Research

***Diaporthe nebulae* sp. nov. and First Report of *D. cynaroidis*, *D. novem*, and *D. serafinae* on Grapevines in South Africa**Palesa Lesuthu,^{1,3} Lizel Mostert,² Christoffel F. J. Spies,^{1,2} Providence Moyo,² Thierry Regnier,³ and Francois Halleen^{1,2,†}¹Plant Protection Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa; ²Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa; and ³Department of Biotechnology and Food Technology, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa**Abstract**

Diaporthe species cause Phomopsis cane and leaf spot as well as Phomopsis dieback on grapevines. Symptoms of Phomopsis dieback have increasingly been observed over the past few years. In order to assess the current status of *Diaporthe* on grapevines in the Western Cape Province of South Africa, isolations were made from dormant grafted nursery vines, dormant rootstock canes, and dying or dead spurs of field vines. Cultures identified as *Diaporthe* based on morphological features were further identified to species level by sequencing the internal transcribed spacers (ITS) 1 and 2 and 5.8S rRNA and, for a representative subsample of isolates, the partial beta-tubulin (*tub2*) and translation elongation

factor 1-alpha (EF1- α) genes. Phylogenetic analysis of the combined ITS, *tub2*, and EF1- α data revealed nine *Diaporthe* species associated with grapevines during this survey. One of these represents a new species, *D. nebulae* sp. nov., and three other species, namely *D. novem*, *D. cynaroidis*, and *D. serafinae*, are reported on grapevines in South Africa for the first time. Species-specific primers were designed for PCR identification of *D. ampelina*, *D. ambigua*, and *D. foeniculina*. Pathogenicity studies conducted on detached grapevine shoots indicated *D. ampelina*, *D. novem*, and *D. nebulae* sp. nov. as the most virulent species.

Species of *Diaporthe* Nitschke are either endophytes, saprobes, or pathogens on a wide range of hosts, and are responsible for several plant diseases, including root and fruit rots, stem cankers, dieback, pod blights, seed decay, wilts, and leaf spots (Díaz et al. 2017; Dissanayake et al. 2015; Guarnaccia and Crous 2017; Guarnaccia et al. 2018; Mostert et al. 2001; Rehner and Uecker 1994; Udayanga et al. 2011). The recent abolishment of dual nomenclature in the *International Code of Nomenclature for algae, fungi, and plants* (ICN) has forced fungal taxonomy to move to a single nomenclature for fungi (Hawksworth 2011; Hawksworth et al. 2011). The generic names *Diaporthe* and *Phomopsis* are no longer used to distinguish different morphs of this genus, and a recent study by Rossman et al. (2015) has recommended that *Diaporthe* should be retained over *Phomopsis* because it was introduced first and represent the majority of species. Species recognition of *Diaporthe* have historically been defined based on host association and morphology (Uecker 1988). Recent studies have, however, shown that various species can be found on a single host and are able to infect a wide range of hosts (Baumgartner et al. 2013; Gomes et al. 2013; Guarnaccia and Crous 2017; Guarnaccia et al. 2016; Mostert et al. 2001; Rehner and Uecker 1994; Santos and Phillips 2009; Thompson et al. 2011, 2015; Van

Niekerk et al. 2005). Therefore, identification and description of species based on host association and morphology is no longer reliable within the genus (Gomes et al. 2013; Udayanga et al. 2014a, b). *Diaporthe* species associated with grapevines are now studied worldwide using the combination of morphology, pathogenicity, and molecular data (Dissanayake et al. 2015; Guarnaccia et al. 2018; Mostert et al. 2001; Rehner and Uecker 1994; Urbez-Torres et al. 2013; Van Niekerk et al. 2005). On this host, *Diaporthe ampelina* has been found to be the predominant and most virulent *Diaporthe* species in several grape-producing countries in the world (Baumgartner et al. 2013; Dissanayake et al. 2015; Guarnaccia et al. 2018; Mostert et al. 2001; Pscheidt and Pearson 1989; Rehner and Uecker 1994; Urbez-Torres et al. 2013; Van Niekerk et al. 2005). For many years, this pathogen was associated with ‘dead-arm’ disease until it was eventually discovered that *Eutypa lata* and not *D. ampelina* caused dead arm disease of grapevine (Berrysmith 1962; Moller and Kasimatis 1981; Pearson and Goheen 1994; Urbez-Torres et al. 2013). However, Urbez-Torres et al. (2013) recently confirmed *D. ampelina* as grapevine canker causing agent, proposing the name Phomopsis dieback. In addition to Phomopsis dieback, *D. ampelina* is also the causal agent of Phomopsis cane and leaf spot, a disease that causes serious losses due to shoots breaking off at the base, dieback, loss of vigor, stunting, reduced bunch set, and fruit rot. Infected canes show brown to black necrosis with oval shaped lesions on the first three to six basal internodes. Clusters may develop rachis necrosis, which results in brown, shriveled berries close to harvest. Leaf symptoms include small, pale green to yellow spots with necrotic centers (Guarnaccia et al. 2018; Pearson and Goheen 1994; Urbez-Torres et al. 2013; Wilcox et al. 2015). Halleen et al. (2003) and Fourie and Halleen (2004a) reported *D. ampelina* to occur and invade rootstock mother plants, propagation material, and grafted vines in South African nursery fields. A total yield loss of between 20 and 30% has been reported as a result of infection by this fungus on grapevines (Pscheidt and Pearson 1989; Urbez-Torres et al. 2013).

In South Africa, Phomopsis cane and leaf spot was first noticed in 1935 (Du Plessis 1938), and *Diaporthe* species have since then also

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been acknowledged as grapevine trunk disease pathogens causing cankers and dieback on vines (Mostert et al. 2001; Van Niekerk et al. 2005; White et al. 2011a, b). Mostert et al. (2001) found 10 *Diaporthe* spp. to occur on South African grapevines, and of these, *D. ampelina*, *D. kyushuensis*, *D. amygdali*, and *D. australafricana* are confirmed as being pathogenic to this host. In a later study, Van Niekerk et al. (2005) found 15 *Diaporthe* spp. occurring on grapevines in South Africa, with *D. ampelina* and *D. amygdali* being the most virulent. Moreover, eight distinct species of the 15 were unidentified (*Phomopsis* spp. 1-8). *Phomopsis* sp. 1 has since been identified as *D. foeniculina* (Santos and Phillips 2009) and *Phomopsis* sp. 6 as *D. eres* (Udayanga et al. 2014b), based on published phylogenies.

Correct identification of *Diaporthe* spp. is an essential step toward meaningful studies on epidemiology and disease management. The ITS region is often useful in the identification of *Diaporthe* spp., but the intraspecific variation observed in ITS sequences of several *Diaporthe* spp. can cause confusion in species recognition when used alone (Farr et al. 2017; Santos et al. 2010). Recent publications have advocated the use of beta-tubulin (*tub2*), calmodulin (*CAL*), histone H3 (*HIS*), internal transcribed spacers (*ITS*), and the translation elongation factor 1- α (*EF1- α*) for phylogenetic analyses in *Diaporthe* (Guarnaccia and Crous 2017; Guarnaccia et al. 2018; Santos et al. 2017; Udayanga et al. 2012). Of these five regions, the *EF1- α* is considered to be most useful for species identification (Santos et al. 2017). Udayanga et al. (2014b) also made use of the actin region (*ACT*) to assist in the resolution of phylogenetic relationships in the *D. eres* species complex, but the usefulness of this region in resolving relationships among other species in the genus has not been determined.

Molecular identification assays have become very popular as fast and sensitive alternatives to conventional culture-based techniques for identifying plant pathogens in infected plant tissues. Specific PCR assays using genus-specific primers for *Diaporthe* such as Phf/Phr (Prasad et al. 2006) and PF1/PF2 (Girish et al. 2009) have been reported. Species-specific primers have been designed for the identification and detection of *D. phaseolorum*, *D. longicolla*, and *D. azadirachtae* (Vedashree et al. 2015; Zhang et al. 1997); however, species-specific primers to identify additional *Diaporthe* spp. on grapevines are lacking.

