Diversity of Diatrypaceae Species Associated with Dieback of Grapevines in South Africa, with the Description of *Eutypa cremea* sp. nov.

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Abstract

Recent studies in grape-growing areas including Australia, California, and Spain have revealed an extensive diversity of Diatrypaceae species on grapevines showing dieback symptoms and cankers. However, in South Africa, little is known regarding the diversity of these species in vineyards. The aim of this study was, therefore, to identify and characterize Diatrypaceae species associated with dieback symptoms of grapevine in South Africa. Isolates were collected from dying spurs of grapevines aged 4 to 8 years old, grapevine wood showing wedge-shaped necrosis when cut in cross section as well as from perithecia on dead grapevine wood. The collected isolates were identified based on morphological characters and phylogenetic analyses of the internal transcribed spacer region (ITS) and β -tubulin gene. Seven Diatrypaceae species were identified on grapevine, namely *Cryptovalsa ampelina*, *C. rabenhorstii*, *Eutypa consobrina*,

E. lata, *E. cremea* sp. nov., *Eutypella citricola*, and *E. microtheca*. The dying spurs yielded the highest diversity of species when compared with the wedge-shaped necrosis and/or perithecia. *C. ampelina* was the dominant species in the dying spurs, followed by *E. citricola*, whereas *E. lata* was the dominant species isolated from the wedge-shaped necroses and perithecia. These results confirm *E. lata* as an important grapevine canker pathogen in South Africa, but the frequent association of *C. ampelina* with spur dieback suggests that this pathogen plays a more prominent role in dieback than previously assumed. In some cases, more than one species were isolated from a single symptom, which suggests that interactions may be occurring leading to decline of grapevines. *C. rabenhorstii*, *E. consobrina*, *E. citricola*, *E. microtheca*, and *E. cremea* are reported for the first time on grapevine in South Africa.

An increase in the number of Diatrypaceae species isolated from grapevine cankers has recently been reported from different grape-growing countries worldwide (Díaz et al. 2011; Luque et al. 2012; Paolinelli-Alfonso et al. 2015; Pitt et al. 2010; Rolshausen et al. 2014; Trouillas and Gubler 2004; Trouillas et al. 2010, 2011). For many decades, canker diseases on grapevine have been attributed to the Diatrypaceae species, *Eutypa lata* (Pers.) Tul. & C. Tul (Carter 1988; Ferreira 1987; Moller and Kasimatis 1978). In *Eutypa* dieback, grapevines become infected when ascospores of *E. lata* enter fresh wounds, resulting from pruning or mechanical damage. Ascospores, which are the primary form of inoculum of *E. lata*, are produced from mature perithecia occurring within stroma found on dead wood, during rainfall periods. The ascospores germinate in xylem vessels and the fungus subsequently colonizes the wood (Moller and Carter 1965; Moller and Kasimatis 1978).

Recent studies, however, have reported the existence of at least 17 Diatrypaceae species from cankers on grapevines. These species include *Cryptosphaeria pullmanensis* Glawe, *Cryptovalsa ampelina* (Nitschke) Fuckel, *Cryptovalsa rabenhorstii* (Nitschke) Sacc., *Diatrype oregonensis* (Wehm.) Rappaz, *D. stigma* (Hoffm.) Fr., *Diatrype whitmanensis* J.D. Rogers & Glawe, *Diatrypella verruciformis* (Ehrh.) Nitschke, *Diatrypella vulgaris* Trouillas, W.M. Pitt & Gubler, *Eutypa laevata* (Nitschke) Sacc., *Eutypa leptoplaca* (Mont.) Rappaz, *Eutypella citricola* Speg., *Eutypella leprosa* (Pers. ex Fr.) Berl., *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler, *Eutypella scoparia* (Schwein.: Fr.) Ellis & Everh., and *Eutypella vitis* (Schwein.: Fr.) Ellis & Everh. (Catal et al. 2007; Díaz et al. 2011; Luque et al. 2012; Mostert et al. 2004; Paolinelli-Alfonso et al. 2015; Pitt et al. 2010; Rolshausen et al. 2006, 2014; Schilder et al. 2003; Trouillas and Gubler 2004; Trouillas et al. 2010, 2011). Studies have even reported that some of the newly reported species including *C. ampelina*, *E. citricola*, *E. microtheca*, *E. laevata*, an unidentified *Eutypa* sp., and an unidentified

Diatrypella sp. occurred more abundantly and were more widely distributed geographically than *E. lata*, which was originally thought to be the most abundant and widely distributed species (Pitt et al. 2010; Rolshausen et al. 2014; Trouillas et al. 2011). These findings raised questions as to whether these newly discovered Diatrypaceae fungi are a threat to the sustainability of the grapevine industry and have led researchers to investigate their role as canker pathogens and the need to take them into account in global efforts to manage canker diseases of grapevine. Results of pathogenicity trials of several of these Diatrypaceae taxa on grapevine have further led researchers to postulate that *E. lata* is not the sole Diatrypaceae species associated with grapevine cankers and dieback symptoms, but several species of Diatrypaceae are also involved in the trunk disease complex as canker pathogens (Jordan and Schilder 2005; Pitt et al. 2013; Rolshausen et al. 2014; Trouillas and Gubler 2010).

The trunk disease complex is composed of grapevine canker diseases, which include *Eutypa* dieback and diseases caused by species in the Botryosphaeriaceae and Diaporthales. Trunk diseases are responsible for the shortened lifespan of vineyards and severe losses as a result of vineyard re-establishment and reduced yields throughout the world. Symptoms include cankers and other dieback symptoms such as stunted shoot, shortened internodes, bud mortality, leaf chlorosis, sectorial wood necrosis, shoot dieback, and cane bleaching.

Similar to other grape-growing countries, canker pathogens are important in South Africa. Recently, poor budburst and spur dieback in Sauvignon blanc and Cabernet Sauvignon vineyards, including those as young as 4 years old, have been observed in Western Cape Province of South Africa. Spurs of the affected vines either produce weak shoots, which die soon after bud break, or the spurs do not produce new shoots at all in spring. These observations are disturbing considering young vineyards are also affected. The question that arises concerns the extent to which Diatrypaceae are involved in the phenomenon.

Although studies in other countries have provided evidence of the occurrence and pathogenicity of several diatrypaceous fungi on grapevine, the information presently available about the occurrence and identity of the species of Diatrypaceae associated with dieback of grapevine in South Africa is still limited. In fact, only four Diatrypaceae species have been isolated from grapevine, namely *E. lata*, *C. ampelina*,

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an unidentified *Eutypa* sp., and a *Eutypella* species (Mostert et al. 2004; Safodien et al. 2005). The overall aim of this study was, therefore, to identify Diatrypaceae species associated with dieback of grapevines. The first objective of this study was to determine whether Diatrypaceae species are associated with dying spurs in Cabernet Sauvignon and Sauvignon blanc vineyards severely affected by poor budburst and spur dieback. The second objective of the study was to identify Diatrypaceae species associated with the wedge-shaped necrosis on numerous wine grape cultivars showing dieback symptoms, in Western Cape Province of South Africa. The third objective was to carry out surveys for perithecia of species of Diatrypaceae on grapevine in South Africa.

