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Diversity, phylogeny and pathogenicity of Botryosphaeriaceae on non-native *Eucalyptus* grown in an urban environment: A case study

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ABSTRACT

The Botryosphaeriaceae are opportunistic pathogens mostly of woody plants, including *Eucalyptus*. These fungi can cause cankers and die-back diseases on non-native *Eucalyptus* trees in South African plantations. Botryosphaeriaceae were isolated from diseased and asymptomatic twigs and leaves from 20 *Eucalyptus* spp. grown in a Pretoria, South Africa arboretum and its surroundings. The isolates were initially grouped based on conidial morphology and Internal Transcribed Spacer (ITS) rDNA PCR-RFLP profiles. They were further identified using DNA sequence data for the ITS rDNA and translation elongation factor 1-α (TEF-1α) gene regions and tested for pathogenicity. Five species were identified including *Botryosphaeria dothidea* and four *Neofusicoccum* species namely *Neofusicoccum parvum*; *N. cryptoaustrale* and *N. ursorum* that were recently described from plant tissues collected as a part of the current study; and *Neofusicoccum eucalypti* (Winter) Maleme, Pavlic & Slippers comb. nov. The latter species is recorded for the first time on *Eucalyptus* in South Africa. Most of the identified species were collected from the leaves of 17 different *Eucalyptus* spp. *Neofusicoccum parvum* was most commonly isolated (72% of all isolates) followed by *B. dothidea* species complex (17%). With exception of *N. parvum* which was isolated from majority of *Eucalyptus* spp. the other species were isolated from limited number of *Eucalyptus* species indicating host-preferences. All the isolated Botryosphaeriaceae species produced lesions on inoculated *Eucalyptus grandis* plants that were significantly larger than those associated with the controls.

1. Introduction

The Botryosphaeriaceae (Botryosphaeriales, Dothideomycetes) are among the most common fungi associated with diseases of trees and shrubs in both native and non-native environments worldwide (Slippers and Wingfield, 2007; Slippers et al., 2009). These fungi are typically associated with symptoms such as branch and stem cankers, die-back as well as leaf and tip blights. Species of Botryosphaeriaceae commonly exist in asymptomatic plant tissues as endophytes or latent pathogens, causing disease symptoms at the onset of stressful environmental conditions (Slippers and Wingfield, 2007; Mehl et al., 2013). Their cryptic nature as endophytes combined with increasing occurrences of extreme weather conditions due to climate change makes these fungi threatening to economically and environmentally important woody plants globally (Desprez-Loustau et al., 2006).

The taxonomy of the Botryosphaeriaceae has been confused in the past. Identification was commonly achieved based on morphological characteristics or the host plants on which species were found. The many overlapping morphological characteristics among different species of the Botryosphaeriaceae and the fact that some morphological features change with age has also resulted in a substantially misleading taxonomy for these fungi. Recent taxonomic studies, combining morphological characters and multigene phylogenies, have led to extensive revisions of the taxonomy of the Botryosphaeriaceae (Crous et al., 2006; Liu et al., 2012; Phillips et al., 2013; Slippers et al., 2013). Based on these analyses, the identities of Botryosphaeriaceae species known from culture has been revised, and they have been placed in 17 genera currently recognised in this family (Phillips et al., 2013; Slippers et al., 2013).

Eucalyptus trees have been planted as non-natives in many parts of the world, including South Africa. It has been previously suggested that the global movement of these trees has also resulted in the introduction of pathogens into new areas via planting stock or seed (Wingfield et al., 2001, 2015). In this regard, species of Botryosphaeriaceae have been

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found on the seeds of *Eucalyptus* and other tree species (Lupo et al., 2001; Gure et al., 2005). Their association with seeds and their presence in asymptomatic plant tissues provides evidence that species of Botryosphaeriaceae can be expected to be easily moved unnoticed into new areas together with *Eucalyptus* (Slippers and Wingfield, 2007; Slippers et al., 2009).

Species of Botryosphaeriaceae have wide host ranges and they can move between native and introduced tree species (Slippers and Wingfield, 2007; Sakalidis et al., 2011). For example, no restrictions to gene flow between non-native *Eucalyptus globulus* plantations and native eucalypt forests in Western Australia could be found in the canker pathogen *Neofusicoccum australe* (Burgess et al., 2006). Similarly, all species of Botryosphaeriaceae identified from the native *Syzygium cordatum* in South Africa, were found to be more pathogenic on *Eucalyptus*, with a several of these species overlapping in occurrence between the two hosts (Pavlic et al., 2007). Consequently, *Eucalyptus* can be expected to acquire new species of Botryosphaeriaceae from the surrounding trees in a new area, and to provide a source of species to native plant communities.

An arboretum of 20 different *Eucalyptus* spp. has been established in Pretoria, South Africa, in 2001, to provide a food-source for Koala Bears at the nearby National Zoological Gardens of South Africa (www.nzg.co.za). Canker and die-back symptoms were observed on these trees and an attempt was made to identify and characterize species of Botryosphaeriaceae on these trees, as well as on apparently healthy *E. camaldulensis* trees growing near the arboretum. This was achieved using (ITS) rDNA PCR-RFLP profiles, DNA sequence data for the ITS rDNA and translation elongation factor 1-α (TEF-1α) gene regions of cultures isolated from these trees. Inoculations were also conducted to consider the pathogenicity of the identified species.

2. Materials and methods

2.1. Isolates

Isolations were made from 20 *Eucalyptus* spp. in the Pretoria Zoological Garden (www.nzg.co.za) arboretum in Pretoria, South Africa (Fig. 1a), as well as from surrounding eucalypt trees planted as ornamentals in the area (Table 1). The arboretum consisted of 12 blocks, each of them having 20 rows (each row representing one *Eucalyptus* species) of 11 trees. Three trees (Tree 1, 5 and 10 of each row) were sampled from three of the blocks (block 1, 6 and 7), thus having in total 9 trees sampled per each *Eucalyptus* sp. In addition, twenty-five *Eucalyptus camaldulensis* trees surrounding the arboretum were sampled. Twig die-back on terminal leader shoots (Fig. 1a) and main stem cankers, identified as cracks in the bark exuding kino (Fig. 1b, c), were observed on approximately 10% of these trees. Cankers were spread widely on the trunks of some trees that appeared reddish in colour due to the extensive production of kino, indicating variation in susceptibility between *Eucalyptus* spp. (Fig. 1b). The trees were sampled during March and April 2005. Isolations were made from diseased and asymptomatic (visually healthy) twigs, and from asymptomatic leaves collected from 205 trees, using the protocol described by Pavlic et al. (2004). All the resulting cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.2. Morphological characteristics

Isolates were induced to sporulate on sterilized pine needles (Smith et al., 2001) placed on 2% water agar (WA) (Agar; Biolab, South Africa) and incubated at 25 °C under near-UV light. Pycnidia formed on pine needles after two to three weeks of incubation. Masses of conidia oozing from the pycnidia were mounted in 85% lactic acid on microscopic slides and examined using a light microscope. Images were captured using an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd., Munich, Germany). Conidia (20–50) and 50 conidiogenous cells were measured for each isolate. Colony morphology and colour were noted for cultures grown on 2% malt extract agar (MEA) (Biolab, South Africa) at 25 °C and culture colours were defined using the colour charts of Rayner (1970). Growth studies were conducted for selected isolates at temperatures ranging from 10 to 35 °C at 5 °C intervals in the dark. Two dishes were prepared for each isolate and two measurements of colony diameter perpendicular to each other were made daily until growth reached the edges of the 90 mm plates.

