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Lasiodiplodia species associated with dying *Euphorbia ingens* in South Africa

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Various species of *Euphorbia* occur in South Africa, including herbaceous, succulent and woody types. The largest of the succulent *Euphorbia* spp. in South Africa is *Euphorbia ingens*. These trees have been dying at an alarming rate in the Limpopo province during the course of the last 15 years. Investigations into the possible causes of the death have included the possible role of fungal pathogens. Amongst the fungi isolated from diseased trees were species in the Botryosphaeriaceae. The aim of this study was to identify these fungi using morphology and DNA sequence data of two gene regions (TEF 1-α and ITS). Results showed that *Lasiodiplodia theobromae* and *L. mahajangana* were present, representing the first report of *Lasiodiplodia* species on a succulent *Euphorbia* species. Pathogenicity studies showed that these *Lasiodiplodia* species can cause infections on healthy *E. ingens* trees, implicating them as contributors to the decline of *E. ingens.*

Keywords: Botryosphaeriaceae, candelabra trees, climate change, insect infestations, tree diseases

Introduction

The genus *Euphorbia* includes more than 2100 species worldwide. *Euphorbia* species are known to vary dramatically in morphology and range from large woody trees to shrub-like herbaceous and succulent cactus-like plants (Palgrave et al. 2002, PBI Euphorbia project, www.euphorbiaceae.org). There is a great diversity of woody to succulent euphorbias in southern Africa, with the largest of these species being *Euphorbia ingens* E.Meyer ex Boissier (Palgrave et al. 2002, Gildenhuys 2006). *Euphorbia ingens* and similar species are characterised by woody main stems and fleshy succulent branches, giving the trees a candelabrum shape (van Wyk and van Wyk 1997, Palgrave et al. 2002, Gildenhuys 2006). *Euphorbia ingens* is known only to occur in Africa with high densities in southern Africa (Palgrave et al. 2002, Gildenhuys 2006).

In the last 15 years, there have been alarming reports of large-scale decline and death of *E. ingens* trees in South Africa. Mortality of these trees has been particularly severe in the Limpopo province. Symptoms associated with the death of trees include greying and spots on the succulent branches, infestation by branch and stem boring insects, and brown to blue discolouration of the internal tissues of the branches and woody main stems (Roux et al. 2008, 2009). Preliminary investigations into the cause of this disease have yielded a variety of fungi, including species of Botryosphaeriaceae (Roux et al. 2008, 2009).

The Botryosphaeriaceae are known as opportunistic pathogens that cause cankers and death of numerous tree species, especially after periods of drought, frost, hail damage and other environmental conditions leading to stress (Punithalingam 1980, Slippers and Wingfield 2007). They are also known to be endophytes, infecting healthy trees and only causing disease after the onset of stress (Smith et al. 1996). In South Africa, fungi in the Botryosphaeriaceae are common, and often cause disease of especially commercially grown plantation trees (Laughton 1937, Swart et al. 1985, Smith et al. 1996, Roux and Wingfield 1997), native tree species such as *Pterocarpus angolensis* DC. (Mehl et al. 2011) and *Syzygium* species (Pavlic et al. 2007). A variety of species are also known to occur on, and cause disease of, fruit trees in the genera *Malus*, *Pyrus*, *Prunus*, *Populus*, *Syzygium* and *Vitis* (van Niekerk et al. 2004, Damm et al. 2007, Pavlic et al. 2007, Slippers et al. 2007). There are, however, no reports of Botryosphaeriaceae from succulent *Euphorbia* species.

The aim of this study was to identify species of Botryosphaeriaceae collected during studies of dying *E. ingens* trees in the Limpopo province of South Africa. We also tested the pathogenicity of the isolates on healthy *E. ingens* trees to consider their possible involvement in tree death.

Materials and methods

Collection of samples and isolations

Isolates were collected from diseased *E. ingens* (Figure 1a) at four sites in the Limpopo province during 2009. Isolations were made from blue-black discoloured wood (Figure 1b) in the main woody stems of the trees, as well as from necrotic tissue and insect tunnels in the