Materials and Methods

Sampling and isolate collection. Four- to eight-year-old Sauvignon blanc and Cabernet Sauvignon vineyards were sampled in 2013 and 2014 to determine whether Diatrypaceae species were associated with dying spurs. Sampling was carried out in different locations in Western Cape Province to represent areas with different environmental conditions, namely Stellenbosch, Grabouw, Constantia, Durbanville, and Robertson. Sampling was conducted in 19 Sauvignon blanc and 17 Cabernet Sauvignon vineyards. Ten dying spurs were collected from each vineyard, individually placed in bags, and taken to the laboratory for processing. In the laboratory, spurs were split open through the pruning wounds to reveal internal symptoms and were then surface sterilized by submerging in 70% ethanol for 30 s, 2.5% sodium hypochlorite for 2 min, and again in 70% ethanol for another 30 s. Subsequently, wood pieces were taken from different necrotic areas as well as from the border between live and dead tissue at the pruning wound and plated onto potato dextrose agar (PDA-C; Biolab, Midrand, South Africa) plates containing chloramphenicol (250 mg/liter). Plates were incubated at 24°C, exposed to approximately 12 h of day light and 12 h of darkness, and inspected daily for fungal growth for 4 weeks. Isolates of presumed Diatrypaceae species were transferred onto new PDA-C plates for further identification.

To identify Diatrypaceae species associated with wedge-shaped cankers, vineyards were selected on the basis of the presence of dieback symptoms, which included dying spurs, stunted shoots, shortened internodes, dwarfed leaves with tattered margins, and cankers. Cordons and trunks of such vines were cut in cross section and wood showing the wedge-shaped necrosis was then taken to the laboratory for isolations. Samples were collected from 10 cultivars, namely Cabernet Sauvignon, Chardonnay, Chenin blanc, Merlot, Pinot noir, Pinotage, Sauvignon blanc, Semillon, Shiraz, and Viognier. Sampling was done in vineyards aged ≥ 10 years old, located in nine different locations in Western Cape Province. Isolations were carried out by cutting small wood pieces from the border between live and dead tissue of the wedge-shaped necrosis and plating onto PDA-C as described above.

Dead grapevine wood with black, charcoal-like stroma with fruiting bodies resembling those of *E. lata* was also collected. Depending on availability of diseased wood, one to 10 samples per vineyard block were randomly collected from the different sampling areas. Isolations from collected samples were achieved according to Trouillas et al. (2010). The stromata were sliced open, under a Nikon SMZ 1500 stereo microscope, using a scalpel to reveal perithecial contents. A drop of sterile water was then added to the opened perithecia. Perithecial contents were then collected with a needle, mounted in water on a microscopic slide, and examined under a light microscope (Nikon Y-TV55) for morphological identification. By pipetting, the perithecial contents on the slides were mixed with 50 μ l of sterile water and transferred onto water agar (WA; Biolab, Midrand, South Africa) plates. Single germinated ascospores were transferred to PDA-C plates within 48 h and the plates were incubated at 24°C for 15 to 21 days. Thereafter, fungal identification was carried out.

Morphological characterization of sexual morphs. This technique was used to characterize isolates of species producing fruiting bodies on grapevine wood. Perithecial contents were collected as described above and mounted in 10% lactic acid, examined microscopically, and images were captured with a Nikon DS-Ri2 camera on a Nikon Y-TV55 light microscope at 1,000 $\times$ magnification. Images of stromata were captured using the Nikon DS-Ri2 camera on a Nikon SMZ 1500

stereo microscope. Measurements were made for two or three representative samples of each species; 5% and 95% confidence intervals were calculated from the measurements of 50 ascospores, 20 asci (length = spore bearing part and width = width at the widest point), and 10 perithecia (diameter). Characteristics such as morphology of perithecia, asci, and ascospores; number of ascospores per ascus, as well as color of ascospores were noted for each species as described by Rappaz (1987), Glawe and Rogers (1984), and Trouillas et al. (2011).

Morphological and cultural characterization of asexual morphs.

For characterization of the asexual states, one or two isolates per species were characterized, depending on the sporulation of the isolates. Three PDA-C plates per isolate were incubated at 24°C for 30 days and visual observations of the colony color were made every 3 days. The conidia size from each PDA-C plate was measured and the average conidia sizes of the plates, on which the respective isolate sporulated, was calculated.

DNA isolation, amplification, and sequencing of fungal isolates.

DNA was extracted from actively growing cultures using the method described by Damm et al. (2008). Because *C. ampelina* and *E. lata* were anticipated to be the most common species, all isolates were first subjected to amplification using species-specific primers to detect these species. The species-specific primer pair, Camp1/Camp2R designed by Luque et al. (2006), was used to detect *C. ampelina*, while the primer pair EL1/EL4 (Catal et al. 2007) was used for the detection of *E. lata*. A touch-down PCR protocol was used for the amplification of *C. ampelina* and was carried out in a 25- μ l reaction containing the following end concentrations: 1 $\times$ PCR buffer, 1 mM MgCl₂, 0.2 μ M of each deoxyribonucleotide triphosphates (dNTPs), 1 mM of each primer, and 0.65 U Taq polymerase. The touch-down PCR protocol consisted of two phases: The first phase included an initial denaturing at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s. Phase 2 consisted of 10 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. The EL1/EL4 PCR reaction was performed in a total volume of 25 μ l consisting of 1 $\times$ PCR buffer, 3.5 mM MgCl₂, 0.8 mM dNTPs, 0.5 μ M of each primer, 0.65 U Taq Polymerase (Bioline, U.S.A.), and 1 μ l DNA template. PCR amplification was conducted using the following thermal regime: initial denaturing for 2 min at 94°C; 35 cycles of denaturing at 94°C for 1 min, followed by annealing at 62°C for 30 s, elongation at 72°C for 1 min; final extension at 72°C for 5 min.

For isolates in which amplification could not be obtained using species-specific primers, the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal RNA gene were amplified using primers ITS1/ITS4 (White et al. 1990). Twenty isolates of *C. ampelina* and 25 isolates of *E. lata*, randomly chosen, were also included in this PCR to verify specificity of the species-specific primers. Amplification and sequencing of the ITS region was performed as described by Moyo et al. (2016). Identification of the isolates was based on subjecting the ITS sequence data to the megablast function of the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database (www.ncbi.nlm.nih.gov). Depending on the number of isolates identified by the BLAST search, up to 11 representative isolates of each species, including *E. lata* and *C. ampelina*, were selected for amplification of the partial β -tubulin gene. The partial β -tubulin gene was amplified and sequenced using the primer set Bt2a/Bt2b (Glass and Donaldson 1995) as described by Moyo et al. (2016).