2.3. DNA extraction and PCR amplification

Single conidial or single hyphal tip cultures were grown on 2% MEA at 25 °C for 7 days. The mycelium was scraped directly from the medium and transferred to Eppendorf tubes (1.5 ml) and 300 µl of an extraction buffer (200 mM Tris-HCl pH 8.5, 150 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. A modified phenol:chloroform method for DNA extraction was followed (Raeder and Broda, 1985). The resulting DNA pellets were re-suspended in 30 µl sterile SABAX water. RNAse (1 mg μl^{-1}) was added to DNA suspensions and left overnight at the room temperature for RNA degradation. DNA electrophoresis was performed on a 1.5% agarose gel, stained with ethidium bromide. Bands were visualised under ultra-violet light. DNA concentration was estimated against a λ standard size marker.

The ITS region was amplified using primers ITS 1 and ITS 4 (White et al., 1990) and a portion of the TEF-1α gene region, was amplified with primer set EF-AF and EF-BR (Sakalidis et al., 2011). The reaction mixture contained 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California), 10× PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM dNTPs and 10 mM of each primer. The reaction mixture was made up to the final volume of 25 µl with sterile water. The following PCR program was used: denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing temperature at 55 °C for 45s, elongation at 72 °C for 1½ min and a final elongation step at 72 °C for 5 min. The PCR amplicons were viewed on a 1% agarose gel, stained with ethidium bromide under UV-light. To estimate the band sizes, a 100 bp marker XIV (Roche Molecular Biochemicals, Almeda, California) was used.

2.4. PCR-RFLP analysis

A PCR-RFLP technique was used on the ITS amplicons to group all the isolates resembling Botryosphaeriaceae based on culture morphology. ITS rDNA amplicons were digested with the restriction enzymes (RE) *Hha*I that recognises the same sequences as *Cfo*I that had been previously used to distinguish species of the Botryosphaeriaceae (Slippers et al., 2007). The PCR-RFLP reaction mixture for each reaction consisted of 20 µl PCR product, 0.3 µl RE *Hha*I and 2.5 µl of the matching enzyme buffer (Fermentas, South Africa). The reaction mixture was incubated at 37 °C overnight. Digested fragments were separated on a 3% agarose gel run at a low voltage (60 V) for 1 h.

2.5. DNA sequencing and phylogenetic analysis

Representative isolates from each of three groups identified based on PCR-RFLP analyses were sequenced using the primers that were used for the PCR amplification. The sequences were compared to those of known Botryosphaeriaceae obtained from GenBank, with a focus on those previously isolated from *Eucalyptus* (Table 1). Sequencing of the purified products was carried out by using ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer). Nucleotide sequences were analysed and edited using SEQUENCE NAVIGATOR version 1.0.1. (Perkin- Elmer Applied Bio-Systems, Foster City, CA) software. Online software, MAFFT version 7 under E-INS-i algorithm was used for alignments (Katoh and Standley, 2013). Maximum likelihood analyses using 10,000 rapid bootstrap inferences (command f–a) under the

Fig. 1. The Pretoria arboretum that includes 20 *Eucalyptus* species. Twig die-back on terminal leader shoot is indicated by arrows (a). Main stem cankers spread widely on the trunks of a tree that appeared reddish in colour due to the extensive production of kino (b). Cankers are identified as cracks in the bark exuding kino (c).

GTRGAMMA model were run in RAxML version 8.1.20 (Stamatakis, 2014) for each dataset. Trees were displayed using FigTree version 1.3.1 (A. Rambaut: http://tree.bio.ed.ac.uk/software/figtree).

2.6. Pathogenicity

Thirteen isolates representing the five species of the Botryosphaeriaceae identified in this study were used in pathogenicity tests in a greenhouse (Table 1). The selected isolates represented two or three of the fastest growing isolates. The isolates selected for pathogenicity tests were grown on MEA at 25 °C under continuous near-fluorescent light for seven days prior to inoculation. Two-year-old *Eucalyptus grandis* clone (ZG-14) trees were maintained in a greenhouse for approximately three weeks prior to inoculations to allow them to acclimatize. The greenhouse was exposed to natural day and night conditions and maintained at a constant temperature of approximately 25 °C. Each of the selected 13 isolates was inoculated onto the stems of ten trees. For controls, 30 trees were inoculated with sterile MEA plugs. For inoculations, wounds were made on the stems of trees approximately 250 mm above the soil level using 8 mm diameter cork borer. Plugs were prepared from seven-day-old cultures using the same size cork borer. The plugs were placed into wounds with the mycelium facing the exposed cambium and sealed with laboratory film (Parafilm "M", Pechiney Plastic Packaging, Chicago, U.S.A) to prevent desiccation and contamination. Lesion lengths were measured six weeks after inoculation. Re-isolation of the fungi from resulting lesions was done by cutting small pieces of the wood from the edge of lesions and plating these on 2% MEA at 25 °C. The entire trial was repeated once to verify the pathogenicity of all isolates under the same conditions. Lesion lengths that developed six weeks after inoculation were used as a measure of the pathogenicity. SAS® version 8.2 (2001) was used for statistical analysis of the data.

3. Results

3.1. Morphological characteristics

Ninety-two isolates were obtained from *Eucalyptus* spp. considered in this study. Forty-four of the 92 isolates produced asexual fruiting structures on WA overlaid with sterilized pine needles. No sexual structures were observed in any of the cultures. Two groups were identified based on conidial morphology and length to width (L/W) ratios. The first group of isolates (76 in total) had hyaline conidia that were aseptate, smooth with granular contents, fusiform to ellipsoid with apices rounded, occasionally (some of them) becoming brown and 1–2-septate with age, with a L/W ratio < 4.0 (2.8–3.9). The isolates in the second group (16 in total) had hyaline, aseptate, narrowly to irregularly fusiform conidia, with a L/W ratio ≥ 4.0. Based on these characteristics these two groups can be linked to two genera in Botryosphaeriaceae, namely *Neofusicoccum* (group I), and *Botryosphaeria* (group II).

3.2. PCR-RFLP analysis

All 92 isolates were used for PCR-RFLP analyses. Three profiles (restriction length polymorphism fingerprints) were observed after digesting ITS rDNA PCR product with RE *Hha*I, indicating three groups for all the isolates. The seventy-six isolates from morphological group I were linked to two PCR-RFLP profiles with 66 having profile A and 10 profile B. The sixteen isolates from the morphological group II were all linked to the profile C.