The total number of *Diaporthe* species on grapevines has been increasing worldwide ever since Mostert et al. (2001) and Van Niekerk et al. (2005) published their studies more than 10 years ago. This raised questions about the current status of *Diaporthe* spp. on grapevines in South Africa. During the past 3 years, unknown *Diaporthe* isolates were regularly recovered from grapevines in the Western Cape Province of South Africa. It was therefore necessary to reassess the status of *Diaporthe* species on South African grapevines. To this end, the aims of this study were to (i) identify *Diaporthe* spp. associated with grapevine dieback symptoms in the Western Cape Province, (ii) relocate unidentified isolates of Van Niekerk et al. (2005) to known species where possible, (iii) characterize species not previously reported on grapevines in South Africa in terms of pathogenicity and morphology, and (iv) develop species-specific PCR assays for the most common *Diaporthe* spp. identified.

Materials and Methods

Sample collection and fungal isolation. Dormant grafted nursery vines ($n = 700$) and dormant rootstock propagation material ($n = 74$) were collected from various viticultural regions of the Western Cape Province over a period of 3 years to obtain isolates of *Diaporthe* species. In addition, dying spurs ($n = 360$) were also collected from established vineyards in the different grapevine producing areas of the Western Cape Province, over a period of 2 years. Samples were cut into transverse and longitudinal sections to reveal internal symptoms such as black streaking, brown streaking, or V-shaped necrosis (White et al. 2011b). Cuttings were then surface sterilized and isolations were made onto potato dextrose agar (PDA, Biolab, South Africa) containing chloramphenicol (250 mg/liter), following the protocol described by Moyo et al. (2016). Plates were then incubated at 25°C and monitored every 1 to 3 days for a period of 4 weeks for growth of trunk disease pathogens. Throughout this 4-week period,

subcultures of fungal growth resembling *Diaporthe* were cultured on fresh PDA plates in order to obtain pure cultures. All cultures were maintained as agar plugs in sterile water and stored at 4°C at the Nietvoorbij campus of the Agricultural Research Council.

DNA extraction, PCR amplification, and sequencing. The isolation protocol described by Damm et al. (2008) was used to extract DNA from aerial mycelium of actively growing PDA cultures of *Diaporthe*-like isolates. The ITS region was amplified for all isolates and sequenced in one direction to facilitate preliminary species-level identifications. Based on these preliminary results, representative isolates were selected for sequencing of double-strand ITS, beta-tubulin (*tub2*), and *EF1- α* regions. ITS amplifications were performed using the universal fungal primers ITS1 and ITS4 (White et al. 1990). The partial *tub2* region was amplified using the primers T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995). The primers EF1-728F and EF2 (Carbone and Kohn 1999) were used to amplify part of the translation elongation factor 1- α (*EF1- α*). The methods described by Moyo et al. (2016) for PCR amplification, purification, and sequencing of the PCR products was followed for ITS, *tub2*, and *EF1- α* . Sequences obtained were evaluated, edited, and assembled (ITS, *tub2*, and *EF1- α* for selected isolates only) using Geneious version R8.0.5 (<https://www.geneious.com>, Kearse et al. 2012).

Phylogenetic analysis. Initial species identification was done by using megablast searches of the GenBank nucleotide database (www.ncbi.nlm.nih.gov). ITS, *tub2*, and *EF1- α* reference sequences representing relevant *Diaporthe* species and *Diaporthella corylina* (CBS 121124) as outgroup were obtained from GenBank (Table 1). *Diaporthe* isolates obtained during this study were aligned with reference sequences in Geneious R8.0.5 using the L-INS-i algorithm of MAFFT V7.017 (Katoh et al. 2009) and manually adjusted where necessary. Preliminary phylogenetic analysis with single strand ITS sequences of all the grapevine *Diaporthe* isolates containing the ITS reference sequences was conducted to identify representative strains for inclusion in the combined ITS-*tub2*-*EF1- α* phylogeny. ITS, *tub2*, and *EF1- α* alignments for the combined phylogeny were aligned separately as described above and concatenated in Geneious R8.0.5. Maximum likelihood phylogenetic analysis of the combined ITS-*tub2*-*EF1- α* dataset was performed using PhyML 3.1 (Guindon et al. 2010) under the HKY+I+G model, which was among the top two best models estimated in jModeltest2 (Darriba et al. 2012) using the Bayesian information criterion for each of the individual alignments (ITS, *tub2*, *EF1- α*). Maximum likelihood bootstrap support was calculated from 1,000 replicates. Bayesian analyses were performed using PhyloBayes-MPI v. 1.7 (Lartillot et al. 2013) under CAT-GTR settings. Two independent chains were run for 4,800 cycles, of which every 10th point was saved. Of the 480 saved points, the first 120 were discarded as burn-in prior to assessing convergence using the bpcomp and tracecomp commands. The effective population sizes after running these commands were larger than 250 and maxdiff values were less than 0.25, indicating acceptable convergence as per the guidelines set out in the PhyloBayes-MPI manual. Trees were viewed and edited using Figtree 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Sequences obtained from GenBank are listed by their accession numbers, followed by taxon names (Fig. 1), while newly generated sequences are listed by their isolate numbers. Newly generated sequences have been deposited in GenBank (Table 1), alignments and phylogenies in TreeBASE (Submission number: 20684).

Morphological characterization. To determine morphological characteristics of new *Diaporthe* species, isolates were plated onto three different types of media: PDA, malt extract agar (MEA), and water agar (WA) containing 4-cm-long pieces of double autoclaved pine needles or grapevine shoots to induce sporulation. The plates were then incubated at 25°C near ultraviolet light in a 12-h light-darkness regime for 4 weeks to induce sporulation. When fruiting structures were observed, they were mounted in 70% lactic acid onto a microscope slide and observed under 1,000 $\times$ magnification. The size, shape, and type of conidia were recorded. Thirty spore measurements were taken for each isolate (length and width), the minimum

and maximum ranges of spore dimensions were recorded, and the averages determined.

Pathogenicity. Four *Diaporthe* species, namely: *D. novem*, *D. serafiniae*, *D. cynaroidis*, and a new species (*D. nebulae*) were selected to evaluate their potential as pathogens of grapevines since they were identified for the first time on South African grapevines. *D. ampelina* was used as a positive control since it is known to be highly virulent, and PDA media were used as negative control. Three different isolates per species were selected for the five *Diaporthe* species, plated out onto PDA media, and incubated at 25°C for 4 weeks.

One hundred and eighty dormant pruned shoots were collected from a 4-year-old Bukettraube (*Vitis vinifera*) vineyard at the Nietvoorbij campus of the Agricultural Research Council and subjected to hot water treatment following the protocol of Fourie and Halleen (2004b). This treatment significantly reduced the incidence of latent pathogen infections as well as any superficial pathogens that may be present on the canes. After this treatment, shoots were wounded between two nodes (2 mm deep) using a 3-mm cork-borer. Plugs were made from the outer margin of colonized agar of the five *Diaporthe* spp. including the positive control, and uncolonized agar (negative

Table 1. List of *Diaporthe* isolates collected in this study and reference strains (from GenBank) studied and included in the phylogenetic analysis