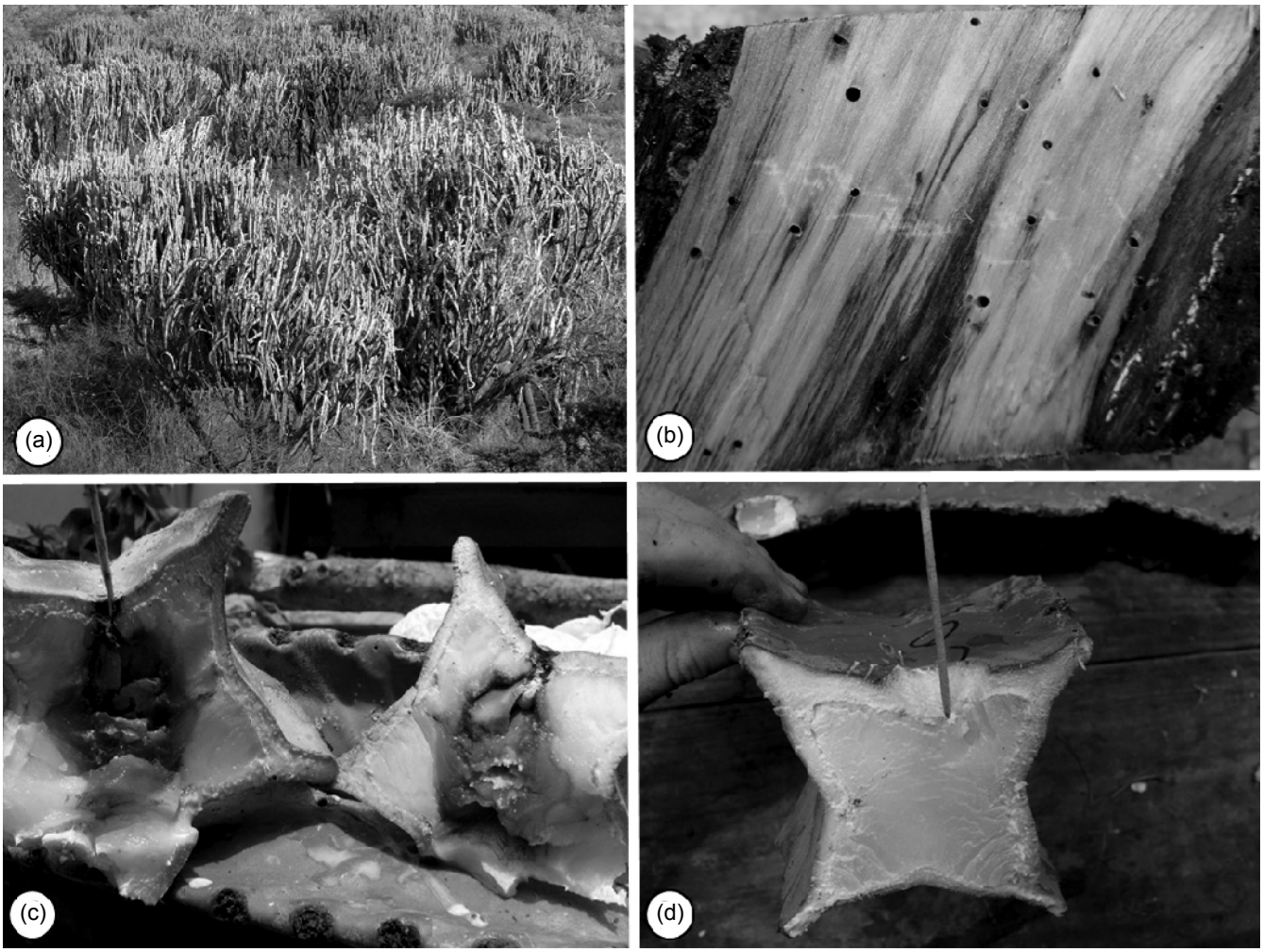

Figure 1: Disease symptoms on *Euphorbia ingens* trees. (a) Dying *Euphorbia ingens* trees near Mokopane in the Limpopo province. (b) Blue stain in wood from which *Lasiodiplodia mahajangana* was isolated. (c) Internal lesion produced by *Lasiodiplodia theobromae* (CMW36766) on the succulent branches of *Euphorbia ingens* during the pathogenicity trial. (d) Healthy control inoculation showing no disease development

succulent branches. Isolations were also made from insects collected from rotting succulent branches and the woody main stems. Direct isolations were made from the plant material taken from the leading edges of lesions using a sterile scalpel. These tissue samples were plated on 2% malt extract agar (MEA; 15 g agar and 20 g malt extract per litre distilled water; Biolab, Merck, Midrand, South Africa) with streptomycin (0.4 g l^{-1}; Sigma-Aldrich, St Louis, USA). Isolations were made from insects by crushing them onto water agar (15 g l^{-1} distilled water; Biolab) and incubating the plates for six weeks at 20 °C. Cultures from insects were purified by transferring mycelium to fresh 2% MEA. A second set of isolates was obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, which were collected previously from similar symptoms (Roux et al. 2008, 2009) on diseased *E. ingens* trees in the Limpopo province. Purified single-spore isolates from plant tissue and insects were deposited in the CMW culture collection.

Culture and morphological characteristics

Isolates were plated onto 1.5% water agar (WA; 15 g l^{-1} distilled water; Biolab) containing sterilised pine needles to induce the formation of fruiting bodies. Cultures were incubated at 20 °C under near-ultraviolet (UV) light. Characteristic fungal structures (conidia, conidiogenous cells, paraphyses and conidiomata) were viewed using a Zeiss light microscope fitted with an Axiocam digital camera with Axiovision 3.1 software (Carl Zeiss, Germany). The fungal structures were placed on glass microscope slides and mounted in 75% lactic acid. Colours of resultant cultures were determined using the colour notations of Rayner (1970).

DNA extraction and polymerase chain reaction amplification

Isolates, representing different collection sites and culture morphology, were grown for 6 d on 2% MEA, prior to DNA extraction. DNA extraction followed the protocol of Möller et al. (1992), after mycelium was scraped from the surfaces of the cultures and freeze-dried for 24 h in 2 ml

Eppendorf tubes. DNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

The polymerase chain reaction was used to amplify the internal transcribed spacer (ITS) regions and the 5.8S gene using the primers ITS1 and ITS4 (White et al. 1990) and the translation elongation factor 1-α (TEF 1-α) gene region using the primers EF1-F and EF1-R (Jacobs et al. 2004). PCRs were done using an Applied Biosystems Veriti thermocycler (Applied Biosystems, Foster City, USA) following the protocols described by Mohali et al. (2007).