3.3. DNA sequencing and analysis

Twenty-nine representative isolates from all three groups identified based on the PCR-RFLP fingerprints (profile A = 13, profile B = 10,

Table 1
Isolates representing species of the Botryosphaeriaceae considered in a phylogenetic study and pathogenicity trial.

Species	Isolate ID[a,b]	Other ID[a,b]	Host	Country	Collector/isolator	ITS	EF
Neofusicoccum parvum	*ATCC58191*	*CMW 9081*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236888
	CMW 10122		*Eucalyptus grandis*	South Africa	H. Smith	AF283681	AY236882
	CPC22757		*E. obliqua*	Thailand	T. Trakunyingcharoen	KM006435	KM006466
	CMW 23792[c]		*E. dorrigoensis*	South Africa	H.M. Maleme	FJ752736	FJ752702
	CMW 20736[c]		*E. robusta*	South Africa	H.M. Maleme	FJ752730	FJ752704
	CMW 20727		*E. microcorys*	South Africa	H.M. Maleme	FJ752735	/
	CMW 20719		*E. ovata*	South Africa	H.M. Maleme	FJ752724	/
	CMW 20724		*E. saligna*	South Africa	H.M. Maleme	FJ752726	/
	CMW 20722		*E. microcorys*	South Africa	H.M. Maleme	FJ752727	/
	CMW 20720		*E. saligna*	South Africa	H.M. Maleme	FJ752728	FJ752703
	CMW 20726		*E. robusta*	South Africa	H.M. Maleme	FJ752729	/
	CMW 20735[c]		*E. nicholii*	South Africa	H.M. Maleme	FJ752733	/
	CMW 20725		*E. scorparia*	South Africa	H.M. Maleme	FJ752725	/
	CMW 20730		*E. tereticornis*	South Africa	H.M. Maleme	FJ752731	/
	CMW 20733		*E. tereticornis*	South Africa	H.M. Maleme	FJ752734	/
	CMW 20734		*E. tereticornis*	South Africa	H.M. Maleme	FJ752732	/
N. cordaticola	*CBS 123634*	*CMW 13992*	*Syzygium cordatum*	South Africa	D. Pavlic	EU821898	EU821868
	CBS 123635	CMW 14056	*S. cordatum*	South Africa	D. Pavlic	EU821903	EU821873
N. brasiliense	*CMM1338*		*Mangifera indica*	Brasil	M.W. Marques	JX513630	JX513610
	CMM1285		*M. indica*	Brasil	M.W. Marques	JX513628	JX513608
N. batangarum	*CBS 124924*	*CMW 28363*	*Terminalia catappa*	Cameroon	D. Begoude, J. Roux	FJ900607	FJ900653
	CBS 124923	CMW 28320	*T. catappa*	Cameroon	D. Begoude, J. Roux	FJ900608	FJ900654
Neofusicoccum* sp. *karanda	MUCC247	WAC12396	*E. grandis* × *E. camaldulensis*	Australia	T. Burgess	EU301028	EU339513
	MUCC125		*E. dunnii*	Australia	G. Hardy	EU339525	EU339514
N. ribis	*CBS 115475*	*CMW 7772*	*Ribes* sp.	USA	B. Slippers, G. Hudler	AY236935	AY236877
	CBS 121.26	CMW 7054	*Ribes rubrum*	USA	N. E. Stevens	AF241177	AY236879
N. kwambonambiense	*CBS 123639*	*CMW 14023*	*S. cordatum*	South Africa	D. Pavlic	EU821900	EU821870
	CBS 123641	CMW 14140	*S. cordatum*	South Africa	D. Pavlic	EU821919	EU821889
	MUCC157		*Eucalyptus dunnii*	Australia	T. Burgess	EU339522	EU339516
	CMW 37399		*E. grandis*	South Africa	M. Gryzenhout	JQ744566	JQ744587
N. umdonicola	*CBS 123645*	*CMW 14058*	*S. cordatum*	South Africa	D. Pavlic	EU821904	EU821874
	CBS 123646	CMW 14060	*S. cordatum*	South Africa	D. Pavlic	EU821905	EU821875
N. occulatum	*CBS 128008*	*MUCC227*	*Eucalyptus grandis*	Australia	T. Burgess	EU301030	EU339509
	WAC12395	MUCC286	*Eucalyptus pellita*	Australia	T. Burgess	EU736947	EU339511
N. algeriense	*CBS 137504*	*ALG1*	*Vitis vinifera*	Algeria	A. Berraf-Tebbal	KJ657702	KJ657715
	ALG9		*V. vinifera*	Algeria	A. Berraf-Tebbal	KJ657704	KJ657721
N. andinum	*CBS 117453*	*CMW 13455*	*Eucalyptus* sp.	Venezuela	S. Mohali	AY693976	AY693977
	CBS 117452	CMW 13446	*Eucalyptus* sp.	Venezuela	S. Mohali	DQ306263	DQ306264
N. nonquaesitum	*CBS 126655*	*PD484*	*Umbellularia californica*	USA	F. P. Trouillas	GU251163	GU251295
	PD301		*Vaccinum corymbosum*	Chile	E. X. Briceno, J.G. Espinoza, B.A. Latorre	GU251164	GU251296
N. arbuti	*CBS 116131*		*Arbutus menziesii*	USA	M. Elliott	AY819720	KF531792
	CBS 117090		*A. menziesii*	USA	M. Elliott	AY819724	KF531791
N. macroclavatum	*CBS 118223*	*WAC12444*	*Eucalyptus globulus*	Australia	T. Burgess	DQ093217	DQ093217
	WAC12445	CMW 15948	*E. globulus*	Australia	T. Burgess	DQ093218	DQ093219
N. eucalyptorum	*CBS 115791*	*CMW 10125*	*E. grandis*	South Africa	H. Smith	AF283686	AY236891
	CMW 10126		*E. grandis*	South Africa	H. Smith	AF283687	AY236892
	CMW 11705		*E. nitens*	South Africa	B. Slippers	AY339256	AY339264
N. eucalypticola	*CBS 115679*	*CMW 6539*	*Eucalyptus grandis*	Australia	M.J. Wingfield	AY615141	AY615133
	CMW 6217	CBS 115766	*Eucalyptus rossii*	Australia	M.J. Wingfield	AY615143	AY615135
N. mangiferae	CBS 118531	CMW 7024	*Mangifera indica*	Australia	G.I. Johnson	AY615185	DQ093221
	CBS 118532	CMW 7797	*M. indica*	Australia	G.I. Johnson	AY615186	DQ093220
N. vitifusiforme	*CBS 110887*	*STE-U 5252*	*Vitis vinifera*	South Africa	J.M. van Niekerk	AY343383	AY343343
	CBS 110880	STE-U 5050	*V. vinifera*	South Africa	J.M. van Niekerk	AY343382	AY343344
N. eucalypti	**CMW 24460**		*E. pilularis*	South Africa	H.M. Maleme	FJ752737	FJ752706
	CMW 23791[c]		*Eucalyptus* sp.	South Africa	H.M. Maleme	FJ752738	FJ752705
	CMW 24571		*E. paniculata*	South Africa	H.M. Maleme	FJ752739	FJ752707
	CMW 15952		*E. diversicolor*	Australia	T. Burgess, K.L. Goei	DQ093194	DQ093215
	CMW 15953		*E. diversicolor*	Australia	T. Burgess, K.L. Goei	DQ093195	DQ093216
	WAC12401	PD293	*E. pauciflora*	Australia	P. J. Keane	AY744372	GU251305
	WAC12402	PD294	*E. camaldulensis*	Australia	G. Whyte	AY744373	GU251306
	WAC12398	CMW 15198	*E. diversicolor*	Australia	T. I. Burgess, K.L. Goei	AY744371	DQ093214
	CBS 121767	CMW 25409	*Acacia mellifera*	Namibia	F.J.J van der Walt, J. Roux	EU101302	EU101347
N. protearum	CMW 39282		*A.karroo*	South Africa	F. Jami	KF270043	KF270013
	CMW 39280		*A.karroo*	South Africa	F. Jami	KF270041	KF270011
	CMW 39281		*A.karroo*	South Africa	F. Jami	KF270042	KF270012
N. ursorum	***CBS 122811***	***CMW 24480*** [c]	*Eucalyptus camaldulensis*	South Africa	H.M. Maleme	FJ752746	FJ752709
	CMW 23790[c]		*Eucalyptus camaldulensis*	South Africa	H.M. Maleme	FJ752745	FJ752708
N. mediterraneum	*CBS 121718*	*PD312*	*Eucalyptus* sp.	Greece	P.W. Crous, M.J. Wingfield, A.J.L. Phillips	GU251176	GU251308
	PD2		*Eucalyptus* sp.	USA	T.J. Michailides	GU251178	GU251310
	CBS 121558		*Olea europea*	Italy	F. Salvatore	GU799463	GU799462
N. viticlavatum	*CBS 112878*	*STE-U 5044*	*Vitis vinifera*	South Africa	F. Halleen	AY343381	AY343342