Phylogenetic analysis. The phylogenetic analyses of the individual ITS and β -tubulin sequences, as well as of the combined dataset (ITS and β -tubulin), were performed using maximum likelihood (ML) and Bayesian analyses. The ITS and β -tubulin sequences, respectively, of representative isolates obtained in this study were aligned with sequences of Diatrypaceae retrieved from GenBank (Table 1) as well as the outgroup *Hypoxylon fragiforme*. Sequences were aligned with MAFFT v7.017 (Katoh et al. 2002) in Geneious v8.1 (<http://www.geneious.com>; Kearse et al. 2012) using the default parameters. The alignments were run through Gblocks v0.91b, using default parameters (Castresana 2000) to exclude poorly aligned positions from subsequent phylogenetic inferences. The concatenated alignment was deposited in TreeBASE under study number S20662 (<http://purl.org/phylo/treebase/phyloids/study/TB2:S20662>).

Maximum likelihood analyses were performed using PhyML (Guindon and Gascuel 2003) in Geneious v8.1 under the general time reversible (GTR) model. Both the proportion of invariable sites and gamma distribution parameter were estimated and bootstrap support values were calculated from 1,000 replicates. Clades with bootstrap support $\geq 70\%$ were considered significant (Hillis and Bull 1993). Bayesian analyses were done with Mr Bayes v3.2.2 (Huelsenbeck and Ronquist 2001) using the Markov Chain Monte Carlo (MCMC) method. Four MCMC chains were run simultaneously and trees were saved every 100th generation. The first 25% of generated trees were discarded as the burn-in phase of the analyses. Posterior probabilities were assigned after a 50% majority-rule. Clades with Bayesian posterior probability $\geq 95\%$ were considered significant (Alfaro et al. 2003).

Results

Sampling and isolate collection. A total of 360 dying spurs (Fig. 1A) were collected in this study, with 190 collected from Sauvignon

blanc and 170 from Cabernet Sauvignon vineyards. Isolations were performed from different symptoms observed inside the dying spurs (Fig. 1B to F). Subsequently, 68 (36%) and 71 (42%) spurs collected from Sauvignon blanc and Cabernet Sauvignon, respectively, yielded Diatrypaeae isolates. Two hundred and ninety-six samples showing wedge-shaped necrosis (Fig. 1G) were collected from vineyards and 72% of these samples yielded Diatrypaeae isolates. A total of 81 grapevine wood pieces with perithecia of Diatrypaeae species were collected from vineyards in Western Cape Province.

Identification of Diatrypaeae isolates. Maximum likelihood and Bayesian analyses of the individual ITS and β -tubulin sequences, as well as of the combined data set, generated trees with similar topology with regards to the clades/subclades representing species isolated in this study. The ML tree of the combined dataset is presented in Figure 2 with ML bootstrap support values and Bayesian posterior probability scores at the nodes. In this tree, isolates obtained in this study were distinguished as belonging to seven species, residing in three

Table 1. List of Diatrypaeae isolates collected in this study and reference strains from GenBank, used for phylogenetic analyses

Species	Host	Origin	Strain number	GenBank number	
				ITS	β -tubulin
<i>Cryptosphaeria ligniota</i>	<i>Populus tremula</i>	Switzerland	CBS 273.87	KT425233	KT425168
<i>Cryptosphaeria ligniota</i>	<i>Populus tremuloides</i>	Colorado, U.S.A.	ATCC46315	KT425234	KT425169
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>	California, U.S.A.	DSIERRA600	KT425184	KT425119
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus balsamifera</i>	Australia	NSW03PO	KT425237	KT425172
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus</i> sp.	Oregon, U.S.A.	OREG100	KT425188	KT425123
<i>Cryptosphaeria pullmanensis</i>	<i>Populus trichocarpa</i>	Washington, U.S.A.	ATCC52655	KT425235	KT425170
<i>Cryptosphaeria pullmanensis</i>	<i>Populus nigra</i> 'italica'	California, U.S.A.	DWIN100	GQ293930	GQ294015
<i>Cryptosphaeria subcutanea</i>	<i>Salix borealis</i>	Norway	CBS 240.87	KT425232	KT425167
<i>Cryptosphaeria subcutanea</i>	<i>Salix myrsinifolia</i>	Norway	DSUB100A	KT425189	KT425124
<i>Cryptovalsa ampelina</i>	<i>Fraxinus latifolia</i>	California, U.S.A.	DMO100	GQ293908	GQ293978
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	New South Wales, Australia	HVVIT04	HQ692558	HQ692459
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8113	KY111659	KY111618
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8114	KY111660	KY111619
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8115	KY111661	KY111620
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8116	KY111662	KY111621
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Paarl, WC, South Africa	STEU 8117	KY111663	KY111622
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8118	KY111664	KY111623
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Riebeeck-Wes, WC, South Africa	STEU 8119	KY111665	KY111624
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Durbanville, WC, South Africa	STEU 8120	KY111666	KY111625
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8121	KY111667	KY111626
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8122	KY111668	KY111627
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Western Australia	WA07CO	HQ692620	HQ692522
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Western Australia	WA08CB	HQ692619	HQ692523
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8112	KY111628	KY111607
<i>Diatrype brunneospora</i>	<i>Acacia longifolia</i> subsp. <i>sophorae</i>	Australia	CNP01	HM581946	HQ692478
<i>Diatrype oregonensis</i>	<i>Quercus kelloggii</i>	California, U.S.A.	DPL200	GQ293940	GQ293999
<i>Diatrype stigma</i>	<i>Quercus</i> sp.	Irish Republic	CBS 211.87	AJ302438	–
<i>Diatrypella verruciformis</i>	<i>Quercus</i> sp.	California, U.S.A.	DCH500	GQ293926	GQ293991
<i>Diatrypella vulgaris</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF03	HQ692590	HQ692505
<i>Diatrypella vulgaris</i>	<i>Vitis vinifera</i>	New South Wales, Australia	CG8	HQ692595	HQ692502
<i>Eutypa consobrina</i>	<i>Arundo donax</i>	Almeria, Spain	F091.961	AJ302447	–
<i>Eutypa consobrina</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8092	KY111651	KY111596
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Bonnievale, WC, South Africa	STEU 8081	KY111655	KY111597
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Durbanville, WC, South Africa	STEU 8082	KY111656	KY111598
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8083	KY111657	KY111599
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Darling, WC, South Africa	STEU 8084	KY111642	KY111600
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Slanghoek, WC, South Africa	STEU 8085	KY111658	KY111601
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8086	KY111645	KY111602
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8087	KY111646	KY111603
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8088	KY111647	KY111604
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8089	KY111648	KY111605
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8090	KY111649	KY111587
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Hermanus, WC, South Africa	STEU 8091	KY111650	KY111606
<i>Eutypa cremea</i>	<i>Arundo donax</i>	France	CBS 286.87	AY684225	AY684203
<i>Eutypa crustata</i>	<i>Ulmus</i> sp.	France	CBS 210.87	AJ302448	DQ006968
<i>Eutypa laevata</i>	<i>Salix</i> sp.	Switzerland	CBS 291.87	HM164737	HM164771

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genera, namely *Eutypa*, *Eutypella*, and *Cryptovalsa*. Eight isolates clustered as a strongly supported group (100%/1.00, ML bootstrap support and Bayesian posterior probability scores) with *E. citricola* isolates. Ten isolates grouped with strong support (99%/1.00) with *C. ampelina* isolates obtained from grapevine and Oregon ash in Australia and California, respectively. Five isolates clustered with *E. microtheca* (74%/0.94) isolates from Australia, whereas one isolate grouped with strong support (100%/1.00) with *C. rabenhorstii* isolates obtained from grapevine in Australia. Five isolates clustered with strong support (99%/1.00) with *E. lata* isolates from California and Australia. One isolate grouped with a *Eutypa consobrina* (Mont.) Rappaz isolate with 100%/1.00 support. Eleven isolates grouped with the French isolate CBS 286.87 from *Arundo donax*, designated as *E. leptoplaca*. This group represents a previously misidentified taxon, which is closely related to *Eutypa tetragona* (Duby) Sacc. The isolate CBS 286.87 and the 11 isolates obtained in this study, being phylogenetically distinct from other *E. leptoplaca* isolates, indicate an undescribed species.