PCR products were viewed using an agarose gel (2%; Whitehead Scientific, Cape Town, South Africa), loaded with GelRed (Anatech, USA), and visualised under UV illumination. The size of the PCR products was estimated using a 100 bp DNA molecular marker (O'RangeRuler™ 100 bp DNA ladder, Fermentas Life Sciences, Vilnius, Lithuania). Sephadex G-50 columns (1 g in 15 ml distilled water; SIGMA, Steinheim, Germany) were used to purify the amplified products in preparation for sequencing.

DNA sequencing

Confirmed PCR products were sequenced with an ABI 3700 DNA analyser (Applied Biosystems) using the Big Dye Cycle Sequencing Kit version 1.1 (Applied Biosystems). Sequences were edited based on forward and reverse sequences using Mega 4.0 (Tamura et al. 2007). To confirm gene identity and obtain related sequences, the correctly edited sequences were checked against the National Centre for Biotechnology Information nucleotide database (www.ncbi.nih.nlm.gov) using BLASTn searches. MAFFT 5.851 (Katoh et al. 2002) was used to align the sequences from this study and those of closely related species obtained from the BLAST results. Phylogenetic analysis of each data set was done using PAUP* 4.0b10 (Swofford 2002) and phylogenetic trees were constructed using random stepwise addition and tree bisection and reconstruction as branch swapping algorithms, based on heuristic searches. Bootstrap and maximum parsimony (MP) analyses were run using 1000 replicates (Felsenstein 1985). A partition homogeneity test was used to determine whether the ITS and TEF 1-α sequence data sets could be combined (Farris et al. 1995, Huelsenbeck et al. 1996). Two separate phylogenetic analyses, one including all the recently described species of *Lasiodiplodia* and another considering only the clades including the isolates from this study, were conducted. Prior to the partition homogeneity test, data sets of individual gene regions were analysed separately. The data sets were rooted with the GenBank sequences of *Botryosphaeria sarmentorum* A.J.L.Phillips, Alves & Luque (CBS 12041) and *Lasiodiplodia gonubiensis* Pavlic, Slippers & M.J.Wingf. (CMW 14078) (Table 1).

Bayesian analysis was used to determine the posterior probabilities of each data set (ITS and TEF 1-α) based on the Markov chain Monte Carlo method. jModelTest 0.1.1 (Posada 2008) was used to determine the most appropriate nucleotide substitution model. The best-fitting models for the ITS and TEF 1-α data sets, based on the Akaike information criterion, were determined for the complete analysis (TPM1: ITS, K80+G: TEF 1-α) and the specific analysis (TIM2+G: ITS, K80+G: TEF 1-α). The Bayesian analysis was run on MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) and trees were recorded every 100 generations based on four chains producing 5 000 000 generations. The likelihood data were used in graphical analysis to estimate the burn-in values for each data set. Mega 4.0 (Tamura et al. 2007) was used to produce consensus trees from the two analysed data sets from which the posterior probabilities were determined.

Pathogenicity trials

Two isolates (CMW36766 and CMW36765) obtained in this study were used to inoculate healthy *E. ingens* trees in the North West province. Cultures were first grown on 2% MEA for 5 d and then used to inoculate wooden toothpicks first soaked in malt extract (20 g malt extract l^{-1} distilled water) and then placed on the surface of MEA in Petri dishes. Mycelium-colonised toothpicks, and sterile toothpicks for the controls, were inserted into the succulent branches (five branches for each isolate) to a depth of 3 mm. After six weeks the results were determined by measuring the surface lesions, cambium lesions and internal lesions after cutting branches in half at the point of inoculation. Isolations were made on MEA from inoculated tissue to comply with Koch's postulates. To determine significance between means, a Student's *t*-test was done with $P < 0.05$ as being significant. Since there was no variance in the controls, the data for each isolate were Bonferroni-corrected for multiple comparisons ($\alpha = 0.05$). All tests were conducted using JMP 9.0.2 (SAS Institute 2011).

Results

Collection of samples and isolations

Fungal isolates resembling the Botryosphaeriaceae were obtained from three of the 23 trees sampled in 2009. Five isolates were obtained from the CMW culture collection (collected 2007/8) and originated from diseased trees at the National Game Breeding Centre at Mokopane. Of the eight isolates resembling Botryosphaeriaceae, six isolates originated from Mokopane, one from Capricorn and one from the Louis Trichardt area. Six of these were from diseased plant material and the remaining two were isolated from insects infesting diseased tissue.

Culture and morphological characteristics

Fungal structures showed typical features of *Lasiodiplodia* species, with aseptate, hyaline conidia, becoming dark brown and septate with striations as they matured. Cultures were white with abundant, fluffy aerial mycelium that became an olivaceous grey (23''''b) with time (10 d). Pycnidia were produced after 5 d on WA with sterilised pine needles and were black in colour, unilocular, solitary, immersed in the media or formed on the top surfaces of the pine needles (Figure 2).