(continued on next page)

Table 1 *(continued)*

Species	Isolate ID[a,b]	Other ID[a,b]	Host	Country	Collector/isolator	ITS	EF
	CBS 112977	STE-U 5041	*V. vinifera*	South Africa	F. Halleen	AY343380	AY343341
N. cryptoaustrale	***CBS 1122813***	***CMW 23785*** [c]	*Eucalyptus* sp.	South Africa	H.M. Maleme	FJ752742	FJ752713
	CMW 23786[c]		*E. saligna*	South Africa	H.M. Maleme	FJ752744	FJ752714
	CMW 23787		*E. dorrigoensis*	South Africa	H.M. Maleme	FJ752743	FJ752711
	CMW 23784		*Eucalyptus* sp.	South Africa	H.M. Maleme	FJ752741	FJ752712
	CMW 20738[c]		*E. citriodora*	South Africa	H.M. Maleme	FJ752740	FJ752710
	CAD023		*Vitis vinifera*	Italy	A. Deidda	KJ638328	KJ638346
N. australe	*CMW 6837*		*Acacia* sp.	Australia	M.J. Wingfield	AY339270	AY339270
	CMW 37396		*Eucalyptus grandis*	South Africa	M. Gryzenhout	JQ744576	JQ744597
	CMW 15951		*E. diversicolor*	Australia	T. Burgess, K.L. Goei	DQ093201	DQ093225
N. luteum	*CBS 110299*		*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259091	AY573217
	CBS 110497		*V. vinifera*	Portugal	A.J.L. Phillips	EU673311	EU673277
Botryosphaeria dothidea	*CBS 115476*	*CMW 8000*	*Prunus* sp.	Switzerland	B. Slippers	AY236949	AY236898
	MUCC500		*Eucalyptus marginata*	Australia	K. Taylor	EF591915	EF591968
	MUCC501		*E. marginata*	Australia	K. Taylor	*EF591916*	*EF591969*
	CMW 20717		*E. citriodora*	South Africa	H.M. Maleme	FJ752749	FJ752720
	CMW 20732		*E. citriodora*	South Africa	H.M. Maleme	FJ752750	FJ752721
	CMW 20728		*E. saligna*	South Africa	H.M. Maleme	FJ752747	FJ752722
	CMW 20739[c]		*E. microcorys*	South Africa	H.M. Maleme	FJ752751	FJ752719
	CMW 20718		*E. tereticornis*	South Africa	H.M. Maleme	FJ752748	FJ752723
	CMW 23783		*E. dorrigoensis*	South Africa	H.M. Maleme	FJ752752	FJ752718
B. auasmontanum	*CBS 121769*	*CMW 25413*	*Acacia mellifera*	Namibia	F.J.J. van der Walt, J. Roux	EU101303	EU101348
B. scharifii	*CBS 124703*	*IRAN 1529C*	*Mangifera indica*	Iran	J. Abdollahzadeh	JQ772020	JQ772057
	CBS 124702	IRAN 1543C	*M. indica*	Iran	J. Abdollahzadeh, A. Javadi	JQ772019	JQ772056
B. ramosa	*CBS 122069*	*CMW 26167*	*Eucalyptus camaldulensis*	Australia	T.I. Burgess, M.J. Wingfield	EU144055	EU144070
B. agaves	*CBS 133992*	*MFLUCC 11-0125*	*Agave* sp.	Thailand	R. Phookamsak	JX646791	JX646856
	MFLUCC 10-0051		*Agave* sp.	Thailand	P. Chomnuti	JX646790	JX646855
B. corticis	*CBS 119047*	*CAP 197*	*Vaccinium corymbosum*	USA	P.V. Oudemans	DQ299245	EU017539
	ATCC 22927		*Vaccinium* sp.	USA	R.D. Millholland	DQ299247	EU673291
B. fabicerciana	*CBS 127193*	*CMW 27094*	*Eucalyptus* sp.	China	M.J. Wingfield	HQ332197	HQ332213
	CMW 27108		*Eucalyptus* sp.	China	M.J. Wingfield	HQ332200	HQ332216
B. fusispora	*MFLUCC 10-0098*		*Entada* sp.	Thailand	S. Boonmee	JX646789	JX646854

[a] Culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ATCC = American Type Culture Collection, Fairfax, VA, USA; CAP = Culture collection of A. J. L. Phillips, Lisbon, Portugal; CBS = Centraalbureau voor isolates Schimmelcultures, Utrecht, Netherlands; CPC = Culture Collection of P.W. Crous, housed at CBS; MFLUCC = Mae Fah Luang University Culture Collection;, Chiang Rai, Thailand; MUCC = Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie prefecture, Japan; IRAN = Iranian Fungal Culture Collection, Iranian Research Institute of Plant Pathology, Iran; PD = Culture collection, University of California, Davis, USA; CAD = Collection A. Deidda; STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; WAC = Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia; CMM = Phytopathogenic Fungi of the Universidad Federal Rural de Pernambuco; ALG = Personal culture collection A. Berraf-Tebbal.