Species	Strain/ isolate	Host	ITS accession	tub2 accession	EF1- α accession
<i>Diaporthella corylina</i>	CBS 121124	<i>Corylus</i> sp.	KC343004	KC343972	KC343730
<i>Diaporthe alleghaniensis</i>	CBS 495.72	<i>Betulaceae</i>	KC343007	KC343975	KC343733
<i>D. alnea</i>	CBS 146.46	<i>Betulaceae</i>	KC343008	KC343976	KC34373
<i>D. ambigua</i>	CBS 187.87	<i>Helianthus annuus</i>	KC343015	KC343983	KC343741
<i>D. ambigua</i>	CBS 114015	<i>Pyrus communis</i>	KC343010	KC343978	KC343736
<i>D. ambigua</i>	Phom 207	US 8-7	KY511309	-	-
<i>D. ambigua</i>	PMM 1734	<i>Vitis vinifera</i>	KY511339	KY511371	-
<i>D. ampelina</i>	CBS 111888	<i>Vitis vinifera</i>	KC343016	KC343984	KC343742
<i>D. ampelina</i>	CBS 114867	<i>Vitis vinifera</i>	KC343017	KC343985	KC343743
<i>D. ampelina</i>	Phom 206	<i>Vitis</i> sp. (rootstock)	KY511308	KY511341	MH708549
<i>D. ampelina</i>	Phom 209	<i>Vitis</i> sp. (rootstock)	KY511310	KY511342	MH708548
<i>D. ampelina</i>	Phom 214	<i>Vitis</i> sp. (rootstock)	KY511311	KY511343	MH708547
<i>D. ampelina</i>	Phom 275	<i>Vitis champinii</i>	KY511319	KY511353	-
<i>D. ampelina</i>	Phom 281	<i>Vitis</i> sp. (rootstock)	KY511322	KY511354	MH708542
<i>D. amygdali</i>	CBS 126679	<i>Prunus dulcis</i>	KC343022	KC343990	KC343748
<i>D. anacardii</i>	CBS 720.97	<i>Anacardium occidentale</i>	KC343024	KC343992	KC343750
<i>D. arecae</i>	CBS 535.75	<i>Citrus</i> sp.	KC343033	KC344001	KC343759
<i>D. australafricana</i>	CBS 111886	<i>Vitis vinifera</i>	KC343038	KC344005	KC343764
<i>D. beckhausii</i>	CBS 138.27	<i>Viburnum</i> sp.	KC343041	KC344009	KC343767
<i>D. bicincta</i>	CBS 121004	<i>Juglans</i> sp.	KC343618	KC344102	KC343860
<i>D. castaneae- mollissimae</i>	DNP 128	<i>Lithocarpus glabra</i>	JF957786	JX275438	JX275401
<i>D. chamaeropsis</i>	CBS 753.70	<i>Spartium junceum</i>	KC343049	KC344017	KC343775
<i>D. cinerascens</i>	CBS 719.96	<i>Ficus carica</i>	KC343050	KC344018	KC343776
<i>D. citri</i>	AR 3405	<i>Citrus</i> sp.	KC843311	KC843187	KC843071
<i>D. citrichinensis</i>	ZJUD 34	<i>Citrus sinensis</i>	JQ954648	KC357459	JQ954648
<i>D. cuppatea</i>	CBS 117499	<i>Aspalathus linearis</i>	KC343057	KC344025	KC343783
<i>D. cynaroidis</i>	CBS 122676	<i>Protea cynaroides</i>	KC343058	KC344026	KC343784
<i>D. cynaroidis</i>	PMM 436	<i>Vitis vinifera</i>	KY511326	KY511358	MH708541
<i>D. cynaroidis</i>	PMM 437	<i>Vitis vinifera</i>	KY511327	KY511359	-
<i>D. cynaroidis</i>	PMM 1658	<i>Vitis vinifera</i>	KY511332	KY511364	-
<i>D. cytospora</i>	AR 5148	<i>Citrus sinensis</i>	KC843309	KC843223	KC843118
<i>D. endophytica</i>	CBS 133811	<i>Schinus terebinthifolius</i>	KC343065	KC344033	KC343791
<i>D. eres</i>	CBS 101742	<i>Fraxinus</i> sp.	KC343073	KC344041	KC343799
<i>D. eres</i>	PMM 1690	<i>Vitis vinifera</i>	KY511338	KY511370	-
<i>D. eres</i>	AR 5193	<i>Ulmus laevis</i>	KJ210529	KJ420799	KJ210550
<i>D. eres</i>	AR 5196	<i>Ulmus laevis</i>	KJ210533	KJ420817	KJ210554
<i>D. eres</i>	AR 5223	<i>Acer nugundo</i>	KJ210528	KJ420830	KJ210549
<i>D. eres</i>	CBS 109767	<i>Acer</i> sp.	DQ491514	KC344043	KC343801
<i>D. eres</i>	CBS 439.82	<i>Cotoneaster</i> sp.	FJ889450	JX275437	KC343816
<i>D. eres</i>	DP 0667	<i>Juglandaceae</i>	KC843328	KC843229	KC843121
<i>D. eres</i>	LCM 114.01 _a	<i>Ulmus</i> sp.	KJ210521	KJ420787	KJ210545
<i>D. eres</i>	DLR 12 _a	<i>Vitis vinifera</i>	KJ210518	KJ420783	KJ210542
<i>D. eugeniae</i>	CBS 444.82	<i>Eugenia aromatica</i>	KC343098	KC344066	KC343824
<i>D. fibrosa</i>	CBS 113830	<i>Rhamnus cathartica</i>	KC343100	KC344068	KC343825
<i>D. foeniculina</i>	CBS 116957	<i>Pyrus pyrifolia</i>	KC343103	KC344071	KC343829
<i>D. foeniculina</i>	CBS 123209	<i>Foeniculum vulgare</i>	KC343105	KC344073	KC343831
<i>D. foeniculina</i>	Phom 224	<i>Vitis champinii</i>	KY511312	-	MH708546
<i>D. foeniculina</i>	PMM 463	<i>Vitis vinifera</i>	KY511328	KY511352	-
<i>D. foeniculina</i>	PMM 480	<i>Vitis vinifera</i>	KY511329	-	MH708540
<i>D. foeniculina</i>	PMM 1638	<i>Vitis vinifera</i>	KY511330	KY511362	MH708551
<i>D. foeniculina</i>	PMM 1679	<i>Vitis vinifera</i>	KY511335	KY511367	-
<i>D. foeniculina</i>	PMM 1680	<i>Vitis vinifera</i>	KY511336	KY511368	MH708539
<i>D. fusicola</i>	CGMCC3.17087	<i>Lithocarpus glabra</i>	KF576281	KF576305	KF576256
<i>D. ganjae</i>	CBS 180.91	<i>Cannabis sativa</i>	KC343112	KC344080	KC343838
<i>D. heliciis</i>	AR 5211	<i>Hedera helix</i>	KJ210538	KJ420828	KJ210559

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control) using a 2-mm cork-borer. The plugs were inoculated into the wounds and sealed with Parafilm (Parafilm M, Bemis). Inoculated shoots were incubated in moist chambers (airtight plastic boxes: 29 × 23.5 × 5.5 cm) in the dark. Each container had one inoculated shoot of each of the 18 treatments. Each treatment was replicated 5 times, and the entire experiment was repeated once. After 5 weeks, shoots were split longitudinally, the internal lesion length was measured, and small pieces of wood tissue (1 × 2 mm) were plated out onto PDA media as above to confirm colonization by the inoculated isolates. The results were statistically analyzed in SAS (version 9. 2)

using analysis of variance (ANOVA) to determine the least significant differences. Percentage of fungal reisolation was also determined.

Designing of PCR based species-specific primers. Species-specific PCR primers were designed for *D. ampelina*, *D. foeniculina*, and *D. ambigua*. These species together with *D. novem* were the most abundant; however, the pathogenic status of *D. novem* on grapevines was unknown; therefore primers were not designed for this species. All the primers were designed using Primer3 (<https://sourceforge.net/projects/primer3>) in Geneious R8.0.5. The primers were designed to anneal to unique regions of the *tub2* sequence for each target species.

Table 1. (Continued from previous page)