DNA sequence analyses

The ITS and TEF 1-α data sets were combined based on a value of $P = 0.350$ (complete *Lasiodiplodia* species group, 43 taxa) and $P = 0.140$ (specific *Lasiodiplodia* species clade, 29 taxa) obtained from the partition homogeneity test done in PAUP (Figure 3 and 4). The MP analyses of the individual gene region data sets did not give good

Table 1: Isolates of Botryosphaeriaceae used in this study and obtained from *Euphorbia ingens* and GenBank

Species	Culture number	Host	Origin	GenBank accession number	
				ITS	TEF 1-α
Botryosphaeria sarmentorum	CBS120.41	*Pyrus communis*	Norway	AY573207	AY573224
Lasiodiplodia citricola	IRAN1521C	*Citrus* sp.	Iran	GU945353	GU945339
Lasiodiplodia citricola	IRAN1522C	*Citrus* sp.	Iran	GU945354	GU945340
Lasiodiplodia crassispora	CMW13488	*Eucalyptus urophylla*	Venezuela	DQ103552	DQ103559
Lasiodiplodia crassispora	WAC12533	*Santalum album*	Australia	DQ103550	DQ103557
Lasiodiplodia crassispora	UCD27Co	Grapevine	USA	GU799457	GU799488
Lasiodiplodia gilanensis	IRAN1501C	Unknown	Iran	GU945352	GU945341
Lasiodiplodia gilanensis	IRAN1523C	Unknown	Iran	GU945351	GU945342
Lasiodiplodia gonubiensis	CBS115812	*Syzygium cordatum*	South Africa	DQ458892	DQ458877
Lasiodiplodia gonubiensis	CMW14078	*Syzygium cordatum*	South Africa	AY639594	DQ103567
Lasiodiplodia hormozganensis	IRAN1498C	*Mangifera indica*	Iran	GU945356	GU945344
Lasiodiplodia hormozganensis	IRAN1500C	*Oleo* sp.	Iran	GU945355	GU945343
Lasiodiplodia iraniensis	IRAN921C	*Mangifera indica*	Iran	GU945346	GU945334
Lasiodiplodia iraniensis	IRAN1502C	*Juglans* sp.	Iran	GU945347	GU945335
Lasiodiplodia mahajangana	CMW36765	*Euphorbia ingens*	South Africa	JN098457	JN098464
Lasiodiplodia mahajangana	CMW27801	*Terminalia catappa*	Madagascar	FJ900595	FJ900641
Lasiodiplodia mahajangana	CMW27818	*Terminalia catappa*	Madagascar	FJ900596	FJ900642
Lasiodiplodia mahajangana	CMW27820	*Terminalia catappa*	Madagascar	FJ900597	FJ900643
Lasiodiplodia margaritacea	CBS122519	*Adansonia gibbosa*	Australia	EU144050	EU144065
Lasiodiplodia margaritacea	CBS122065	*Adansonia gibbosa*	Australia	EU144051	EU144066
Lasiodiplodia parva	CBS494.78	Cassava-field soil	Colombia	EF622084	EF622064
Lasiodiplodia parva	CBS456.78	Cassava-field soil	Colombia	EF622083	EF622063
Lasiodiplodia parva	CBS356.59	*Theobroma cacao*	Sri Lanka	EF622082	EF622062
Lasiodiplodia plurivora	STEU-5803	*Prunus salicina*	South Africa	EF445362	EF445395
Lasiodiplodia plurivora	STEU-4583	*Vitis vinifera*	South Africa	AY343482	EF445395
Lasiodiplodia pseudotheobromae	CBS116459	*Gmelinea arborea*	Costa Rica	EF622077	EF622057
Lasiodiplodia pseudotheobromae	CBS374.54	*Coffea* sp.	Zaire	EF622080	EF622059
Lasiodiplodia pseudotheobromae	CBS447.62	*Citrus aurantium*	Suriname	EF622081	EF622060
Lasiodiplodia rubropurpurea	CBS118740	*Eucalyptus grandis*	Australia	DQ103553	DQ103571
Lasiodiplodia rubropurpurea	CMW15207	*Eucalyptus grandis*	Australia	DQ103554	DQ103572
Lasiodiplodia theobromae	CMW36766	*Euphorbia ingens*	South Africa	JN098457	JN098465
Lasiodiplodia theobromae	CMW37026	*Euphorbia ingens*	South Africa	JN098458	JN098466
Lasiodiplodia theobromae	CMW26225	*Euphorbia ingens*	South Africa	JN098459	JN098467
Lasiodiplodia theobromae	CMW26592	*Euphorbia ingens*	South Africa	JN098460	JN098468
Lasiodiplodia theobromae	CMW26593	*Euphorbia ingens*	South Africa	JN098461	JN098469
Lasiodiplodia theobromae	CMW26594	*Euphorbia ingens*	South Africa	JN098462	JN098470
Lasiodiplodia theobromae	CMW26595	*Euphorbia ingens*	South Africa	JN098463	JN098471
Lasiodiplodia theobromae	CBS111530	Unknown	Unknown	EF622074	EF622054
Lasiodiplodia theobromae	CMW30105	*Syzygium cordatum*	Zambia	FJ747642	FJ871116
Lasiodiplodia theobromae	CMW30104	*Syzygium cordatum*	Zambia	FJ747641	FJ871115
Lasiodiplodia theobromae	CMW28317	*Terminalia catappa*	Cameroon	FJ900602	FJ900648
Lasiodiplodia theobromae	CMW28319	*Terminalia catappa*	Cameroon	FJ900603	FJ900649
Lasiodiplodia theobromae	IRAN1233C	*Mangifera indica*	Iran	GU973868	GU973860
Lasiodiplodia theobromae	IRAN1496C	*Mangifera indica*	Iran	GU973869	GU973861
Lasiodiplodia venezuelensis	WAC12539	*Acacia mangium*	Venezuela	DQ103547	DQ103568
Lasiodiplodia venezuelensis	WAC12540	*Acacia mangium*	Venezuela	DQ103548	DQ103569