[b] Isolates sequenced in this study are given in bold and isolates connected to type material are given in italics.

[c] Isolates used in the pathogenicity tests.

and profile C = 6 isolates), were selected for ITS r DNA sequencing of which nineteen (representing each species identified based on the ITS r DNA sequences) were successively sequenced for TEF-1 α (Table 1). Sequences of approximately 550 bp (ITS rDNA) and 300 bp (TEF-1α) were obtained. For phylogenetic analyses, the final nucleotide matrices consisted of 22 and 85 ITS rDNA sequences for genera *Botryosphaeria* and *Neofusicoccum*, respectively (ITS data not shown). There were 22 concatenated ITS + TEF-1α sequences for *Botryosphaeria* and 73 concatenated ITS + TEF-1α sequences for *Neofusicoccum*. Of these, thirteen to six, respectively, represented isolates obtained in this study, while other sequences were those for known species of *Neofusicoccum* and *Botryosphaeria*, mostly representing those previously isolated from *Eucalyptus* (Table 1). Best maximum likelihood (ML) trees for *Botryosphaeria* were rooted against *N. parvum* and *N. ribis*. For *Neofusicoccum*, *B. dothidea* and *B. cortices* were used as outgroups. Final ML Optimization Likelihood for the *Neofusicoccum* concatenated ITS + TEF-1α dataset was −2051.439865, and −3098.108649 for *Botryosphaeria* dataset. All isolates obtained in this study grouped into five different clades representing *N. parvum*, *Dichomera eucalypti*, *N. ursorum* and *N. cryptoaustrale* (Fig. 2) and *Botryosphaeria dothidea* (Fig. 3). The two subclades were observed within *N. parvum* clade in phylogenetic analyses of combined TEF-1α and ITS rDNA sequence data sets (Fig. 2), however there was no congruency between phylogenetic analyses of individual data sets. Therefore, the isolates within this clade were identified as *N. parvum*. The isolates of *B. dothidea* from *Eucalyptus* in this study grouped within two sub-clades, while the two isolates obtained from *Eucalyptus* in Australia formed a third sub-clade (Fig. 3). These three sub-clades were observed in phylogenetic analyses of the ITS rDNA sequences (not shown) and of combined TEF-1α and ITS rDNA sequence data sets (Fig. 3). Four fixed nucleotides were identified between ITS sequences in two sub-clades that included sequences obtained in this study, while there was no variation among TEF-1α sequences. More isolates from these group and additional gene regions should be sequenced to confirm there is more than one species among these isolates. Based on analyses in this study they can be treated as *B. dothidea sensu lato*, or *B. dothidea* complex. With the use of the PCR-RFLP profiles and DNA sequence data, the number of isolates per species obtained from both *Eucalyptus* in the arboretum, as well as in the surrounding *E. camaldulensis* could be confirmed for all 92 isolates. The number of isolates representing the various Botryosphaeriaceae and their distribution on *Eucalyptus* spp. in the arboretum, as well as in the surrounding *E. camaldulensis* is presented in Fig. 4.

3.4. Taxonomy

Dichomera eucalypti was grouped firmly in the *Neofusicoccum* clade in this and other recently published studies (Barber et al., 2005; Burgess et al., 2005; Crous et al., 2006; Slippers et al., 2013; Phillips et al.,

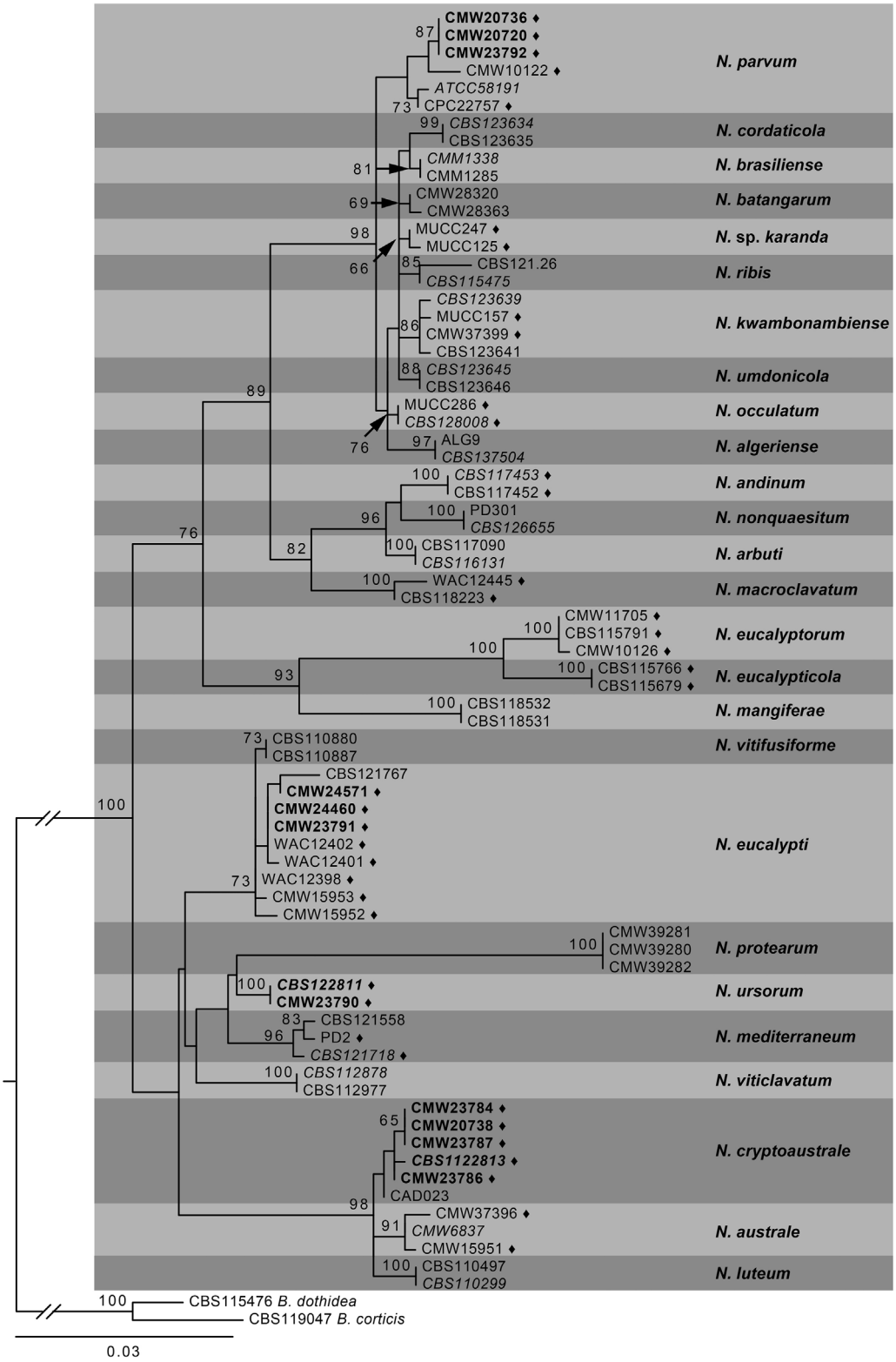

Fig. 2. Phylogenetic tree obtained from the combined sequence datasets of the ITS rDNA and EF-1α loci for *Neofusicoccum* species. Bootstrap values ≥ 65 based on 10,000 bootstrap replicates are shown. Isolates sequenced in this study are in bold, isolates related to type specimens are in italics and isolates from *Eucalyptus* are marked as ♦. The tree is rooted to *Botryosphaeria dothidea* and *B. corticis*.