Species	Strain/ isolate	Host	ITS accession	<i>tub2</i> accession	EF1- α accession
<i>D. hickoriae</i>	CBS 145.26	<i>Carya glabra</i>	KC343118	KC344086	KC343844
<i>D. impulsua</i>	CBS 141.27	<i>Sorbus americana</i>	KC343122	KC344090	KC343848
<i>D. inconspicua</i>	CBS 133813	<i>Maytenus ilicifolia</i>	KC343123	KC344091	KC343849
<i>D. infecunda</i>	CBS 133812	<i>Schinus terebinthifolius</i>	KC343126	KC344094	KC343852
<i>D. longicolla</i>	CGMCC3.17089	<i>Glycine max</i>	KF576267	KF576291	KF576242
<i>D. longicolla</i>	HM 347711	-	KF576268	KF576292	-
<i>D. longispora</i>	CBS 194.36	<i>Ribes</i> sp.	KC343135	KC344103	KC343861
<i>D. lusitanicae</i>	CBS 123212	<i>Foeniculum vulgare</i>	KC343136	KC344104	KC343862
<i>D. manihotia</i>	CBS 505.76	<i>Manihot utilisima</i>	KC343138	KC344106	KC343864
<i>D. nebulae</i>	Phom 240	<i>Vitis</i> sp. (rootstock)	KY511315	KY511346	MH708543
<i>D. nebulae</i>	PMM 1678	<i>Vitis vinifera</i>	KY511334	KY511366	-
<i>D. nebulae</i>	PMM 1681	<i>Vitis vinifera</i>	KY511337	KY511369	MH708552
<i>D. neilliae</i>	CBS 14427	<i>Spiraea</i> sp.	KC343144	KC344112	KC343870
<i>D. cf. nobilis</i>	CBS 113470	<i>Castanea sativa</i>	KC343146	KC344114	KC343872
<i>D. cf. nobilis</i>	CBS 200.39	<i>Laurus nobilis</i>	KC343151	KC344119	KC344119
<i>D. cf. nobilis</i>	CBS 116954	<i>Pyrus pyrifolia</i>	KC343148	KC344116	KC343874
<i>D. cf. nobilis</i>	CBS 116953	<i>Pyrus pyrifolia</i>	KC343147	KC344115	KC343873
<i>D. cf. nobilis</i>	CBS 338.89	<i>Hedera helix</i>	KC343152	KC344120	KC343878
<i>D. cf. nobilis</i>	CBS 587.79	<i>Pinus pantepella</i>	KC343153	KC344121	KC343879
<i>D. novem</i>	DPO 378	<i>Glycine max</i>	KC343157	KC344125	KC343883
<i>D. novem</i>	CBS 127270	<i>Glycine max</i>	KC343156	KC344124	KC343882
<i>D. novem</i>	Phom 201	<i>Vitis champinii</i>	KY511307	KY511340	MH708550
<i>D. novem</i>	Phom 232	<i>Vitis</i> sp. (rootstock)	KY511314	KY511345	MH708544
<i>D. novem</i>	Phom 257	<i>Vitis</i> sp. (rootstock)	KY511319	KY511351	-
<i>D. novem</i>	Phom 264	<i>Vitis</i> sp. (rootstock)	KY511320	KY511352	-
<i>D. novem</i>	Phom 290	<i>Vitis</i> sp. (rootstock)	KY511323	KY511355	-
<i>D. novem</i>	Phom 297	<i>Vitis</i> sp. (rootstock)	KY511325	KY511357	-
<i>D. oncostoma</i>	CBS 100454	<i>Robinia pseudoacacia</i>	KC343160	KC343402	KC343886
<i>D. oncostoma</i>	CBS 589.78	<i>Robinia pseudoacacia</i>	KC343162	KC344130	KC343888
<i>D. ovoicicola</i>	CGMCC 3.17092	<i>Lithocarpus glabra</i>	Kf576264	Kf576288	KF576239
<i>D. phaseolorum</i>	CBS 116019	<i>Caperonia palustris</i>	KC343175	KC344143	KC343901
<i>D. pustulata</i>	CBS 109742	<i>Acer pseudoplatanus</i>	KC343185	KC344153	KC343911
<i>D. pterocarp</i>	MFLUCC.10571	<i>Pterocarpus indicus</i>	JQ619899	JX275460	JX275416
<i>D. rudis</i>	AR 3422	<i>Laburnum anagyroides</i>	KC843090	KC843254	KC843090
<i>D. rudis</i>	P86g1	<i>Vitis vinifera</i>	AY485750	JX2754554	-
<i>D. serafiniae</i>	BRIP 55665	<i>Helianthus annuus</i>	KJ197274	KJ197254	KJ197236
<i>D. serafiniae</i>	Phom 225	<i>Vitis</i> sp. (rootstock)	KY511313	KY511344	MH708545
<i>D. serafiniae</i>	Phom 242	<i>Vitis</i> sp. (rootstock)	KY511316	KY511347	-
<i>D. serafiniae</i>	Phom 247	<i>Vitis</i> sp. (rootstock)	KY511317	KY511348	-
<i>D. serafiniae</i>	Phom 249	<i>Vitis</i> sp. (rootstock)	KY511318	KY511349	-
<i>D. serafiniae</i>	Phom 255	<i>Vitis</i> sp. (rootstock)	-	KY511350	-
<i>D. subclavata</i>	ZJUD 83	<i>C. grandis shatianyou</i>	KJ490618	KJ490439	KJ490497
<i>D. vaccinii</i>	CBS 160.32	<i>Vaccinium macrocarpon</i>	KC343228	KC344196	KC343954
<i>D. vaccinii</i>	DF 5032	<i>Vaccinium corymbosum</i>	AF317570	KC843225	JQ807380
<i>Phomopsis</i> sp. 1	STEU 5573	<i>Vitis vinifera</i>	AY485724	-	-
<i>Phomopsis</i> sp. 2	STEU 2674	<i>Vitis vinifera</i>	AF230761	-	-
<i>Phomopsis</i> sp. 3	STEU 4407	<i>Vitis vinifera</i>	AY485725	-	-
<i>Phomopsis</i> sp. 4	STEU 5464	<i>Vitis vinifera</i>	AY485726	-	-
<i>Phomopsis</i> sp. 5	STEU 5346	<i>Vitis vinifera</i>	AY485728	-	-
<i>Phomopsis</i> sp. 5	PMM 1657	<i>Vitis vinifera</i>	KY511331	KY511363	-
<i>Phomopsis</i> sp. 5	PMM 1660	<i>Vitis vinifera</i>	KY511333	KY511365	-
<i>Phomopsis</i> sp. 6	STEU 5135	<i>Vitis vinifera</i>	AY485735	-	-
<i>Phomopsis</i> sp. 7	STEU 5495	<i>Vitis vinifera</i>	AY485742	-	-
<i>Phomopsis</i> sp. 8	STEU 5462	<i>Vitis vinifera</i>	AY485743	-	-

Fig. 1. Maximum likelihood phylogenetic tree of *Diaporthe* spp. based on combined ITS-tub2-EF1- α sequence data. Bootstrap support values $\geq 65\%$ (calculated from 1,000 replicates) and Bayesian posterior probabilities ≥ 0.70 are shown. The scale bar shows the number of substitutions per site. Species identified in the current study are in bold, and the clades are highlighted. Van Niekerk et al. (2005) isolates all begin with STE-U. Ex-type and ex-epitype isolates are indicated with a square. *Diaporthella corylina* used as outgroup.

Two forward primers and two reverse primers were designed for each species. The primers were selected that had low hairpin and/or self-dimer stability, G/C content of 30 to 80%, and melting temperature of ~60°C. Different combinations of primer pairs were evaluated for specificity, and eventually one species-specific set was selected per species. The species-specific primer sets designed and selected for each species are listed in Table 2. The optimal annealing temperatures of the primers were determined by performing a gradient PCR with annealing temperatures varying from 55 to 65°C. PCR reactions were performed in 10-µl reactions containing 1 µl of template DNA, 0.08 µM of each primer, and 1× Kapa Taq ReadyMix PCR master mix. The standardized amplification regime for the primers was initial denaturation at 94°C for 5 min followed by 30 cycles of 30 s at 94°C, 30 s of annealing at 63°C, and 30 s at 72°C, and a final extension step at 72°C for 5 min. In order to validate the primers, the specificity of the primer pairs was tested against 14 other *Diaporthe* spp. and species of other fungi often associated with grapevines, such as *Phaeoacremonium minimum* (FH-P 576), *Diplodia seriata* (FH-B 594), *Eutypa lata* (PMM 1756), *Ilyonectria lirioidendri* (FH-C 695), *Cadophora luteo-olivacea* (FH-Cad 500), *Cryptovalsa ampelina* (PMM 1761), *Fusarium solani* (FH-Fus 5), and Basidiomycetes, i.e., *Fomitiporia capensis* (FH 136) and *Phellinus resupinatus* (FH 195). The PCR products were separated and visualized as above mentioned but using a 1.5% (wt/vol) agarose gel. DNA for some recently described *Diaporthe* species closely related to the target species were not available for specificity testing, e.g., *D. baccae* (related to *D. foeniculina*), *D. hispaniae*, and *D. hungariae* (both related to *D. ampelina*). For these species, direct sequence comparisons were made to investigate the potential for species-specific primers to also amplify these species.

Results

Sample collection and fungal isolation. *Diaporthe* isolates were recovered from all three grapevine material types sampled. A total of 140 *Diaporthe* isolates were identified, 38 isolates were obtained from dormant rootstock propagation material, 40 from dormant grafted nursery vines, and 62 from dying or dead spurs. Isolates were obtained from black and brown streaking, V-shaped necrosis, as well as brown internal necrosis.