resolution in terms of species identity and with $P > 0.05$, trees were combined for this study. The MP analysis for the combined data sets for the complete *Lasiodiplodia* group (655 characters, 9% of characters parsimony informative) and the specific *Lasiodiplodia* species clade (649 characters, 2% of characters parsimony informative) generated seven (TL = 182, CI = 0.780, RI = 0.832, RC = 0.649) and 100 trees (TL = 53, CI = 0.849, RI = 0.855, RC = 0.726), respectively, with similar topology for both groups. Both of the combined data sets had strong Bayesian support with statistically significant values. However, bootstrap analysis produced trees with limited resolution, possibly due to similarity in the DNA sequences investigated, not resolving the final identity of the species. Bayesian analysis produced trees with high resolution and was used as the final model to identify the species (Douady et al. 2003). Burn-in values were obtained for all analyses (burn-in values: complete analysis 52, specific analysis 122).

In the final phylogenetic analyses, the data set including all described *Lasiodiplodia* spp. gave rise to seven clades, whereas three clades emerged for the data set containing only selected *Lasiodiplodia* spp. The complete *Lasiodiplodia* data set did not show a good resolution but indicated that the isolates from this study resided in clade one. Analyses of the reduced *Lasiodiplodia* data set showed that isolates represented *Lasiodiplodia mahajangana* Begoude, Jol.Roux

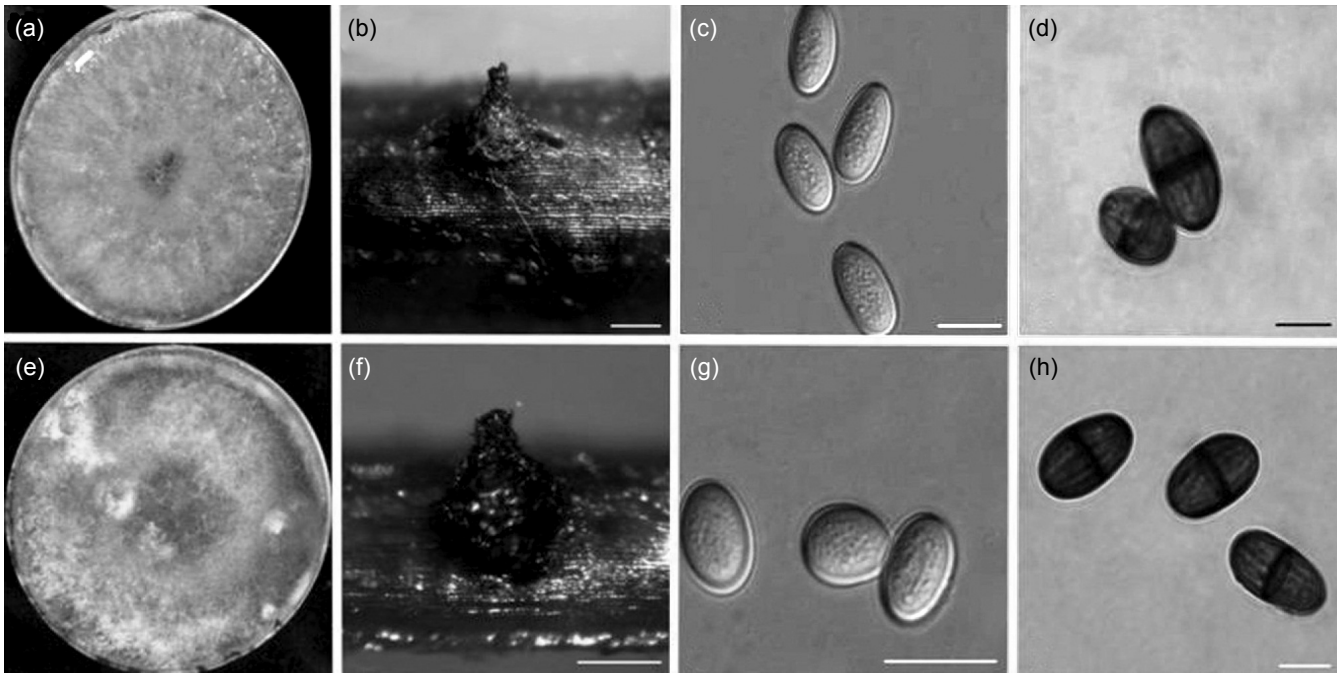

Figure 2: *Lasiodiplodia mahajangana* and *L. theobromae* culture and conidial morphology. (a) Culture morphology of *L. mahajangana*. (b) Pycnidium of *L. mahajangana*, with short neck, on sterile pine needle. (c) Immature conidia of *L. mahajangana* with typical ellipsoid to ovoid shape. (d) Mature conidia of *L. mahajangana* being one septate with characteristic striations. (e) Culture morphology of *L. theobromae*. (f) Pycnidium of *L. theobromae*. (g) Immature conidia of *L. theobromae*. (h) Mature conidia of *L. theobromae*. Bars: b and f = 200 μm; c and g = 20 μm; d and h = 10 μm

& Slippers (CMW36765) and *Lasiodiplodia theobromae* (Patouillard) Griffon & Maubl (CMW26225, CMW26592, CMW26593, CMW26594, CMW26595, CMW36766 and CMW37026) with strong Bayesian support (Figures 3 and 4). *Lasiodiplodia mahajangana* was isolated from blue stain in the wood from one tree (Figure 1b) near the Capricorn Toll Plaza (23°21.910′ S, 29°44.621′ E), whereas *L. theobromae* was isolated from diseased plant material (CMW26225, CMW26592, CMW26593, CMW26594 and CMW26595) from Mokopane (24°10.291′ S, 29°01.131′ E) as well as the insects *Cyrtogenius africus* Wood (CMW36766) and *Cossonus* Claireville (CMW37026) from Mokopane and Last Post Private Nature Reserve (23°17.738′ S, 29°55.467′ E) (Louis Trichardt site), respectively.