2013). *Neofusicoccum* is represented by species with *Fusicoccum*-like conidia sometimes having *Dichomera*-like synanamorphs (Crous et al., 2006). *Fusicoccum*-like conidia were observed in this study as opposed to muriform, globose conidia observed in the previous studies. Based on these morphological observations and the phylogenetic grouping of our isolates with isolates from Barber et al. (2005) (Fig. 2), which were morphologically linked to the epitype, this taxon was transferred to *Neofusicoccum* as *Neofusicoccum eucalypti* (Winter) Maleme, Pavlic & Slippers comb. nov. (Maleme, 2008).

3.5. Pathogenicity

The data were analyzed separately for each of the two trials and because there was no significant difference between them, these data were subsequently combined. All isolates inoculated on the *Eucalyptus grandis* clone ZG-14 produced lesions after six weeks that were

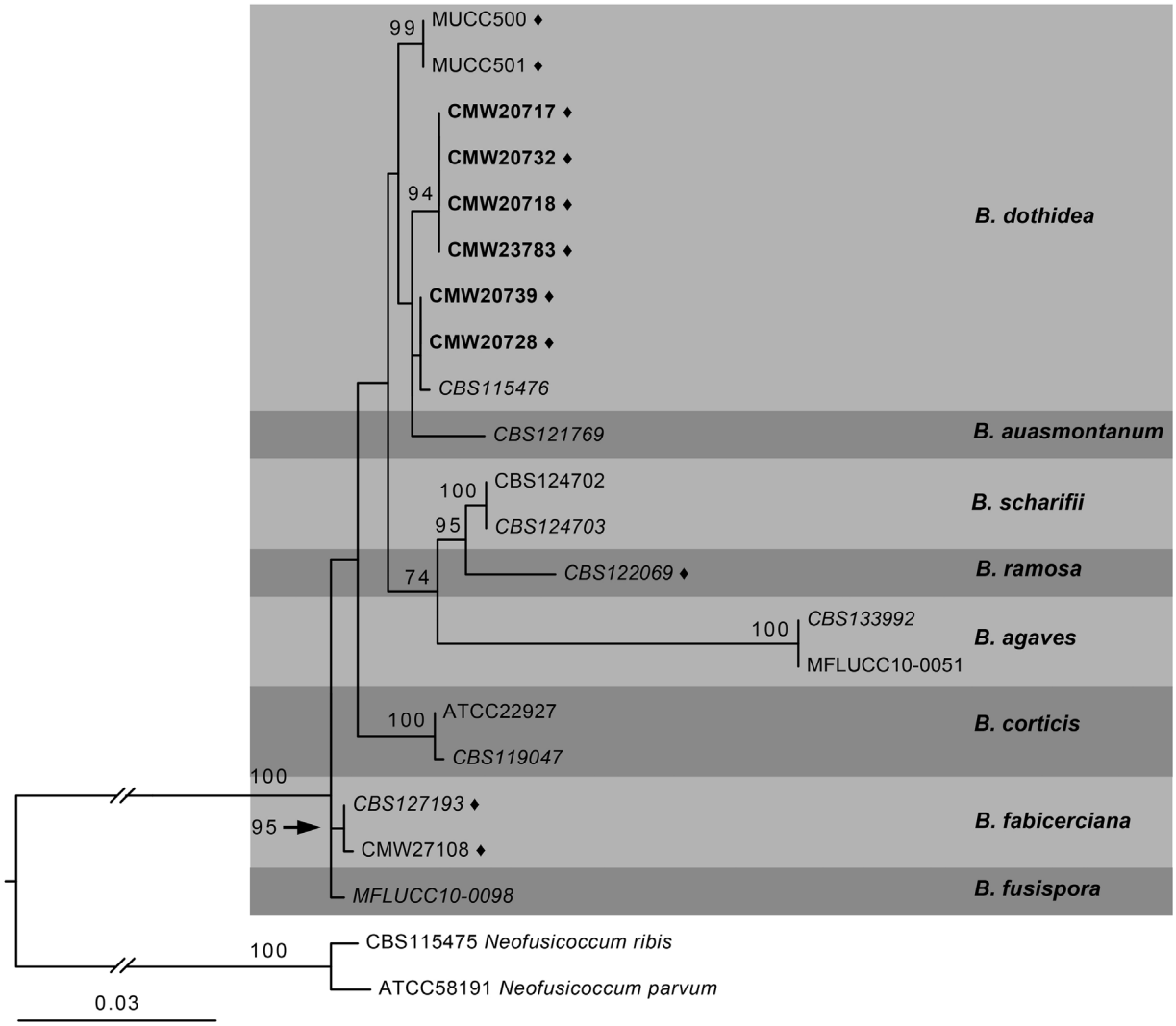

Fig. 3. Phylogenetic tree obtained from the combined sequence datasets of the ITS rDNA and EF-1α loci for *Botryosphaeria* species. Bootstrap values ≥ 65 based on 10,000 bootstrap replicates are shown. Isolates sequenced in this study are in bold, isolates related to type specimens are in italic and isolates from *Eucalyptus* are marked as ♦. The tree is rooted to *Neofusicoccum parvum* and *N. ribis*.

significantly larger than those of the controls (R-square = 0.58, Coefficient variable = 39.7, Root MES = 16.2), confirming their pathogenicity on this host (Fig. 5). The isolates were recovered by re-isolations from the lesions. Although some minor lesion development was observed on some of the control trees, no Botryosphaeriaceae could be re-isolated from these lesions.

The most aggressive of the isolates was a single isolate of *N. eucalypti* (CMW 24571) and two isolates of *N. cryptoaustrale* (CMW 23785 and CMW 23786). The lesions produced by these isolates on one *Eucalyptus* clone were significantly longer than those of all other isolates used in the inoculation tests but some isolates of these species had lower levels of aggressiveness (Fig. 5). On average, *B. dothidea* isolates were the least aggressive, while *N. cryptoaustrale* and *N. parvum* the most aggressive of the species tested (Fig. 5).