Phylogenetic analysis. The combined maximum likelihood tree (ITS, *tub2*, and EF1-α) revealed nine clades containing *Diaporthe* isolates recovered from South African grapevines during this study (Fig. 1). The first clade (99% bootstrap support, 1.00 posterior probability) consisted of six grapevine isolates which grouped together with *D. foeniculina* ex-type (CBS 111553) and two other isolates identified as *D. foeniculina* (CBS 116957 and CBS 123209) and *Phomopsis* sp. 1 (STE-U 5573). The second clade (77% bootstrap support, 0.97 posterior probability) contained three grapevine isolates representing a unique and previously unsequenced species of *Diaporthe* (*D. nebulae*) which is closely related to *D. anacardii* and an unidentified species previously recovered from grapevines in South Africa (*Phomopsis* sp. 7). The third clade contained one grapevine isolate that grouped with *D. eres* (AR 5223, AR 5193, CBS 101742, and CBS 109769) and *Phomopsis* sp. 6 isolate STE-U 5135 with a bootstrap support of <65% and posterior probability <0.70. The fourth clade (100% bootstrap support, 1.00 posterior probability) consisted of three grapevine isolates that grouped together with *Diaporthe cynaroidis* ex-type (CBS 122676). The fifth

clade consisted of two grapevine isolates which grouped with *Phomopsis* sp. 5 (STE-U 5346) with a bootstrap support of 80% and posterior probability of 1.00. The sixth clade (97% bootstrap support, 1.00 posterior probability) contained five grapevine isolates that grouped together with *D. ampelina* isolate CBS 111888 (ex-type) and CBS 114867. The seventh clade (95% bootstrap support, 1.00 posterior probability) consisted of seven grapevine isolates that grouped with the ex-type of *Diaporthe novem* strain CBS 127270. The eighth clade (84% bootstrap support, 0.99 posterior probability) consisted of five grapevine isolates that grouped with *D. serafiniae* (BRIP 55665) ex-type. The ninth clade (100% bootstrap support, 1.00 posterior probability) consisted of two isolates from grapevines that grouped with *D. ambigua* ex-type (CBS 114015). Of the remaining isolates of Van Niekerk et al. (2005), *Phomopsis* sp. 7 (STE-U 5495) was closely related to *D. anacardii* (CBS 720. 97) and the new species found in the current study (*D. nebulae*). *Phomopsis* sp. 4 (STE-U 5646) grouped together with *D. eres* (AR5196), with a support value of <65% and posterior probability <0.70. *Phomopsis* sp. 3 (STE-U 4407) was related to *D. ganjae* (CBS 180. 91) with a bootstrap support value of 72% and posterior probability of 1.00. *Phomopsis* sp. 8 (STE-U 5462) grouped with *D. arecae* (CBS 535.75) with 99% bootstrap support and posterior probability of 1.00, and *Phomopsis* sp. 2 (STE-U 2674) grouped with *D. oncostoma* (CBS 100454 and CBS 589. 76) with a bootstrap support value of 100% and posterior probability of 1.00.

Taxonomy. *Diaporthe nebulae* Lesuthu; L. Mostert; C.F.J. Spies; Moyo; and Halleen, sp. nov. – MycoBank MB820340; Figure 2.

Etymology. Latin, from *nebula* meaning cloud, named because of its cloudy cultural growth. *Conidiomata* on WA with pine needles pycnidial, black, abundant on pine needles, mostly solitary, some aggregate, up to 337 µm diam, exuding creamy conidial droplets from the ostioles. *Conidiophores*, hyaline, smooth, branched, straight to curved, densely aggregated, 1–3 septate (14–) 22 (–33) × (1.5–) 2 (–3) µm. *Conidiogenous cells*, hyaline, subcylindrical, rarely tapering toward the apex (4–) 13 (–20) × (1–) 2 (–3) µm. *Alpha conidia*, abundant, aseptate, hyaline, smooth, acutely rounded apex, tapering toward both endings, bi- or multiguttulate, wide, ranging from (7–) 16 (–18) × (3–) 5 (–7) µm. *Beta conidia*, hyaline, smooth, slightly curved, some spindle-shaped, (10) 42 (–60) × (2–) 3 (–4) µm. *Gamma conidia*, not observed.

Culture characteristics. Colony diameter was 70 mm after 14 days at 25°C on PDA, white to off-white on the surface, and white with yellow patches underneath.

Material examined. SOUTH AFRICA, Western Cape Province, Constantia, on wood of *Vitis vinifera*, 16 April 2014, P. Moyo, holotype PREM 62198 (dried culture of PMM 1681), ex-holotype CBS 142721 = STE-U 8525 = PMM 1681; Western Cape Province, Stellenbosch, on wood of *Vitis vinifera*, 18 April 2014, P. Moyo, STE-U 8526 = PMM 1678; Western Cape Province, Wellington, on rootstock cuttings (*Vitis* sp.), 20 June 2013, F. Halleen, STE-U 8527 = Phom 240.

Notes. Morphological differences were observed when comparing the new species (*D. nebulae*) isolates with those of the published closely related species, *D. anacardii* (Gomes et al. 2013) and *Phomopsis* sp. 7 (Van Niekerk et al. 2005). *D. nebulae* produced wider alpha conidia, longer conidiophores, and conidiogenous cells (Fig. 2). This species is currently known only from the southern side of the Drakenstein Mountains of the Western Cape (Boland area).

Table 2. Species-specific primer pairs designed and used in this study for the detection and identification of *Diaporthe ambigua*, *D. ampelina*, and *D. foeniculina*

Primer name	Primer sequence	Target species	Amplicon size
Dambigua_F1	5'-GTCTACAACCGCGACACT-3'	<i>D. ambigua</i>	350 bp
Dambigua_R2	5'-GGAGGCCTGGGCGATAAATG-3'	<i>D. ambigua</i>	350 bp
Damp_F1	5'-GCGTCAGATTGCTAAYACCAACC-3'	<i>D. ampelina</i>	500 bp
Damp_R2	5'-CGATAACGCATTAGCAAGGTTTCG-3'	<i>D. ampelina</i>	500 bp
Dfoen_F1	5'-GTACGCACCTCCTATTCCTGTC-3'	<i>D. foeniculina</i>	500 bp
Dfoen_R1	5'-CTGTAGATGGTAACAACGTGGCT-3'	<i>D. foeniculina</i>	500 bp

Pathogenicity. The analysis of variance (ANOVA) for mean lesion lengths for the two pathogenicity experiments showed significant interaction; therefore the two experiments could not be combined. Individual isolates within the species performed differently in the two experiments, suggesting intraspecific variation in pathogenicity. In the first experiment, *D. ampelina* isolates (FH-Phom 206, FH-Phom 209, and FH-Phom 214) formed the longest

Fig. 2. *Diaporthe nebulae* (a) conidiomata sporulating on PDA, (b) longitudinal section through conidiomata, (c) section through coniomatal wall, (d, e, f, g) conidiophore and conidiogenous cells, (h) alpha conidia, (i) alpha and beta conidia, (j) front and back of 14-day-old culture on PDA and WA. Scale bars: a = 500 μ m; b-c = 100 μ m; d-i = 10 μ m.

lesions (58.6–60.6 mm), followed by one isolate (PMM 1678) of *D. nebulae* (44.8 mm) (Table 3). However, the lesions formed by *D. nebulae* isolate PMM 1678 was not significantly shorter than lesions formed by the *D. ampelina* isolates. Isolates of *D. cynaroidis* (FH-Phom 436, FH-Phom 437, and PMM 1658) formed the shortest lesions (4.8–15.7 mm), which were not significantly different from the negative control. All the other isolates, *D. novem* (FH-Phom 201, FH-Phom 232, FH-Phom 257), *D. serafiniae* (FH-Phom 242, FH-Phom 249, FH-Phom 255), and *D. nebulae* (FH-Phom 240, PMM 1681) formed moderate lesions (20.6–36.7 mm) that were not significantly different from each other, but significantly longer than the negative control, and significantly shorter than those formed by the *D. ampelina* isolates. All the species were successfully reisolated from the shoots and were identified as being the same as the ones inoculated. The average percentage of reisolation was 79% for *D. ampelina*, 93% for *D. novem*, 97% for *D. nebulae*, 91% for *D. serafiniae*, 71% for *D. cynaroidis*. *Diaporthe* species were also recovered from 2% of the samples receiving the PDA negative control treatment (Table 3).