Pathogenicity trials

Lasiodiplodia mahajangana (CMW36765) and *L. theobromae* (CMW36766) produced lesions on the exterior, cambium and internal core of healthy *E. ingens* branches (Figure 1c). The most severe damage caused by the fungi was in the internal core of the succulent branches, which was rotten. Lesions on the exterior were conspicuous at the point of inoculation with necrotic tissue and a black discharge. *Lasiodiplodia mahajangana* and *L. theobromae* lesions were brown and circular at the points of inoculation with necrotic tissue in the internal core. The control also had small brown circular lesions at the point of inoculation but had no signs of discolouration in the cambium or internal core (Figure 1d) of the succulent branches. Statistical analysis did not show significant differences in pathogenicity between species except for the cambium lesion length with *P* values of 0.025 (df = 12.51), 0.1303 (df = 18.00) and 0.4261 (df = 14.06) for the cambium lesion, internal lesion depth and internal lesion width data, respectively (Figure 5). Isolations from the sites of inoculation yielded *L. theobromae* and *L. mahajangana* based on characteristic morphological features.

Discussion

Results of this study showed that two species of *Lasiodiplodia*, *L. theobromae* and *L. mahajangana*, are associated with die-back symptoms on *E. ingens*. These fungi were identified based on morphological characteristics and DNA sequence comparisons. They are both well known from trees in southern Africa (Crous et al. 2000, Burgess et al. 2003, Pavlic et al. 2007, Begoude et al. 2010), but have not previously been reported from *E. ingens*.

Lasiodiplodia mahajangana is a recently described species from healthy branches of *Terminalia catappa* L. in Madagascar (Begoude et al. 2010). The current study represents only the second report of this fungus and very limited information is, therefore, available regarding its possible origin or importance. In this study *L. mahajangana* was isolated from blue stain in the wood of the main stem of an *E. ingens* tree. Blue stain is a common symptom of wood infected by species in the Botryosphaeriaceae, resulting from the dark colour of the mycelium of these fungi (Slippers and Wingfield 2007). This study thus suggests that *L. mahajangana* has a potentially wide distribution in Africa, including a diverse host range. Its impact and importance will, however, only become known with further surveys and more in-depth studies.