Taxonomy. *Cryptovalsa ampelina* (Nitschke) Fuckel, Symbolae Mycologicae, Beiträge zur Kenntnis der Rheinischen Pilze: 212, 1870 (Fig. 3). Stromata poorly developed and embedded in bark and/or wood. Perithecia globose to ovoid, (439–) 456 to 862 (–941) μ m diam., necks infrequently observed, protruding singly or in groups. Asci polysporous, clavate, long stipitate, with an apical invagination, (64–) 73 to 140 (–147) $\times$ (8–) 9 to 13 (–14) μ m. Ascospores allantoid, pale brown, (7–) 8 to 12 (–14) $\times$ 2 to 3 μ m. Colonies are white and the underside is cream on PDA-C. Conidia were not observed.

Material examined. South Africa, Western Cape Province, Durbanville, on dead wood of *Vitis vinifera*, 20 June 2013, P. Moyo, culture number STEU 8120.

Notes. A higher maximum ascus length was observed in our study compared with that reported by Trouillas et al. (2011) and Mostert et al. (2004) of 135.80 μ m and 125 μ m, respectively.

Eutypa cremea Moyo, F. Halleen, L. Mostert, sp. nov., MycoBank MB 819824 (Fig. 4).

Table 1. (Continued from previous page)

Species	Host	Origin	Strain number	GenBank number	
				ITS	β -tubulin
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, U.S.A.	EAMS100	HM164730	HM164764
<i>Eutypa lata</i>	<i>Populus nigra</i> ‘italica’	South Australia	SAPN01	HQ692616	HQ692500
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8093	KY111652	KY111613
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Somerset West, WC, South Africa	STEU 8094	KY111653	KY111614
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Barrydale, WC, South Africa	STEU 8095	KY111643	KY111615
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8096	KY111654	KY111616
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8097	KY111644	KY111617
<i>Eutypa lata</i> var. <i>aceri</i>	<i>Acer campestre</i>	France	CBS 217.87	HM164734	HM164768
<i>Eutypa lejoplaca</i>	<i>Acer pseudoplatanus</i>	Switzerland	020202-5	AY684221	AY684196
<i>Eutypa leptoplaca</i>	<i>Frangula alnus</i>	Switzerland	CBS 287.87	AY684226	AY684204
<i>Eutypa leptoplaca</i>	<i>Cyssus hypoglauca</i>	Australia	CBS 288.87	AY684227	AY684205
<i>Eutypa maura</i>	<i>Acer pseudoplatanus</i>	Switzerland	CBS 219.87	AY684224	DQ006967
<i>Eutypa petrakii</i> var. <i>petrakii</i>	<i>Prunus spinosa</i>	Switzerland	CBS 244.87	HM164735	HM164769
<i>Eutypa sparsa</i>	<i>Populus</i> sp.	Switzerland	3802-3a	AY684200	AY684219
<i>Eutypa tetragona</i>	<i>Laburnum alpinum</i>	Switzerland	190802-3	AJ684223	AY684202
<i>Eutypa tetragona</i>	<i>Sarothamnus scoparius</i>	France	CBS 284.87	DQ006923	DQ006960
<i>Eutypella australiensis</i>	<i>Acacia longifolia</i> subsp. <i>sophorae</i>	Australia	CNP03	HM581945	HQ692479
<i>Eutypella cerviculata</i>	<i>Alnus glutinosa</i>	Switzerland	CBS 221.87	AJ302468	–
<i>Eutypella citricola</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF01	HQ692589	HQ692521
<i>Eutypella citricola</i>	<i>Citrus sinensis</i>	New South Wales, Australia	HVOT01	HQ692581	HQ692509
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	New South Wales, Australia	HVVIT07	HQ692579	HQ692512
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8098	KY111634	KY111588
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8099	KY111637	KY111589
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8100	KY111638	KY111590
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8102	KY111635	KY111591
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8103	KY111639	KY111592
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8104	KY111636	KY111593
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8105	KY111640	KY111594
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8106	KY111641	KY111595
<i>Eutypella cryptovalsoidea</i>	<i>Ficus carica</i>	New South Wales, Australia	HVFIG02	HQ692573	HQ692524
<i>Eutypella leprosa</i>	<i>Tilia</i> sp.	Switzerland	CBS 276.87	AJ302463	–
<i>Eutypella microtheca</i>	<i>Ulmus procera</i>	South Australia	ADEL300	HQ692560	HQ692528
<i>Eutypella microtheca</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF02	HQ692569	HQ692533
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	New South Wales, Australia	T2R2S7	HQ692566	HQ692532
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8107	KY111629	KY111608
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8108	KY111630	KY111609
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8109	KY111631	KY111610
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8110	KY111632	KY111611
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Calitzdorp, WC, South Africa	STEU 8111	KY111633	KY111612
<i>Eutypella quaternata</i>	<i>Fagus sylvatica</i>	Switzerland	CBS 278.87	AJ302469	–
<i>Eutypella vitis</i>	<i>Vitis labrusca</i>	Illinois, U.S.A.	ATCC64171	AJ302466	–
<i>Eutypella vitis</i>	<i>Vitis vinifera</i>	Michigan, U.S.A.	MSUELM13	DQ006943	DQ006999
<i>Peroneutypa alsophila</i>	<i>Arthrocnemum fruticosum</i>	France	CBS 250.87	AJ302467	–
<i>Peroneutypa kochiana</i>	<i>Atriplex halimus</i>	Almeria, Spain	F-092,373	AJ302462	–
<i>Peroneutypa scoparia</i>	<i>Robinia pseudoacacia</i>	France	CBS 242.87	AJ302465	–
<i>Peroneutypa scoparia</i>	<i>Robinia pseudoacacia</i>	France	DFMAL100	GQ293962	GQ294029

Etymology. Named after the colony color, which is mostly cream.

