1-(29) × (1)-1.1-1.2-(1.5)	21.5 ± 0.3 × 1.2 ± 0.1	19.1 ± 0.4
Cryptovalsa ampelina	JL476	(20)-22.4-23.6-(28) × (1)-1.2-1.4-(1.5)	23.0 ± 0.3 × 1.3 ± 0.1	18.2 ± 0.4
Cryptovalsa ampelina	JL717	(21)-23.7-24.7-(29) × (1)-1.4-1.5-(1.5)	24.2 ± 0.3 × 1.5 ± 0.1	16.8 ± 0.3
Eutypa lata	JL355	(18)-21.7-22.9-(29) × (1)-1.3-1.4-(1.5)	22.3 ± 0.3 × 1.4 ± 0.1	16.6 ± 0.5
Eutypa lata	JL399	(23)-29.1-30.7-(35) × (1)-1.4-1.6-(2)	29.9 ± 0.4 × 1.5 ± 0.1	20.4 ± 0.4
Eutypa lata	JL407	(21)-24.6-25.7-(31) × (1)-1.1-1.2-(1.5)	25.1 ± 0.3 × 1.2 ± 0.1	22.0 ± 0.6
Eutypa lata	JL411	(19)-22.7-24.0-(29) × (1)-1.1-1.2-(1.5)	23.4 ± 0.3 × 1.1 ± 0.1	21.0 ± 0.4
Eutypa lata	JL427	(25)-33.3-35.0-(41) × (1)-1.1-1.2-(1.5)	34.1 ± 0.5 × 1.1 ± 0.1	30.9 ± 0.7
Eutypa lata	JL431	(29)-33.9-35.7-(42) × (1)-1.1-1.2-(1.5)	34.8 ± 0.5 × 1.1 ± 0.1	31.5 ± 0.8
Eutypa lata	JL432	(21)-26.2-27.6-(32) × (1)-1.3-1.4-(1.5)	26.9 ± 0.3 × 1.4 ± 0.1	19.9 ± 0.4
Eutypa lata	JL479	(19)-21.8-22.9-(28) × (1)-1.4-1.5-(2)	22.3 ± 0.3 × 1.5 ± 0.1	15.4 ± 0.3
Eutypa lata	JL600	(22)-27.4-29.0-(36) × (1)-1.3-1.4-(1.5)	28.2 ± 0.4 × 1.4 ± 0.1	21.0 ± 0.5
Eutypa lata	JL677	(22)-25.0-26.6-(33) × (1)-1.1-1.2-(1.5)	25.8 ± 0.4 × 1.2 ± 0.1	22.5 ± 0.5
Eutypa lata	JL723	(18)-21.4-22.4-(26) × (1)-1.2-1.3-(1.5)	21.9 ± 0.3 × 1.2 ± 0.1	18.3 ± 0.4
Eutypa lata	JL726	(23)-25.8-26.9-(31) × (1)-1.2-1.3-(1.5)	26.3 ± 0.3 × 1.2 ± 0.1	22.1 ± 0.5
Eutypella citricola	JL583	(11)-14.3-15.1-(17) × (1)-1.1-1.2-(1.5)	14.7 ± 0.2 × 1.1 ± 0.1	13.2 ± 0.3
Eutypella citricola	JL734	(12)-15.3-16.0-(19) × (1)-1.0-1.1-(1.5)	15.6 ± 0.2 × 1.1 ± 0.1	14.8 ± 0.2
Eutypella microtheca	JL625	(11)-14.8-15.7-(18) × (1)-1.3-1.4-(1.5)	15.2 ± 0.2 × 1.3 ± 0.1	11.7 ± 0.2
Eutypella microtheca	JL735	(13)-15.5-16.3-(19) × (1)-1.1-1.3-(1.5)	15.9 ± 0.2 × 1.2 ± 0.1	13.8 ± 0.3
Eutypella microtheca	JL738	(13)-15.4-16.1-(18) × (1)-1.0-1.2-(1.5)	15.8 ± 0.2 × 1.1 ± 0.1	14.7 ± 0.3

ens, *E. citricola* and *E. microtheca* are reported for the first time as occurring on grapevines in Spain. To our knowledge, *A. decipiens* is also reported for the first time as occurring on grapevine anywhere. In the present study, *E. lata* was the species most frequently isolated from grapevines in Eastern Spain, which coincides with its prevalence as the most important diatrypaceous pathogen in many grapevine-growing regions of the world (Carter, 1991; Mugnai *et al*., 1996; Larignon and Dubos, 1997; Fischer and Kassemeyer, 2003; Péros *et al*., 2008; Trouillas *et al*., 2010a). *Cryptovalsa ampelina* and *E. microtheca* were detected less frequently than *E. lata*, followed by *Eutypa* sp., *E. citricola* and *A. decipiens*. *Cryptovalsa ampelina*, *E. citricola* and *E. microtheca* seem to be widespread across distant grapevine regions in the world. While *C. ampelina* has been reported from California (Trouillas *et al*., 2010), South Africa and Australia (Mostert *et al*., 2004; Trouillas *et al*., 2011), and Spain (Luque *et al*., 2006), both *E. citricola* and *E. microtheca* have been found also in Australia and California (Trouillas *et al*., 2011). While slight differences in colony form were observed for the studied isolates, morphological characters alone did not allow an unambiguous differentiation of these fungi.