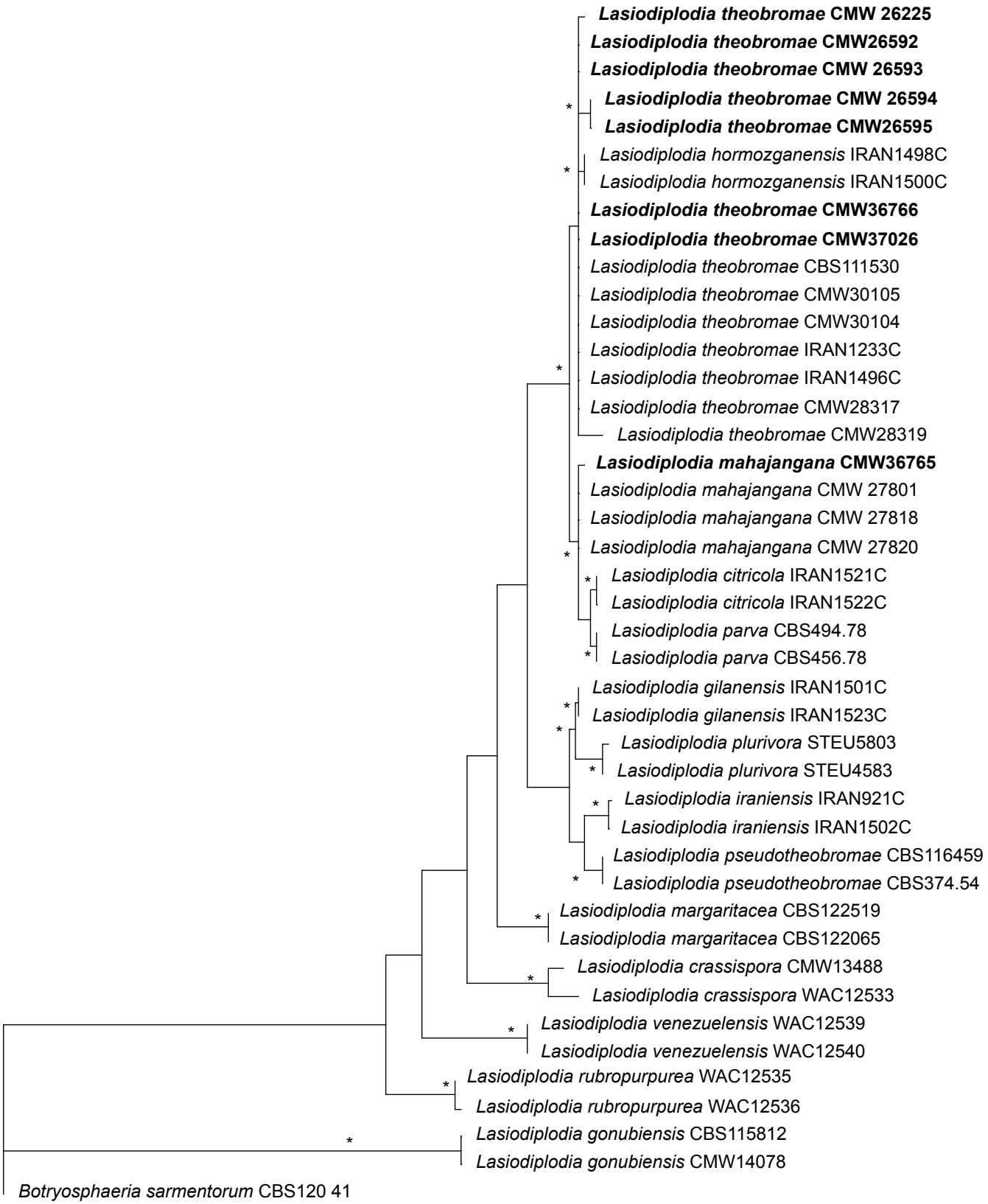

Figure 3: One of the most parsimonious trees obtained from maximum parsimony analysis of the combined sequences of ITS and TEF 1-α (complete) of representative taxa of *Lasiodiplodia*. Isolates in bold were collected in this study and stars at the nodes indicate posterior probabilities higher than 0.90

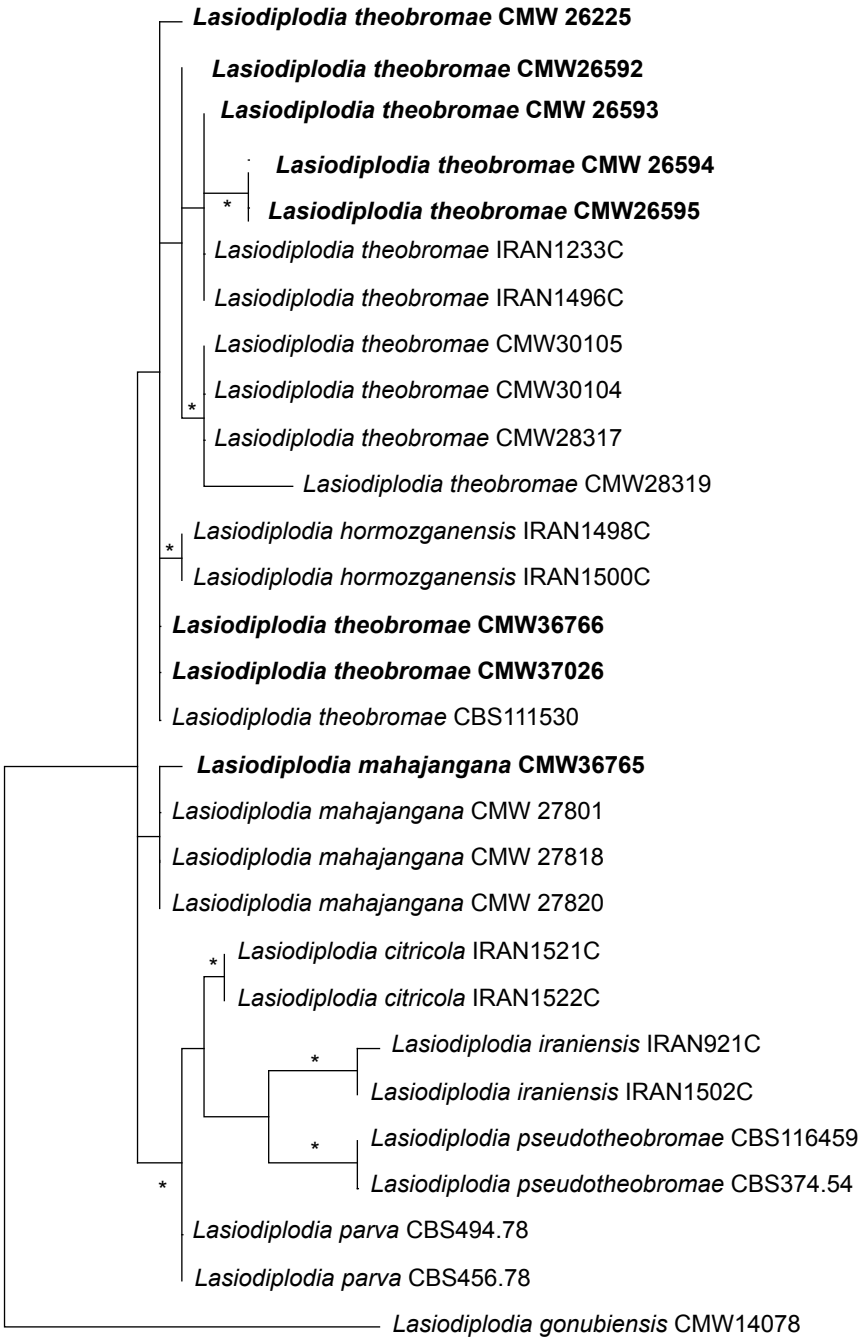

Figure 4: One of the most parsimonious trees obtained from maximum parsimony analysis of the combined sequences of ITS and TEF 1-α (clade specific) of the representative taxa of *Lasiodiplodia*. Isolates in bold were collected in this study and stars at the nodes indicate posterior probabilities higher than 0.90

Lasiodiplodia theobromae was obtained from diseased plant material and insects collected from the internal parts of dying *E. ingens* trees. These insects, *Cyrtogenius africus* (Curculionidae: Scolytinae), and a *Cossonus* sp. (Curculionidae: Cossoninae) were not surface-disinfected and inoculum of *L. theobromae* could have been on their surfaces or related to tissue that they had consumed and so occurring in their guts. The Botryosphaeriaceae typically disperse via rain splash and are not adapted to insect dispersal. However, species such as *L. theobromae* have been isolated previously from insects such as *Hypocryphalus mangiferae* Stebbing (Scolytinae) after surface sterilisation, implying that it might be carried in the gut or mycangia (Masood et al. 2010). Both insect families have previously been associated with succulent *Euphorbia* species (Jordal 2006, 2009).