Type. South Africa, Western Cape Province, Durbanville, Hooggelegen, on dead wood of *V. vinifera*, 11 August 2014, P. Moyo, holotype PREM 61675, culture ex-type CBS 142718 = STEU 8082. Stromata on wood or bark, poorly developed, with a charcoal-like appearance. Perithecia scattered or in rows (Fig. 4A), circular to ovoid, (228–) 232 to 376 (–395) μm diam. Ostioles apparent on surface of substrate, emerging on surface separately, 3 to 4 sulcate. Asci clavate, with apical ring, octosporous, long stipitate, (23–) 26 to 58.5 (–62) $\times$ 5 to 7.5 μm . Ascospores yellowish, allantoid, (5.5–) 6 to 8 $\times$ 1 to 2 μm . Colonies on PDA-C are cream and flat, sometimes with white aerial mycelium. Conidia hyaline, filiform, (19–) 22 to 33 $\times$ 1 to 1.5 μm .

Other material examined. South Africa, Western Cape Province, Darling, on dead wood of *V. vinifera*, 11 November 2013, P. Moyo, culture number STEU 8101 (PREM 61676); Darling, on dead wood of *V. vinifera*, 12 June 2012, F. Halleen, culture number STEU 8084. Algeria, on *Fraxinus* sp., *Sphaeria leptoplaca* PC MC8636; on *Arundo mauritanica*, *S. leptoplaca* PC MC8635; on *A. mauritanica*, *S. consobrina* PC MC5627; on *A. mauritanica*, *S. consobrina* PC MC5626.

Notes. Variation in cultural growth of individual isolates of this species was observed. For example, an isolate could be cream and very flat with no aerial mycelium on one PDA-C plate and be cream with white aerial mycelium on another plate, despite being plated at the same time and exposed to same incubation conditions.

E. cremea is similar to *E. leptoplaca* and *E. tetragona* (*E. tetragona* as described by Rappaz 1987) in having 3 to 4 sulcate ostioles emerging separately, yellowish ascospores, and spherical to ovoid perithecia, but these species differ in the length of the spore bearing part of their asci. *E. cremea* has a higher maximum length of their spore bearing part [(23–) 26 to 58.5 (–62) μm] compared with *E. leptoplaca* (25 to 40 μm) and *E. tetragona* (30 to 45 [–55] μm) (Rappaz 1987). Additionally, *E. tetragona* has larger perithecia (300 to 400 [–500] μm diam.) versus *E. leptoplaca* (150 to 300 μm diam.) (Rappaz 1987) and *E. cremea* (228–) 232 to 376 (–395) μm diam.).

Eutypa lata (Pers.) Tul. & C. Tul., Selecta Fungorum Carpologia: Xylariei- Valsei- Spaeriei 2:56, 1863 (Fig. 5). Stromata on bark and wood. Perithecia oval, (307–) 334 to 497 (–553) μm diam. Ostioles not sulcate. Asci spindle shaped, 8-spored, long stipitate, truncate at

apex, (26–) 32 to 69 (–83) $\times$ 6 to 7 (–8) μm . Ascospores allantoid, 7 to 10 (–13) $\times$ 1 to 2 μm . Colonies cottony white on PDA-C, but later turn gray to black and underside of colonies is cream. Conidia single celled, filiform, (13–) 27 to 43 (–49) $\times$ 1 to 2 μm .

Material examined. South Africa, Western Cape Province, Durbanville, Hooggelegen, on dead wood of *V. vinifera*, 20 June 2013, P. Moyo, culture number STEU 8218.

Eutypella citricola Speg., Anales del Museo Nacional de Buenos Aires 6: 245, 1898 (Fig. 6). Stromata on wood or bark, in pustules unevenly distributed or grouped. Perithecia spherical to ovoid, (280–) 306 to 585 (–603) μm diam., surrounded by white powdery layer. Ostioles apparent on surface, emerging singly or in groups, sulcate. Asci octosporous, clavate, long-stiped, (34–) 36 to 61 (–73) $\times$ (5–) 5.5 to 8 (–9) μm . Ascospores pale yellow, allantoid, (7–) 8 to 10 (–12) $\times$ 2 to 3 μm . Colonies on PDA-C white but turning black with age starting from the center of colony, black color more apparent from beneath the colony. Conidia filiform, (13–) 14 to 19 (–20) $\times$ 1 to 2 μm .

Material examined. South Africa, Western Cape Province, Durbanville, Diemersdal, on dead wood of *V. vinifera*, 18 November 2013, P. Moyo, culture number STEU 8231.

Eutypella microtheca Trouillas, W. M. Pitt & Gubler, Fungal Diversity 49: 217, 2011 (Fig. 7). Stromata on wood and bark, pustular and breaking through the periderm of the host. Perithecia circular to ovoid, surrounded by a white powdery layer, (273–) 307 to 655 (–656) μm diam. Ostioles sulcate, emerging in groups through the cracked periderm. Asci long stipitate, clavate, octosporous, (33–) 35 to 65 (–94) $\times$ 6 to 9 μm . Ascospores pale yellow, allantoid to sub-allantoid, (6–) 7 to 11 (–12) $\times$ 2 to 3 μm . Colonies start as white turning pink in color with age. Conidia were not observed.

Material examined. South Africa, Western Cape Province, Calitzdorp, on dead wood of *V. vinifera*, 1 November 2014, P. Moyo, culture number STEU 8111.

Notes. A higher maximum length of the spore bearing part of asci (94 μm) and a higher ascospore length (12 μm) are reported here compared with 60 and 11 μm , respectively, reported by Trouillas et al. (2011).

Occurrence of sexual morphs in vineyards. The survey of fruiting structures in vineyards revealed five species to produce fruiting

Fig. 1. Disease symptoms observed on dying spurs and cordons/trunks of naturally infected grapevines in Western Cape Province of South Africa. **A**, Four-year-old grapevine showing a dying spur (circled in red) and poor bud burst (shown by yellow arrow) in the field. **B**, Brown streaks extending from a pruning wound of a dying spur. **C**, Brown sectorial necrosis seen from the reverse side of the dying spur presented in **B**. **D**, Brown/black streaks extending from a dying spur which has a wound (yellow arrow) next to it. **E**, Black line (yellow arrow) at the border of dead and live tissue. **F**, Brown streaks in a dying spur. **G**, Wedge-shaped necrosis.

bodies on dead grapevine wood. Fruiting bodies of *E. lata* were found on 51% of the grapevine wood samples collected. Perithecia of *C. ampelina* were found on 38% of the wood pieces, whereas perithecia of *E. cremea*, *E. citricola*, and *E. microtheca* were found on 6, 4, and 1% of the wood pieces, respectively.