In the second experiment, *D. ampelina* isolates (FH-Phom 206, FH-Phom 209, and FH-Phom 214) formed the longest lesions (48.8–65.6 mm). All *D. cynaroidis* isolates (FH-Phom 436, FH-Phom 437, and PMM 1658) and two *D. serafiniae* isolates (FH-Phom 242, FH-Phom 249) formed the shortest lesions (2.3–12.6 mm) that were not significantly different from the negative control. All the other isolates, *D. novem* (FH-Phom 201, FH-Phom 232, FH-Phom 257), *D. nebulae* (FH-Phom 240, PMM 1678, PMM 1681), and *D. serafiniae* (FH-Phom 255) formed moderate lesions (18.7–30.9 mm) which were not significantly different from one another, but significantly longer than the negative control and significantly shorter than those formed by the *D. ampelina* isolates. All the species were again successfully reisolated from the shoots and were identified as being the same as the ones inoculated in the second experiment. The average percentage of reisolation was 48% for *D. ampelina*, 48% for *D. nebulae*, 41% for *D. serafiniae*, 53% for *D. cynaroidis*, 57% for *D. novem*, and no *Diaporthe* species were isolated from the negative control (Table 3).

Designing of PCR-based species-specific primers. Species-specific primer sets were successfully designed for all three target species (*D. ambigua*, *D. ampelina*, and *D. foeniculina*). One primer pair per species was selected. These primer pairs amplified well at all temperatures tested. For *D. foeniculina* Dfoen_F2/ Dfoen_R1, for *D.*

Table 3. Results from two in vitro pathogenicity experiments conducted with selected isolates of *Diaporthe* species and controls on detached Bukettraube shoots

Isolates	Mean lesion lengths (mm) ^y		Reisolation (%) ^z	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
<i>D. ampelina</i> (FH-Phom 206)	57.9 a	65.6 a	70.0	65.0
<i>D. ampelina</i> (FH-Phom 209)	60.6 a	48.8 ab	67.5	20.0
<i>D. ampelina</i> (FH-Phom 214)	58.6 a	56.4 ab	100.0	60.0
<i>D. nebulae</i> (FH-Phom 1678)	44.8 ab	19.8 cde	100.0	82.5
<i>D. nebulae</i> (FH-Phom 1681)	36.7 bc	30.9 c	90.0	27.5
<i>D. nebulae</i> (FH-Phom 240)	23.7 cde	19.6 cde	100.0	35.0
<i>D. novem</i> (FH-Phom 201)	31.6 bcd	31.5 c	92.5	52.5
<i>D. novem</i> (FH-Phom 232)	26.9 cde	32.6 c	90.0	50.0
<i>D. novem</i> (FH-Phom 257)	30.6 bcd	24.0 cd	95.0	67.5
<i>D. serafiniae</i> (FH-Phom 255)	24.1 cde	18.7 cde	90.0	35.0
<i>D. serafiniae</i> (FH-Phom 249)	21.4 cde	12.6 def	92.5	42.5
<i>D. serafiniae</i> (FH-Phom 242)	20.6 cdef	5.3 ef	90.0	45.5
<i>D. cynaroidis</i> (PMM 1658)	15.7 defg	9.5 def	65.0	62.5
<i>D. cynaroidis</i> (FH-Phom 436)	12.7 efg	9.8 def	100.0	62.5
<i>D. cynaroidis</i> (FH-Phom 437)	4.8 fg	2.3 f	47.5	32.5
PDA 1	0.0 g	0.0 f	0.0	0.0
PDA 2	0.0 g	0.0 f	6.7	0.0
PDA 3	0.0 g	0.0 f	0.0	0.0

^y Within each experiment, mean lesion lengths followed by the same letter were not significantly different. (LSD_{exp1} = 16.23, P_{exp1} < 0.05), and (LSD_{exp2} = 15.3, P_{exp2} < 0.05).

^z Reisolation percentage was determined as the number of shoots from which the inoculated fungus was reisolated after a 5-week inoculation period.

ampelina Damp_F1/ Damp_R2, and for *D. ambigua* Dambigua_F1/ Dambigua_R2 were selected. An annealing temperature of 63°C was chosen for all three selected primer combinations, as good amplification of the target species was observed at this temperature. Amplification was not seen in any of the nontarget *Diaporthe* species (Fig. 3) or fungi (results not shown). Sequence comparisons of the species-specific primers with available sequences of *D. hispaniae* (CBS 143351-CPC 30321) and *D. hungariae* (CBS 143353-CPC 30130) that are closely related to the target species *D. ampelina* indicates that the Damp_R2 primer does not match these species. Unfortunately, these GenBank accessions are not long enough to include the potential binding site for Damp_F1, so the specificity of this primer could not be confirmed. Unfortunately, the species-specific primers developed for *D. foeniculina* (Dfoen_F1 and Dfoen_R1) perfectly match the corresponding *tub2* region of *D. baccae* (CBS 136972), which was described after the optimization of this assay.

Discussion

The primary aim of this study was to assess the status of *Diaporthe* species as dieback pathogens on grapevines in the Western Cape, South Africa. Previous *Diaporthe* species identified on South African vineyards were: *D. ambigua*, *D. ampelina*, *D. amygdali*, *D. australafricana*, *D. helianthi*, *D. kyushuensis*, *D. rudis*, and eight other isolates that were not identified to species level. *D. ampelina* and *D. amygdali* were found to be the most virulent (Mostert et al. 2001; Van Niekerk et al. 2005; White et al. 2011a). In the present study, *Diaporthe* isolates were obtained from dormant rootstock grafting material, dormant grafted nursery vines, and dying or dead spurs. A three gene (ITS, *tub2*, and EF1- α) phylogenetic analysis revealed nine species of *Diaporthe* among these isolates: *D. ampelina*, *D. ambigua*, *D. cynaroidis*, *D. eres*, *D. foeniculina*, *D. novem*, *D. serafinae*, *Phomopsis* sp. 5 (Van Niekerk et al. 2005), and a new species here described as *D. nebulae* that is related to *D. anacardii*. Of these, *D. serafinae* and *D. cynaroidis* have not previously been reported from grapevines globally, and *D. novem* has not been reported from this host in South Africa. These first reports add to our knowledge of *Diaporthe* species on grapevines in South Africa.

The present study has also attempted to resolve the identity of isolates previously identified by Van Niekerk et al. (2005). Based on the phylogeny and morphological features, *Phomopsis* sp. 2 (STE-U 2674) grouped strongly with *D. oncostoma* (CBS 589.78 and CBS 100456). The published morphological description of *D. oncostoma* (Gomes et al. 2013) is similar to that reported for *Phomopsis* sp. 2 by Mostert et al. (2001), confirming our phylogenetic identification of *Phomopsis* sp. 2 as *D. oncostoma*. Unfortunately, *Phomopsis* sp. 3 and 8 are only represented by ITS sequence data in the combined phylogeny. More strains and/or sequence data are needed to better resolve these species' identity. *Phomopsis* sp. 5 and *Phomopsis* sp. 7 occupied unique phylogenetic positions in the present study and could not be identified as currently known species. *Phomopsis* sp. 7 was closely related to, but distinct from, *D. anacardii*.