Species delineation in the genus *Lasiodiplodia*, and particularly within what is known as *L. theobromae*, is

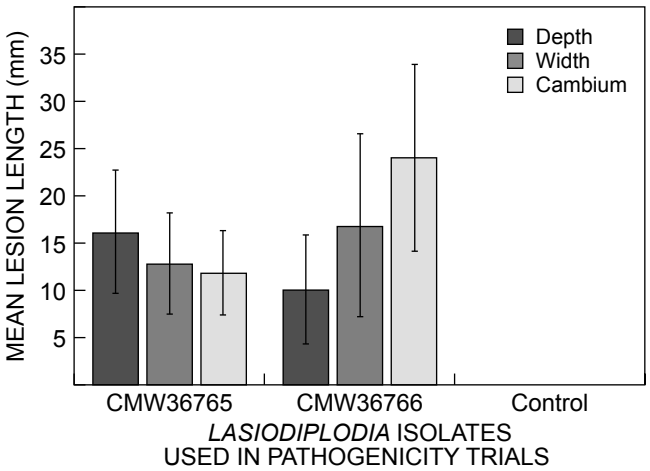

Figure 5: Histogram of mean lesion lengths (mm) resulting from inoculations with isolates of *Lasiodiplodia mahajangana* (CMW36765) and *L. theobromae* (CMW367660) used in the *Euphorbia ingens* pathogenicity trails. Bars indicate 95% confidence limits for each isolate

recognised to be problematic and in need of detailed re-evaluation. In the past three years, eight cryptic species have been described in the genus *Lasiodiplodia*, including several within what was previously known as *L. theobromae* (Alves et al. 2008, Pavlic et al. 2008, Abdollahzadeh et al. 2010, Begoude et al. 2010). Five years ago, only five species of *Lasiodiplodia* were known but this number has now reached 14. Phylogenetic analyses, based on ITS and TEF 1-α gene sequences, in the current study showed poor resolution in the *L. theobromae* clade (Figures 3 and 4) with limited differences between isolates of *L. theobromae* and *L. hormozganensis* Abdollahzadeh, Zare & A.J.L.Phillips. Similarly, conidial sizes for isolates obtained from *E. ingens* differed from those of previous descriptions of *L. theobromae* (Table 2). Additional analyses will be needed to resolve this group more clearly, either by including other gene regions or using specific microsatellite markers to consider the problem at a population level.

Inoculation studies, using *L. theobromae* and *L. mahajangana*, showed that both these fungi have the potential to cause disease on *E. ingens*. Both produced extensive internal rot of the succulent branches of these trees within six weeks. It was not surprising to find that *L. theobromae* and *L. mahajangana* were able to cause disease symptoms on *E. ingens*. Previously, *L. theobromae* was shown to be pathogenic to *Eucalyptus* clones (GC540) (Pavlic et al. 2007), grapevine (Úrbez-Torres et al. 2008, Úrbez-Torres and Gubler 2009) and *T. catappa* (Begoude et al. 2010). Similar to results in the study by Begoude et al. (2010), *L. mahajangana* produced smaller lesions to *L. theobromae* in the present study.

Conclusions

Since environmental and other stress factors play an important role in the epidemiology of diseases caused by fungi in the Botryosphaeriaceae, the symptoms observed on dying *E. ingens* trees in South Africa could, at least in part, be attributed to *L. theobromae* and *L. mahajangana*. A study by van der Linde (2011) indicated that increased temperature and decreased rain in the study areas in the Limpopo province over the last 40 years may be a possible stress factor for *E. ingens*. It does appear that a link to an environmental and/or an anthropogenic trigger has initiated the sudden and severe decline of these trees, in combination with a variety of pathogens and insects.

This study presents the first report of *Lasiodiplodia* on a succulent *Euphorbia* species. Further and more extensive surveys will be required to fully understand the diversity and distribution of Botryosphaeriaceae on native *Euphorbia* trees and to establish the possible triggers enabling these fungi to attack and thrive on these trees.

Table 2: Conidial measurements comparing *Lasiodiplodia theobromae* isolates from *Euphorbia ingens* and previous studies

Conidial size (μm)	Host	Reference
*20–30 × 10–15	Unknown	Punithalingam (1976)
26.2–28.8 × 14–14.4	Unknown	Alves et al. (2008)
22.5–26 × 12.5–15	*Terminalia catappa*	Begoude et al. (2010)
22.4–24.2 × 12.9–14.3	*Mangifera indica*	Abdollahzadeh et al. (2010)
18.1–21.3 × 11.6–13.3	*Euphorbia ingens*	This study

* Indicates the first description of the anamorph state of *Lasiodiplodia theobromae*

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