Diatrypaceae species and symptom associations. The dying spurs presented a higher diversity of Diatrypaceae than the wedge-shaped cankers on grapevines. From a total of 360 dying spurs processed, seven species, namely *C. ampelina*, *C. rabenhorstii*, *E. cremea*, *E. consobrina*, *E. lata*, *E. citricola*, and *E. microtheca* were

isolated from the two grapevine cultivars sampled from the five different areas within Western Cape Province of South Africa. *C. ampelina* was the most frequently isolated species (46.4% of the total number of Diatrypaceae isolates) followed by *E. citricola* (26.8%), *E. lata* (20.1%), *E. cremea* (4.3%), *E. microtheca* (1.2%), *E. consobrina* (0.6%), and *C. rabenhorstii* (0.6%). Isolations from wedge-shaped necrosis collected from 10 cultivars in eight locations in the Western Cape yielded four species, namely *C. ampelina*, *E. lata*, *E. cremea*, and *E. citricola*. *E. lata* represented the most frequent species detected in the wedge-shaped necrosis (89.2% of all Diatrypaceae

Fig. 2. Maximum likelihood tree derived from the combined analysis of ITS and β -tubulin sequence data. Maximum likelihood bootstrap support values and Bayesian posterior probability scores are given at the nodes. *Hypoxylon fragiforme* served as an outgroup. Isolates obtained from this study are in bold.

isolates obtained), followed by *E. citricola* (8.5%), *E. cremea* (1.4%), and *C. ampelina* (0.9%). The wedge-shaped cankers from which *E. citricola* and *E. cremea* were isolated were often associated with dieback symptoms, and not strictly from grapevine expressing Eutypa dieback symptoms. In both symptom types (dying spurs and wedge-shaped necrosis), a number of samples were infected by more than one species. More than one fungal species was isolated from a single symptom in 17 and 4% of the samples from dying spurs and wedge-shaped necrosis, respectively, which yielded Diatrypaceae species. *C. ampelina* and *E. lata* were found in 83 and 100% of the samples from dying spurs and wedge-shaped necrosis, respectively, which yielded more than one fungal species (Table 2).

Discussion

There has been an increase in the number of studies of the Diatrypaceae occurring on grapevine recently, and the use of DNA sequences makes it easier to identify taxa to generic and species level. In this study, phylogenetic analyses allowed the identification of seven Diatrypaceae species from declining grapevines in South Africa, namely *C. ampelina*, *C. rabenhorstii*, *E. lata*, *E. consobrina*, *E. cremea*, *E. citricola*, and *E. microtheca*. Except for *E. lata* and *C. ampelina*, the taxa found in this study are reported for the first time on grapevine in South Africa.

After finding close resemblance of the type materials of *E. leptoplaca* and *E. consobrina*, Trouillas and Gubler (2004) concluded that the French isolate CBS 286.87 designated as *E. leptoplaca* by Rappaz (1987) could be *E. consobrina*. The close resemblance of the type

materials of these two species was confirmed in this study. However, the phylogenetic results of this study strongly support the separation of this isolate, which clustered with some of the *Eutypa* strains collected in the present study, from isolates of both *E. leptoplaca* and *E. consobrina*. This cluster is described as a new species and as a result, strain CBS 286.87 was reidentified as *E. cremea* in this study. *E. cremea* could be distinguished from *E. consobrina* based on both phylogenetic analyses and morphological characteristics. Colonies of *E. cremea* are cream on PDA-C while those of *E. consobrina* were apricot (data not shown). Furthermore, perithecia of *E. consobrina* collected from non-grapevine material (data unpublished) were larger ([249–] 262 to 451 [–468] μm diam.) and also form larger asci ([25–] 27 to 69 [–82] $\times$ 5 to 7 μm) and ascospores (6 to 10 [–11] $\times$ [1–] 1.5 to 3 μm) than *E. cremea*.

The newly described species, *E. cremea*, was isolated from grapevine samples collected from different areas in Western Cape Province including Bonnievale, Constantia, Darling, Durbanville, Hermanus, Robertson, Slanghoek, and Stellenbosch. This shows that this species occurs in a wide geographical area in Western Cape Province of South Africa and therefore, grapevine appears to be a good host for *E. cremea*. Furthermore, besides its isolation from grapevine, *E. cremea* has been associated with *Arundo donax* from Algeria (Rappaz 1987), which indicates its capability to infect multiple hosts.

The identity of Diatrypaceae species associated with wedge-shaped necrosis and spur dieback on grapevine were investigated and results showed that the frequency of isolation of these species from the two symptom types differed considerably. Wedge-shaped cankers on grapevine

Fig. 3. Morphology of *Cryptovalsa ampelina*. **A**, Stromata showing perithecia cavities. **B**, Group of polysporous asci. **C**, Clavate, polysporous ascus. **D**, Allantoid to suballantoid ascospores. **E**, Colony on PDA-C after 15 days growing at 24°C under intermittent light (12 h).

have, in the past, been associated with *E. lata* (Moller and Kasimatis 1978), and indeed, this species was found to be the dominant species in this symptom type in the current study. The high incidence of isolation of this pathogen indicates that the wedge-shaped necrosis in grapevine wood could be mostly attributed to *E. lata* and that this species is an important grapevine canker pathogen in South Africa. *E. lata* produces toxins (Tey-Rulh et al. 1991) and wood degrading enzymes (English and Davis 1978; Schmidt et al. 1999), which have been implicated in the decline of grapevine. The presence of additional Diatrypaceae species in the same tissue with *E. lata* further compromises the immunity of the vine and possibly enhance the decline of the infected grapevine.

Isolations from dying Sauvignon blanc and Cabernet Sauvignon spurs resulted in seven species of Diatrypaceae being identified, with *C. ampelina* being the most frequently isolated species. *C. ampelina* co-occurred with all the other six species found to infect spurs. However, the frequency in which this species co-occurred with other species was lower than that, in which it occurred alone in both cultivars. The high frequency with which *C. ampelina* was isolated agrees with results of Luque et al. (2014), who found this species to be the most abundant species of the Diatrypaceae in naturally infected grapevine spurs in Spain.

E. lata infects grapevines by means of ascospores, which germinate and colonize the vascular tissue of the trunks and cordons (Moller and Kasimatis 1978, 1980). Mycelium of this fungus is usually not detected in annual canes or the foliage of infected grapevines (Rudelle et al. 2005) and the development of foliar symptoms in Eutypa dieback-affected grapevines has been credited to the phytotoxins produced by *E. lata* inside the wood (Tey-Rulh et al. 1991). While the dieback of spurs and the low isolation frequency of *E. lata* could be explained by the translocation of toxins of this fungus from mature wood to the spurs, the higher incidence of isolation of *C. ampelina* in both cultivars in our study could be explained by the capability of *C. ampelina* to better colonize young wood of spurs compared with *E. lata*. This also makes *C. ampelina* an important causal agent of spur dieback in the Western Cape of South Africa. The isolation of several species of Diatrypaceae in vineyards as young as 4 years old, however, could also suggest that these species are involved in the long process of the development of dieback of grapevines, or they may work synergistically with *E. lata* in symptom development.

A higher number of isolates and diversity of Diatrypaceae species were found in Cabernet Sauvignon compared with Sauvignon blanc vineyards. It would seem that Cabernet Sauvignon is more susceptible

Fig. 4. Morphology of *Eutypa cremea* from grapevine. **A**, Stromata showing perithecia in rows. **B**, Perithecial cavities. **C**, Asci attached to the hymenium. **D**, Mature and immature asci. **E**, Long stalked octosporous ascus. **F**, Allantoid ascospores. **G**, Colony on a 90 mm diam. PDA-C dish after 15 days incubation at 24°C under 12 h of intermittent lighting. **H**, Spore mass oozing from pycnidia after 30 days of incubation at 24°C. **I**, Filiform conidia.