The intraspecific diversity of *E. lata* shown in the phylogenetic analyses was not related to the geographical origin of isolates. In addition, the intraspecific groups within *E. lata* detected in the phylogenetic trees were not consistent across the different DNA phylogenies. These two characteristics of this species were also reported by Trouillas and Gubler

(2010a) in a study on the host range, biological variation and phylogenetic diversity of *E. lata* in California. Furthermore, great variability in conidial length was also observed in the Spanish isolates of *E. lata*, which has also been reported earlier in other regions (Rolshausen *et al*., 2006; Trouillas and Gubler, 2010a). On the other hand, the rest of the taxa showed little variability, both in the phylogenetic analyses and the conidial sizes, but this could be partially explained by the low numbers of isolates studied. However, low genetic variability has also been detected for some diatrypaceous fungi (e.g. *Cryptovalsa*, *E. citricola* and *E. microtheca*) in previous studies (Trouillas and Gubler, 2010b; Trouillas *et al*., 2011). Future research should involve a greater number of isolates for each species to confirm these observations.

Most of the 38 Diatrypaceae isolates studied were obtained from infected wood (*i.e.* necrotic wood) and cankers of diseased vines, thus suggesting a possible pathogenic role of these species on grapevine. The same can be said for the isolates from perithecia, since this fact demonstrates that grapevine can support the whole life cycle of the particular fungus. Trouillas *et al*. (2010) also suggested that the occurrence and ease of isolation of these fungi from the margin of active cankers would indicate that they may participate in the grapevine decline. Trouillas and Gubler (2010b) showed that several diatrypaceous species, including *C. ampelina* and *E. lata*, were able to colonize grapevine wood and cause vascular streaking. While the pathogenicity of *E. lata* has been widely studied (Carter *et al*., 1985; Carter, 1991; Péros *et al*., 1999), the status of *C. ampelina* as a grapevine pathogen is still unclear. Some studies have confirmed the pathogenicity of *C. ampelina* on grapevine (Mostert *et al*., 2004; Luque *et al*., 2006; Trouillas and Gubler, 2010b), but Luque *et al*. (2006) suggested a moderate virulence for this fungus based on the low percentage of fungal recovery. Recently, *A. decipiens* has been associated with a decline of the European hornbeam (*Carpinus betulus* L.) in Italy, and its pathogenicity on this host has been confirmed (Saracchi *et al*., 2008; Rocchi *et al*., 2010). Pathogenicity of *A. decipiens* on grapevine was not assessed in our study, therefore this character still remains to be investigated. Trouillas and Gubler (2004) established the pathogenicity of *Eutypa leptoplaca* on grapevine in Northern California, but this species was not found in our study. Recently, data on the pathogenicity of *Eutypella vitis* and *Diatrypella* sp. on grapevine have been reported from USA (Úrbez-Torres *et al*., 2009, 2011), but little additional information is available on the pathogenicity on grapevine of diatrypaceous fungi different from those cited above. The pathogenicity of Diatrypaceae species has usually been determined only after the assessment of the vascular necroses caused by these fungi in short-term pathogenicity trials (often less than 1 year duration). However, Trouillas and Gubler (2010b) suggested that long-term pathogenicity tests should be carried out to determine conclusively the pathogenicity of these fungi and their ability to produce cankers as well as foliar symptoms. Long-term pathogenicity tests, including several isolates of *Eutypa* sp., *A. decipiens*, *E. citricola* and *E. microtheca* obtained in this study, are currently under way in our laboratory in order to establish the pathogenicity of these lesser-known diatrypaceous fungi on grapevine.

Trouillas *et al*. (2010) stated that some species of Diatrypaceae are opportunistic pathogens that occur naturally on several plant species of the indigenous flora (*i.e.* they are plurivorous), and are capable of infecting introduced agricultural host plants such as grapevine. Therefore, it is important that the pathogenicity of all the Diatrypaceae species associated with grapevine is determined, so that current management strategies for grapevine trunk diseases, which usually only take *E. lata* into consideration, should be refined to include additional species.

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