In our phylogeny, two species known from South African grapevines clustered in the *Diaporthe eres* species complex (Gomes et al. 2013; Lawrence et al. 2015; Udayanga et al. 2014b). Udayanga et al. (2014b) considered this species complex to be redundant and noted that the poorly supported nonmonophyletic grouping could be observed when ITS sequences are included in the combined analysis. They used seven gene areas to resolve the *D. eres* species complex and found CBS 587.79 and CBS113470, together with other isolates to cluster with *D. eres* when ITS is not included. Udayanga et al. (2014b) synonymized several species with *D. eres*. Technically, this clade should be referred to as *D. eres*, but it is not congruent with our phylogeny, e.g., Udayanga et al. (2014b) excludes *D. vaccinii* from *D. eres*, but includes *D. cf. nobilis* strains CBS 587.79 and CBS 113470, while in our phylogeny this concept of *D. eres* is polyphyletic. Our phylogeny concurs more with Lawrence et al. (2015), but this is to be expected in light of our use of the ITS region, and also the lack of CAL and HIS regions. Furthermore, neither Lawrence et al. (2015) nor Udayanga et al. (2014b) included the full set of *D. cf. nobilis* isolates as treated by Gomes et al. (2013) and as

included here. The mean average of alpha conidia of PMM 1690 were (6–)7–9(–10) $\times$ 2–2.5(–3) μ m, which is very similar to those of the isolate *Phomopsis* sp. 6, (6–)7–9(–10) $\times$ 2–2.5 μ m, and *D. eres* (6–)6.5–8.5(–9) $\times$ 3–4 μ m (Van Niekerk et al. 2005; Udayanga et al. 2014b). The fact that PMM 1690 and *Phomopsis* sp. 6 (STE-U 5135) show high phylogenetic and morphological similarity to the ex-type strain of *D. eres* (AR5193 = CBS 1385594) confirms the identity of these strains as *D. eres*. *Phomopsis* sp. 4, however, clustered with *D. eres* strain AR5196 in one of two clades containing *D. cf. nobilis* isolates. Clearly, the taxonomy of the *D. eres/nobilis* clade needs further resolution before the species identity of *Phomopsis* sp. 4 can be elucidated.

The in vitro pathogenicity trials tested the ability of mycelium of selected *Diaporthe* species to infect wounded detached shoots of the cultivar Bukettraube and cause lesions. The interaction between the two experiments was most probably due to *D. serafinae* isolates which caused larger lesions in the first experiment and were considered to be possible pathogens, whereas in the second experiment caused lesions that were not significantly different from the negative control and were considered as weak pathogens. The difference observed may also be due to differences in the lignin content of each individual shoot (Mutawila 2014), since lignin is involved in host resistance against colonization. Our pathogenicity trials suggest that *D. cynaroidis* is a nonpathogen of grapevines, but that *D. novem*, *D. nebulae*, and some isolates of *D. serafinae* are pathogenic to this host. Lawrence et al. (2015) also found *D. novem* to be a pathogen of grapevines, *D. cynaroidis* is a pathogen of protea (Marincowitz et al. 2008), but our study was the first to test for pathogenicity on grapevine. Thompson et al. (2015) found *D. serafinae* on sunflower but did not conduct pathogenicity studies on this host or any other host.

Fig. 3. Species-specific PCR amplification of (A) *Diaporthe ambigua*, (B) *D. foeniculina*, and (C) *D. ampelina* isolates. Lanes 1–2 contained product from the target organism. Lanes 3 and 18 were left open. Lanes 4–17 PCR amplification of all *Diaporthe* spp. identified during surveys of Western Cape vineyards and other alternative hosts planted close to vineyards: (4) *D. novem*, (5) *D. serafinae*, (6) *D. cynaroidis*, (7) *D. eres*, (8) *D. ambigua*, (9) *D. infecunda*, (10) *D. australafricana*, (11) *D. nebulae*, (12) *D. woodii*, (13) *Phomopsis* sp. 7, (14) *Phomopsis* sp. 4, (15) *Phomopsis* sp. 8. Lanes 16 and 17 were the other two of the three species for which species-specific primers were designed. Lane 19 no template control.

Studies have concluded that *Diaporthe* strains can only be identified to species level with the application of molecular techniques (Gomes et al. 2013; Guarnaccia and Crous 2017; Guarnaccia et al. 2018; Santos et al. 2017; Udayanga et al. 2014a, b). In the present study, a PCR-based assay was developed for the rapid and accurate identification of *D. foeniculina*, *D. ampelina*, and *D. ambigua*. However, these assays were not tested against some of their close relatives, i.e., *D. baccae*, *D. chamaeropsis*, and *D. cytosporella* that are closely related to *D. foeniculina*; *D. longispora* that is closely related to *D. ambigua*; and *D. fibrosa*, *D. hispaniae*, and *D. hungariae* that are closely related to *D. ampelina*, because no material was available for such species. With the exception of *D. baccae*, data for these species show mismatches in the primer binding sites, which makes non-specific amplification less likely. Unfortunately, the primer binding sites for the *D. foeniculina* primers perfectly match the corresponding region of *D. baccae*, suggesting that this species would also give a positive result. However, *D. baccae* was recently reported as a grapevine pathogen in Europe (Guarnaccia et al. 2018), suggesting that such a false positive would still be an indication of a grapevine pathogen. *D. baccae* has not been reported in South Africa. This is the first report of species-specific primers for *D. ampelina*, *D. foeniculina*, and *D. ambigua*.

This study identified nine *Diaporthe* species on grapevines in South Africa and clarified the taxonomic status of *Phomopsis* sp. 2 (Van Niekerk et al. 2005). This brings the total number of *Diaporthe* species known on this host in South Africa to 19. The focus of this survey (and that of Van Niekerk et al. 2005) was, however, on *Diaporthe* associated with grapevine wood, i.e., these species are more likely to be involved in Phomopsis dieback. To date, the only *Diaporthe* species affecting green shoots and leaves and causing Phomopsis cane and leaf spot in South Africa is *D. ampelina* (Mostert et al. 2001; Van Niekerk et al. 2005, 2011). Surveys of the species involved in Phomopsis cane and leaf spot are required to verify the *Diaporthe* species associated with this disease and provide a more comprehensive picture of the diversity of *Diaporthe* spp. on grapevines in South Africa.