Fig. 5. Morphology of *Eutypa lata*. **A**, Stromata on wood. **B**, Cross-section of stromata. **C**, Octosporous ascus. **D**, Allantoid ascospores. **E**, Colony on PDA-C after 15 days growing at 24°C under intermittent light (12 h). **F**, Filiform conidia.

Fig. 6. Morphology of *Eutypella citricola*. **A**, Stromata. **B**, Group of asci. **C**, Long-stiped, octosporous ascus. **D**, Colony after 15 days on PDA-C dish (90 mm diam.) incubated at 24°C under intermittent light (12 h).

to these fungi than Sauvignon blanc. Péros and Berger (1994) reported that Cabernet Sauvignon has higher susceptibility to *E. lata* than Sauvignon blanc and this could be the case for the other Diatrypaceae species as well. Apart from species that were isolated fairly frequently from spurs, *C. rabenhorstii* and *E. consobrina* were each isolated only once, which suggests that their presence in grapevine wood might be influenced by certain conditions, such as the occurrence of other woody hosts in the vicinity of vineyards. This assumption is supported by other studies, which found diatrypaceous species occurring on grapevine to also occur in other woody hosts surrounding vineyards and postulated that these hosts act as inoculum sources to vineyards (Trouillas et al. 2010, 2011). Furthermore, the low frequency of isolation of these species in the present study makes it difficult to establish their role in grapevine decline.

While *E. lata* has been known to be the most important diatrypaceous pathogen in many grapevine-producing areas of the world, *C. ampelina*, *E. microtheca*, and *E. citricola* also seem to be geographically widespread, having been reported in areas including Australia (Trouillas et al. 2011), California (Trouillas et al. 2010), Chile (Diaz et al. 2011), Mexico (Paolinelli-Alfonso et al. 2015), and northeast Spain (Luque et al. 2012). Before this study, *C. rabenhorstii* has only been reported on grapevine in Australia (Trouillas et al. 2011). Results from the current study also extend the host range of *E. consobrina* from *Arundo mauritanica* to *Vitis* spp. and further increase the number of Diatrypaceae species found to colonize grapevine wood.

Extensive searches for perithecia in vineyards revealed that five Diatrypaceae species have the ability to complete their life cycle on grapevine tissue. However, perithecia of species such as *E. microtheca*, *E. citricola*, and *E. cremea* were not as prevalent in Western Cape vineyards as those of *C. ampelina* and *E. lata*. Nevertheless, finding stromata of these species on dead grapevine wood suggests that they may be involved in dieback and eventually death of grapevines. The epidemiology and biology of the newly discovered Diatrypaceae species on grapevine is currently unknown. However, the isolation of these species from necrotic tissues below pruning wounds of naturally infected spurs and from wedge-shaped cankers associated with dieback symptoms, in this study, suggests that these species, similar to *E. lata*, infect grapevine via pruning wounds on spurs and cordons and develop inside vascular tissues.

Prevention of the introduction of causal pathogens into the vineyard is the most reliable control strategy against canker diseases. Prevention of infection is achieved by strategies that include the use of pruning wound protectants (Ayres et al. 2017; Sosnowski et al. 2008), avoiding pruning immediately after rainfall (Petzoldt et al. 1983), and sanitation.

Table 2. The diversity of Diatrypaceae fungi isolated from dying spurs of Cabernet Sauvignon (CS) and Sauvignon blanc (SB) vineyards as well as wedge-shaped necrosis on wood pieces collected from different vineyards in Western Cape Province of South Africa

Species isolated	Number of samples		
	Dying spurs		Wedge-shaped necrosis ^a
	SB	CS	
<i>Cryptovalsa ampelina</i>	27	29	1
<i>Eutypa lata</i>	17	7	181
<i>Eutypa cremea</i>	3	1	2
<i>Eutypella citricola</i>	13	17	11
<i>Eutypella microtheca</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Cryptovalsa rabenhorstii</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa lata</i>	5	–	1
<i>Cryptovalsa ampelina</i> + <i>Eutypa consobrina</i>	1	–	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa cremea</i>	–	2	–
<i>Cryptovalsa ampelina</i> + <i>Eutypella citricola</i>	2	7	–
<i>Cryptovalsa ampelina</i> + <i>Eutypella microtheca</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa lata</i> + <i>Eutypella citricola</i>	–	1	–
<i>Eutypa lata</i> + <i>Eutypa cremea</i>	–	–	1
<i>Eutypa lata</i> + <i>Eutypella citricola</i>	–	3	7
<i>Eutypa cremea</i> + <i>Eutypella citricola</i>	–	1	–
Total number of samples yielding Diatrypaceae isolates	68	71	204
Total number of samples collected	190	170	296

^a This symptom type was collected from wood of 10 different grapevine cultivars.

Fig. 7. Morphology of *Eutypella microtheca*. **A**, Stromata with sulcate ostioles protruding on the surface. **B**, Stromata with a white, powdery layer surrounding the perithecial. **C**, Octosporous ascus. **D**, Allantoid ascospores. **E**, Colony after 15 days on 90 mm diam. PDA-C dish incubated at 24°C under intermittent light (12 h).

An in vitro study by Gramaje et al. (2012) showed that fungicides such as fluazinam, carbendazim, and tebuconazole were effective against mycelial growth of species including *C. ampelina*, *E. citricola*, and *E. microtheca*. However, no other prevention strategies have so far been evaluated for the potential to control or prevent infection of grapevine caused by the Diatrypaceae species, including those detected in this study, except for fungicides known to reduce *E. lata* infection. Further research on management strategies against canker diseases, poor budburst, and spur dieback of grapevine need to include all Diatrypaceae species involved in the respective disease complexes.