4. Discussion

Five species of the Botryosphaeriaceae, *Botryosphaeria dothidea*, *Neofusicoccum parvum*, *N. cryptoaustrale*, *N. ursorum*, and *N. eucalypti* (Winter) Maleme, Pavlic & Slippers comb. nov. were identified from 20 *Eucalyptus* spp. planted in an arboretum in Pretoria, and from ornamental *E. camaldulensis* trees surrounding the arboretum. Most isolates were of *N. parvum* and *B. dothidea*. With exception of *N. parvum* which was isolated from majority of *Eucalyptus* spp. the other species were isolated from limited number of *Eucalyptus* species indicating host-preferences. Five identified species were shown to be able to produce lesions longer than those for the controls in artificial inoculations on one *Eucalyptus grandis* clone. *Neofusicoccum eucalypti* is recorded for the first time on *Eucalyptus* in South Africa.

Neofusicoccum parvum was the most commonly isolated species in this study presenting 72% of all isolates. After its first description from kiwifruit in New Zealand (Pennycook and Samuels, 1985), this species has been recorded from more than 90 hosts, mostly woody angiosperms, across the globe (Phillips et al., 2002; Gure et al., 2005; Pavlic et al., 2007; Sakalidis et al., 2011). On *Eucalyptus,* it is commonly reported as a cause of canker and die-back (Nakabonge, 2002; Ahumada, 2003; Gezahgne et al., 2004; Barber et al., 2005; Mohali et al., 2006; Rodas et al., 2009; Chen et al., 2011; Iturritxa et al., 2011; Pillay et al., 2013). In South Africa, *Neofusicoccum parvum* is also known from native Myrtaceae, including *Heteropyxis natalensis* and *Syzygium cordatum* (Smith et al., 2001; Slippers et al., 2004; Pavlic et al., 2007; Pillay et al., 2013; Pavlic-Zupanc et al., 2015). Pavlic et al. (2015) demonstrated that *N. parvum* is dominant and most abundant on *S. cordatum* in habitats influenced by human activity. Thus, its abundance on *Eucalyptus* species in an urban environment is not surprising.

Botryosphaeria dothidea was the second most common species

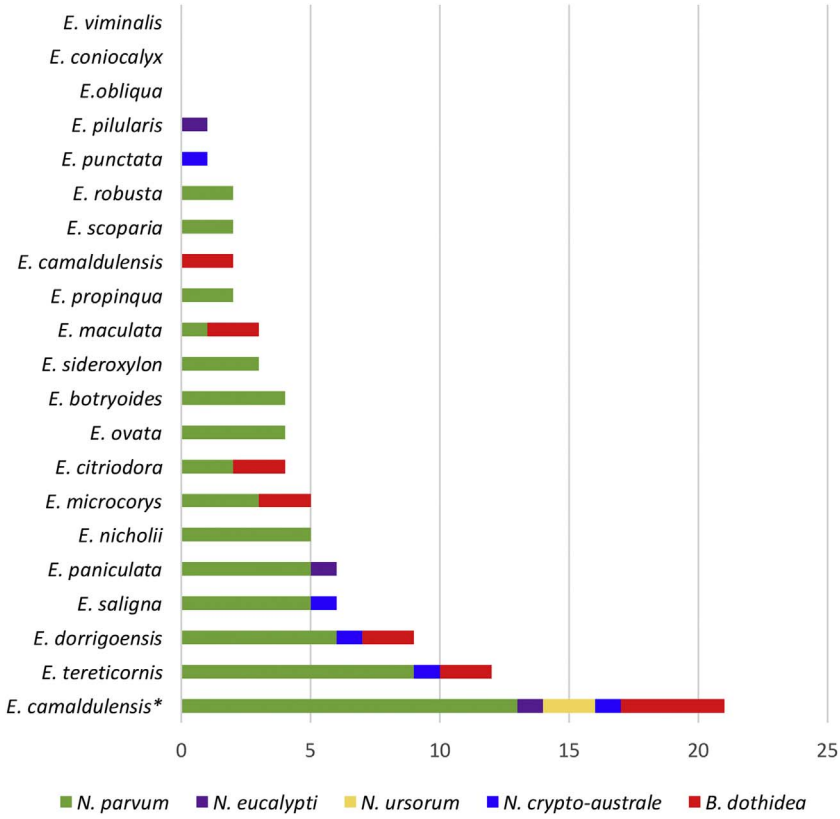

Fig. 4. Distribution of five Botryosphaeriaceae isolated from 20 different *Eucalyptus* spp. in the Pretoria arboretum and from (*) surrounding *E. camaldulensis* trees.

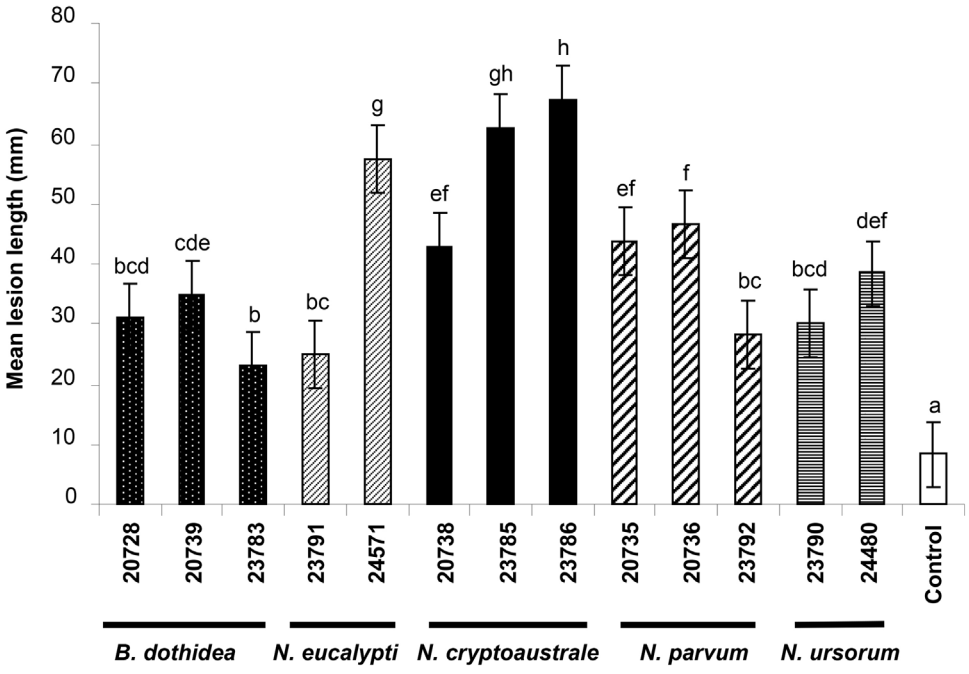

Fig. 5. Mean lesion lengths (mm) for isolates of five species of Botryosphaeriaceae after inoculation on a *Eucalyptus grandis* clone (ZG-14), including *Botryosphaeria dothidea*, *Neofusicoccum eucalypti*, *N. cryptoaustrale*, *N. parvum*, *N. ursorum*. Control inoculations were done with MEA agar. Bars indicate the 95% confidence limit for each isolate.

obtained in this study, representing 17% of all isolates. This fungus has been documented on many hosts worldwide, including *Eucalyptus* (Smith et al., 1996, 2001; Yu et al., 2009; Pérez et al., 2010). Recent studies have indicated that this fungal species is not common on *Eucalyptus* and other closely related hosts in South Africa (Slippers et al., 2004; Pavlic et al., 2007; Pillay et al., 2013). In contrast, *B. dothidea* was the most common species identified on native *Acacia karoo* trees across the country (Jami et al., 2015). A few recent studies have also described *B. dothidea* as one of the most common Botryosphaeriaceae species on a variety of trees grown in native forests and as ornamental in urban habits in Europe (Piškur at al. 2010; Zlatković et al., 2016). Its dominant presence on *Eucalyptus* grown in the urban habitats, may indicate biotic exchange between *Eucalyptus* and diverse community of trees grown as ornamentals in the urban area of Pretoria, many of which have been introduced from other parts of the world.