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Literature Cited

- Baumgartner, K., Fujiyoshi, F., Travadon, R., Castlebury, L. A., Wilcox, W. F., and Rolshausen, P. E. 2013. Characterization of species of *Diaporthe* from wood cankers of grape in Eastern North American vineyards. *Plant Dis.* 97: 912-920.
- Berrysmith, F. 1962. "Dead arm" disease of grapevines. *N.Z. J. Agric.* 105:309-313.
- Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for the speciation studies in filamentous ascomycetes. *Mycologia* 91:553-556.
- 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.
- Darriba, D., Taboada, G. L., Doalla, R., and Posada, D. 2012. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* 9:772.
- Díaz, G. A., Latorre, B. A., Lolas, M., Ferrada, E., Naranjo, P., and Zoffoli, J. P. 2017. Identification and characterization of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* causing a postharvest fruit rot in kiwifruit. *Plant Dis.* 101:1402-1410.
- Dissanayake, A. J., Liu, M., Zhang, W., Chen, Z., Udayanga, D., Chukeatirote, E., Li, X., Yan, J., and Hyde, K. D. 2015. Morphological and molecular characterisation of *Diaporthe* species associated with grapevine trunk disease in China. *Fungal Biol.* 119:283-294.
- Du Plessis, S. J. 1938. The dead-arm disease in vine. *Farming S. Afr.* 2:79-83.
- Farr, D. F., Castlebury, L. A., and Rossman, A. Y. 2017. Morphological and molecular characterisation of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in eastern United States. *Mycologia* 94:494-504.
- Fourie, P. H., and Halleen, F. 2004a. Occurrence of grapevine trunk disease pathogens in rootstock mother plants in South Africa. *Australas. Plant Pathol.* 33:313-315.
- Fourie, P. H., and Halleen, F. 2004b. Proactive control of petri disease of grapevine through treatment of propagation material. *Plant Dis.* 88:1241-1245.
- Girish, K., Bhat, S., and Raveesha, K. A. 2009. PCR-based detection of *Phomopsis azadirachtae* in die-back affected neem seeds. *Arch. Phytopathol. Plant Prot.* 42:626-632.
- 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.
- Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: A genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia* 31:1-41.
- Guarnaccia, V., and Crous, P. W. 2017. Emerging citrus diseases in Europe caused by species of *Diaporthe*. *IMA Fungus* 8:317-334.
- Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., Ezra, D., Fontaine, F., Gramaje, D., Gutierrez-Aguirregabiria, A., Kaliterna, J., Kiss, L., Luque, J., Mugnai, L., Naor, V., Raposo, R., Sandor, E., Vaczy, K. Z., and Crous, P. W. 2018. *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. *Persoonia* 40:135-153.
- Guarnaccia, V., Vitale, A., Cirvilleri, G., Alello, D., Susca, A., Epifani, F., Perrone, G., and Polizzi, G. 2016. Characterisation and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. *Eur. J. Plant Pathol.* 146:963-976.
- Guindon, S., Du-fayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst. Biol.* 59:307-321.
- Halleen, F., Crous, P. W., and Petrini, O. 2003. Fungi associated with healthy grapevine cutting in nurseries, with special reference to pathogens involved in the decline of young vines. *Australas. Plant Pathol.* 32:47-52.
- Hawksworth, D. L. 2011. A new dawn for the naming of fungi: Impacts of decisions made in Melbourne in July 2011 on the future publication and regulation of fungal names. *MycKeys* 1:7-20.
- Hawksworth, D. L., Crous, P. W., Redhead, S. A., et al. 2011. The Amsterdam Declaration on fungal nomenclature. *IMA Fungus* 2:105-112.
- Katoh, K., Asimenos, G., and Toh, H. 2009. Multiple alignment of DNA sequences with MAFFT. *Methods Mol. Biol.* 537:39-64.
- 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.
- Lartillot, N., Rodrigue, N., Stubbs, D., and Richer, J. 2013. PhyloBayes MPI: Phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. *Syst. Biol.* 62:611-615.
- Lawrence, P. D., Travadon, R., and Baumgartner, K. 2015. Diversity of *Diaporthe* species associated with wood cankers of fruit and nut crops in northern California. *Mycologia* 107:926-940.
- Marincowitz, S., Crous, P. W., Groenewald, J. Z., and Wingfield, M. J. 2008. Microfungi occurring on *Proteaceae* in the fynbos. *CBS Fungal Biodiversity Series* 7:1-166.
- Moller, W. J., and Kasimatis, A. N. 1981. Further evidence that *Eutypa armeniaca* – not *Phomopsis viticola* – incites dead arm symptoms on grape. *Plant Dis.* 65:429-431.
- Mostert, L., Crous, P. W., Kang, J., and Phillips, A. J. L. 2001. Species of *Phomopsis* and a *Liberella* sp. occurring on grapevines with specific reference to South Africa: Morphological, cultural, molecular and pathological characterization. *Mycologia* 93:146-167.
- 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.
- Mutawila, C. 2014. Improving pruning wound protection against grapevine trunk disease pathogens. Ph.D. thesis. University of Stellenbosch, Stellenbosch, South Africa.
- O'Donnell, K., and Cigelnik, E. 1997. Two different intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- Pearson, R. C., and Goheen, C. 1994. Phomopsis cane and leaf spot. Pages 17-18 in: *Compendium of Grape Diseases*. W. B. Hewitt and R. C. Pearson, eds. American Phytopathological Society, St. Paul, MN, USA.
- Prasad, M. N. N., Bhat, S. S., Raj, A. P. C., and Janardhana, G. R. 2006. Molecular detection of *Phomopsis azadirachtae*, the causative agent of dieback disease of neem by polymerase chain reaction. *Curr. Sci. India* 91:158-159.
- Pscheidt, J. W., and Pearson, R. C. 1989. Effect of grapevine training systems and pruning practices on occurrence of Phomopsis cane and leaf spot. *Plant Dis.* 73: 825-828.
- Rehner, S. A., and Uecker, F. A. 1994. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis*. *Can. J. Bot.* 72:1666-1674.
- Rossman, A. Y., Adams, G. C., Cannon, P. F., Castlebury, L. A., Crous, P. W., Gryzenhout, M., Jaklitsch, W. M., Mejia, L. C., Stoykov, D., Udayanga, D., Voglmayr, H., and Walker, D. M. 2015. Recommendations of generic names in Diaporthales competing for protection or use. *IMA Fungus* 6:145-154.
- Santos, J. M., Correia, V. G., and Phillips, A. J. L. 2010. Primers for mating-type diagnosis in *Diaporthe* and *Phomopsis*: Their use in teleomorph induction in vitro and biological species definition. *Fungal Biol.* 114:255-270.

- Santos, J. M., and Phillips, A. J. L. 2009. Resolving the complex of *Diaporthe* (*Phomopsis*) species occurring on *Foeniculum vulgare* in Portugal. *Fungal Divers.* 34:111-125.
- Santos, L., Alves, A., and Alves, R. 2017. Evaluating multi-locus phylogenies for species boundaries determination in the genus *Diaporthe*. *PeerJ* 5:e3120.
- Thompson, S. M., Tan, Y. P., Shivas, R. G., Neate, S. M., Morin, L., Bissett, A., and Aitken, E. A. B. 2015. Green and brown bridges between weeds and crops reveal novel *Diaporthe* species in Australia. *Persoonia* 35:39-49.
- Thompson, S. M., Tan, Y. P., Young, A. J., Neate, S. M., Aitken, E. A. B., and Shivas, R. G. 2011. Stem cankers on sunflower (*Helianthus annuus*) in Australia reveal a complex of pathogenic *Diaporthe* (*Phomopsis*) species. *Persoonia* 27:80-89.
- Udayanga, D., Castlebury, L. A., and Rossman, A. Y. 2014a. Species limits in *Diaporthe*: Molecular re-assessment of *D. citri*, *D. cytospora*, *D. foeniculina* and *D. rudis*. *Persoonia* 32:83-101.
- Udayanga, D., Castlebury, L. A., Rossman, A. Y., Chukeatirote, E., and Hyde, K. D. 2014b. Insights into the genus *Diaporthe*: Phylogenetic species delimitation in the *D. eres* species complex. *Fungal Divers.* 67:203-229.
- Udayanga, D., Liu, X., Crous, P. W., McKenzie, E. H. C., Chukeatirote, E., and Hyde, K. D. 2012. A multi-locus phylogenetic evaluation of *Diaporthe* (*Phomopsis*). *Fungal Divers.* 56:157-171.
- Udayanga, D., Liu, X., McKenzie, E. H. C., Chukeatirote, E., Bahkali, A. H. A., and Hyde, K. D. 2011. The genus *Phomopsis*: Biology, applications, species concepts and names of common phytopathogens. *Fungal Divers.* 50:189-225.
- Uecker, F. A. 1988. A world list of *Phomopsis* names with notes on nomenclature, morphology and biology. *Mycol. Memoir* 13:1-231.
- Urbez-Torres, J. R., Peduto, F., Smith, R. J., and Gubler, W. D. 2013. *Phomopsis* dieback: A grapevine trunk disease caused by *Phomopsis viticola* in California. *Plant Dis.* 97:1571-1579.
- Van Niekerk, J. M., Groenewald, J. Z., Farr, D. F., Fourie, P. H., Halleen, F., and Crous, P. W. 2005. Reassessment of *Phomopsis* species on grapevines. *Australas. Plant Pathol.* 34:27-39.
- Van Niekerk, J. M., Halleen, F., and Fourie, P. H. 2011. Temporal susceptibility of grapevine pruning wounds to trunk pathogen infection in South African grapevines. *Phytopathol. Mediterr.* 50 (Suppl.): 139-150.
- Vedashree, S., Sateesh, K. M., Chowdappa, P., and Nirmalkumar, B. J. 2015. Species-specific PCR-based assay for identification and detection of *Phomopsis* (*Diaporthe*) *azadirachtae* causing die-back disease in *Azadirachta indica*. *J. Phytopathol.* 163:818-828.
- White, C. L., Halleen, F., Fischer, M., and Mostert, L. 2011b. Characterisation of fungi associated with esca diseased grapevines in South Africa. *Phytopathol. Mediterr.* 50:204-223.
- White, C. L., Halleen, F., and Mostert, L. 2011a. Symptoms and fungi associated with esca in South African vineyards. *Phytopathol. Mediterr.* 50:S236-S246.
- 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, and J. J. Sninsky, eds. Academic Press Inc., New York.
- Wilcox, W. A., Ellis, M. A., Rawnsley, B., Rossman, A., and Pscheidt, J. 2015. *Phomopsis* cane and leaf spot. Pages 68-71 in: *Compendium of Grape Diseases, Disorders, and Pests*, 2nd ed. W. F. Wilcox, W. D. Gubler, and J. K. Uyemoto, eds. American Phytopathological Society, St. Paul, MN, USA.
- Zhang, A. W., Hartman, G. L., Riccioni, L., Chen, W. D., Ma, R. Z., and Pedersen, W. L. 1997. Using PCR to distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from other soybean fungal pathogens and to detect them in soybean tissues. *Plant Dis.* 81:1143-1149.