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Literature Cited

- Alfaro, M. E., Zoller, S., and Lutzoni, F. 2003. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Mol. Biol. Evol.* 20:255-266.
- Ayres, M. R., Wicks, T. J., Scott, E. S., and Sosnowski, M. R. 2017. Developing pruning wound protection strategies for managing *Eutypa* dieback. *Aust. J. Grape Wine Res.* 23:103-111.
- Carter, M. V. 1988. *Eutypa* dieback. Pages 32-34 in: *Compendium of Grape Diseases*. R. Pearson and A. Goheen, eds. APS Press, St Paul, MN.
- Castresana, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17:540-552.
- Catal, M., Jordan, S. A., Butterworth, S. C., and Schilder, A. M. C. 2007. Detection of *Eutypa lata* and *Eutypella vitis* in grapevine by nested multiplex polymerase chain reaction. *Phytopathology* 97:737-747.
- Damm, U., Mostert, L., Crous, P. W., and Fourie, P. H. 2008. Novel *Phaeoacremonium* species associated with necrotic wood of *Prunus* trees. *Persoonia* 20:87-102.
- Díaz, G. A., Prehn, D., and Latorre, B. A. 2011. First Report of *Cryptovalsa ampelina* and *Eutypella leprosa* associated with grapevine trunk diseases in Chile. *Plant Dis.* 95:490.
- English, H., and Davis, J. R. 1978. *Eutypa armeniaca* in apricot: Pathogenesis and induction of xylem soft-rot. *Hilgardia* 46:193-204.
- Ferreira, J. H. S. 1987. Dieback of grapevines in South Africa. PhD Thesis, University of the Western Cape, South Africa.
- Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.
- Glawe, D. A., and Rogers, J. D. 1984. Diatrypaceae in the Pacific Northwest. *Mycotaxon* 20:401-460.
- Gramaje, D., Ayres, M. R., Trouillas, F. P., and Sosnowski, M. R. 2012. Efficacy of fungicides on mycelial growth of diatrypaceous fungi associated with grapevine trunk disease. *Australas. Plant Pathol.* 41:295-300.
- Guindon, S., and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52:696-704.
- Hillis, D. M., and Bull, J. J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42:182-192.
- Huelsenbeck, J. P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754-755.
- Jordan, S., and Schilder, A. 2005. *Eutypella vitis*, a potential pathogen of grapevines in Michigan. *Phytopathology* 95:S51 (Abstract).
- Katoh, K., Misawa, K., Kuma, K., and Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30:3059-3066.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., and Drummond, A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647-1649.
- Luque, J., Elena, G., Garcia-figueres, F., Reyes, J., Barrios, G., and Legorburu, F. J. 2014. Natural infections of pruning wounds by fungal trunk pathogens in mature grapevines in Catalonia (Northeast Spain). *Aust. J. Grape Wine Res.* 20:134-143.
- Luque, J., Garcia-figueres, F., Legorburu, F. J., Muruamendaraz, A., Armengol, J., and Trouillas, F. P. 2012. Species of Diatrypaceae associated with grapevine trunk diseases in Eastern Spain. *Phytopathol. Mediterr.* 51:528-540.
- Luque, J., Sierra, D., Torres, E., and Garcia, F. 2006. *Cryptovalsa ampelina* on grapevines in N.E. Spain: identification and pathogenicity. *Phytopathol. Mediterr.* 45:S101-S109.
- Moller, W. J., and Carter, M. V. 1965. Production and dispersal of ascospores in *Eutypa armeniaca*. *Aust. J. Biol. Sci.* 18:67-80.
- Moller, W. J., and Kasimatis, A. N. 1978. Dieback of grapevines caused by *Eutypa armeniaca*. *Plant Dis. Rep.* 62:254-258.
- Moller, W. J., and Kasimatis, A. N. 1980. Protection of grapevine pruning wounds from *Eutypa* dieback. *Plant Dis.* 64:278-280.
- Mostert, L., Halleen, F., Creaser, M. L., and Crous, P. W. 2004. *Cryptovalsa ampelina*, a forgotten shoot and cane pathogen of grapevines. *Australas. Plant Pathol.* 33:295-299.
- Moyo, P., Mostert, L., Bester, M., and Halleen, F. 2016. Trunk disease fungi associated with *Diospyros kaki* in South Africa. *Plant Dis.* 100:2383-2393.
- Paolinelli-Alfonso, M., Serrano-Gomez, C., and Hernandez-Martinez, R. 2015. Occurrence of *Eutypella microtheca* in grapevine cankers in Mexico. *Phytopathol. Mediterr.* 54:86-93.
- Péros, J. P., and Berger, G. 1994. A rapid method to assess the aggressiveness of *Eutypa lata* isolates and the susceptibility of grapevine cultivars to *Eutypa* dieback. *Agronomie* 14:515-523.
- Petzoldt, C. H., Sall, M. A., and Moller, W. J. 1983. Factors determining the relative number of ascospores released by *Eutypa armeniaca* in California. *Plant Dis.* 67:857-860.
- Pitt, W. M., Huang, R., Trouillas, F. P., Steel, C. C., and Savocchia, S. 2010. Evidence that *Eutypa lata* and other diatrypaceous species occur in New South Wales vineyards. *Australas. Plant Pathol.* 39:97-106.
- Pitt, W. M., Trouillas, F. P., Gubler, W. D., Savocchia, S., and Sosnowski, M. R. 2013. Pathogenicity of diatrypaceous fungi on grapevines in Australia. *Plant Dis.* 97:749-756.
- Rappaz, F. 1987. Taxonomie et nomenclature des Diatrypacées à ascques octosporées. *Mycol. Helv.* 2:285-648.
- Rolshausen, P. E., Baumgartner, K., Travadon, R., Fujiyoshi, P., Pouzoulet, J., and Wilcox, W. F. 2014. Identification of *Eutypa* spp. causing *Eutypa* dieback of grapevine in eastern North America. *Plant Dis.* 98:483-491.
- Rolshausen, P. E., Mahoney, N. E., Molyneux, R. J., and Gubler, W. D. 2006. Reassessment of the species concept in *Eutypa lata*, the causal agent of *Eutypa* dieback of grapevine. *Phytopathology* 96:369-377.
- Rudelle, J., Octave, S., Kaid-Harche, M., Roblin, G., and Fleurat-Lessard, P. 2005. Structural modifications induced by *Eutypa lata* in the xylem of trunk and canes of *Vitis vinifera*. *Funct. Plant Biol.* 32:537-547.
- Safodien, S., Halleen, F., Crous, P. W., Smit, W. A., and Botha, A. 2005. Molecular characterisation and identification of *Eutypa* spp. from grapevines in South Africa. *Phytopathol. Mediterr.* 44:86-87 (Abstract).
- Schilder, A. M. C., Butterworth, S. C., and Catal, M. 2003. *Eutypa* dieback: progression and detection of the pathogen in grapevines. *Phytopathology* 93: S76 (Abstract).
- Schmidt, C. S., Wolf, G. A., and Lorenz, D. 1999. Production of extracellular hydrolytic enzymes by the grapevine dieback fungus *Eutypa lata*. *J. Plant Dis. Prot.* 106:1-11.
- Sosnowski, M. R., Creaser, M. L., Wicks, T. J., Lardner, R., and Scott, E. S. 2008. Protection of grapevine pruning wounds from infection by *Eutypa lata*. *Aust. J. Grape Wine Res.* 14:134-142.
- Tey-Rulph, P., Philippe, I., Renaud, J. M., Tsoupras, G., De Angelis, P., Fallot, J., and Tabacchi, R. 1991. Eutypine, a phytotoxin produced by *Eutypa lata* the causal agent of dying-arm disease of grapevine. *Phytochemistry* 30:471-473.
- Trouillas, F. P., and Gubler, W. D. 2004. Identification and characterization of *Eutypa leptoplaca*, a new pathogen of grapevine in Northern California. *Mycol. Res.* 108:1195-1204.
- Trouillas, F. P., and Gubler, W. D. 2010. Pathogenicity of Diatrypaceae species in grapevines in California. *Plant Dis.* 94:867-872.
- Trouillas, F. P., Pitt, W. M., Sosnowski, M. R., Huang, R. J., Peduto, F., Loschiavo, A., Savocchia, S., Scott, E. S., and Gubler, W. D. 2011. Taxonomy and DNA phylogeny of Diatrypaceae associated with *Vitis vinifera* and other woody plants in Australia. *Fungal Divers.* 49:203-223.
- Trouillas, F. P., Urbez-Torres, J. R., and Gubler, W. D. 2010. Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia* 102:319-336.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols, a guide to methods and applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and J. White, eds. Academic Press, San Diego.