Three distinct, highly supported lineages were identified for *B. dothidea* isolates in phylogenetic analyses using DNA sequence data for the ITS rDNA and TEF-1α gene regions. Two lineages comprised isolates of *B. dothidea* obtained in this study, while two isolates obtained from *Eucalyptus* in Australia form the third one. The *Botryosphaeria dothidea* –

complex was introduced for the first time by Smith et al. (2001) based on phylogenetic analyses of ITS sequence data obtained for a group of isolates from *Eucalyptus* in South Africa. High levels of variation have been observed among sequences of isolates identified as *B. dothidea* from different woody hosts in numerous studies (Smith et al., 2001; Burgess et al., 2005; Slippers et al., 2007; Inderbitzin et al., 2010). In the latter study, based on a six-locus phylogeny, three lineages were resolved among isolates of *B. dothidea* from a variety of woody hosts including eucalypt. Those lineages were also correlated to distinct morphological characters. Results of the present study also suggest that isolates identified as *B. dothidea* could include cryptic species.

Neofusicoccum cryptoaustrale and *N. ursorum* were recently described from a plant tissue collected as a part of the current study (Crous et al., 2013). *Neofusicoccum cryptoaustrale* as a cryptic sister species to *N. australe* has previously been isolated from *Wollemia nobilis*, a native conifer in eastern Australia (Slippers et al., 2005) and on native *Syzygium cordatum* trees in South Africa (Pavlic et al., 2007). The occurrence of *N. cryptoaustrale* on two different native hosts in Australia and South Africa and on non-native *Eucalyptus* in South Africa, makes it difficult to suggest a possible origin for the fungus. Its existence on *Eucalyptus* spp. in South Africa could be explained by the movement of species of Botryosphaeriaceae between continents on plant material, possibly from Australia where *Eucalyptus* is native. Alternatively, it could had jumped hosts from native *Syzygium cordatum* to introduced *Eucalyptus*, or *vice versa* in South Africa since both hosts were shown to share similar pathogens (Pavlic et al., 2007; Pillay et al., 2013). Two isolates of *Neofusicoccum ursorum* were collected from *E. camaldulensis* growing around the arboretum. This species is currently known only from South Africa, and to the best of our knowledge, has never been reported from any other area or host globally.

Neofusicoccum eucalypti is established in this study as a new combination for *Camarosporium eucalypti*. The taxon was originally described from *Eucalyptus* spp. in Australia as producing globose, subglobose, obovoid, obpyriform, muriform or somewhat fusiform, septate conidia (Sutton, 1975). This was confirmed by Barber et al. (2005) who designated an epitype specimen (and ex-type culture) for '*Dichomera eucalypti*'. The isolates obtained in the present study were identical to the ex-type cultures in ITS rDNA and TEF-1α sequence data, but did not have morphological characteristics described by Sutton (1975) and Barber et al. (2005). They rather produced hyaline, aseptate, fusiform to ellipsoid conidia in culture. This observation, together with the consistent grouping with other species of *Neofusicoccum*, validates our treatment of the fungus in *Neofusicoccum* as *N. eucalypti*. Some other *Neofusicoccum* species (e.g. *N. parvum*, *N. australe*, see Barber et al. (2005)) are also known to produce synanamorphs that are *Dichomera*-like. It remains unclear why some isolates, such as those found in this study, produce only one of the spore forms and not the other.

Neofusicoccum eucalypti is well known from woody tissues, foliage and bark samples of *Eucalyptus* spp. in Australia (Sutton, 1975; Barber et al., 2005; Burgess et al., 2005). The species was not common in this study, with only two isolates of this species identified as endophytes from asymptomatic leaves in the *Eucalyptus* arboretum and one from the surrounding *E. camaldulensis* trees. This is the first report of this fungus on *Eucalyptus* in South Africa. Its occurrence on non-native *Eucalyptus* in South Africa might have been anticipated due to its common association with *Eucalyptus* in Australia and the fact that the trees sampled in this study were generated from seed originating in Australia.

All the isolates tested in pathogenicity trial could infect two-year-old *Eucalyptus grandis* trees and produces lesions significantly longer than controls. The isolates of *N. cryptoaustrale* were the most virulent. Wide host and geographic range, as well as the high level of virulence revealed in this study, makes *N. cryptoaustrale* a potential threat to both native and non-native hosts in South Africa and Australia (Slippers et al., 2005; Pavlic et al., 2007). Although the isolates of *N. eucalypti* varied significantly in virulence, it is noteworthy that one of the isolates was amongst the most virulent in the pathogenicity trial. The presence of *N. eucalypti* in South Africa, albeit at low levels currently, poses a potential threat to *Eucalyptus grandis*. Although most commonly isolated, individual *N. parvum* isolates were pathogenic to *Eucalyptus grandis*, but when compared to other species studied here they were mildly virulent, followed by *N. ursorum*. *Botryosphaeria dothidea* isolates were on average the least virulent. This results are consistent with recent studies about *B. dothidea* and *Neofusicoccum* spp. pathogenicity on Myrtaceae species in South Africa, Venezuela and Colombia (Mohali et al., 2007; Pavlic et al., 2007; Rodas et al., 2009).

Numerous species of the Botryosphaeriaceae have been identified in recent years on *Eucalyptus* by combining both morphological characters and multigene phylogeny (Slippers et al., 2007; Chen et al., 2011; Crous et al., 2013; Pillay et al., 2013). Some are thought to be host specific and/or with a local distribution, such as *B. fabicerciana* and *N. andinum* that have been recorded only on *Eucalyptus* in China and Venezuela, respectively (Mohali et al., 2006; Chen et al., 2011; Phillips et al., 2013). Others have a broad host range and are more widely distributed, such as *N. parvum* that has been documented on *Eucalyptus* in countries such as South Africa, Venezuela, Uganda, China and Spain (Roux et al., 2000, 2001; Mohali et al., 2007; Chen et al., 2011; Iturritxa et al., 2011). The present study adds to this emerging global view of a combination of a few common generalists and some rare species of the Botryosphaeriaceae that infect *Eucalyptus* at any given location, not only in plantation forestry but in urban ecosystems.

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