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The *Eucalyptus* shoot and leaf pathogen *Teratosphaeria destructans* recorded in South Africa

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Species of *Teratosphaeria* include some of the most important fungal pathogens of plantation-grown eucalypt trees. During routine disease surveys, symptoms and signs of leaf spot and blight were observed on the foliage of one-year-old *E. grandis* × *E. urophylla* hybrids in the Zululand region of KwaZulu-Natal, South Africa. These were distinct from those caused by the well-known and leaf-infecting fungus *Teratosphaeria suttonii*, which is not considered an important pathogen in the country. Culture and morphological characteristics as well as DNA sequences for three gene regions were used to compare the fungus isolated from the newly emerging symptoms with those for known *Teratosphaeria* species. DNA sequences were the same as those for *T. destructans* and this was consistent with the distinctive morphology of the asexual spores and the symptoms on leaves. *Teratosphaeria destructans* is an aggressive pathogen and actions will be needed to ensure that it does not impart serious losses to the local forestry industry.

Keywords: *Kirramyces*, leaf spot, *Phaeophleospora*, Teratosphaeriaceae

Introduction

Species of the fungal genus *Teratosphaeria* (Capnodiales, Teratosphaeriaceae) include important stem, shoot and leaf-blight pathogens of plantation-grown eucalypt trees in the tropics and Southern Hemisphere (Crous 1998; Park et al. 2000; Hunter et al. 2011). Of the leaf- and shoot-infecting species, *T. nubilosa* and *T. cryptica* are the most serious pathogens of eucalypt species grown in temperate areas, with *E. globulus* and *E. nitens* reported to be the most susceptible (Hunter et al. 2011). In tropical and subtropical regions of the world, *T. destructans*, *T. pseudoeucalypti* and *T. viscidus* are considered to be most destructive, causing disease on various species including *E. grandis* and its hybrids (Old et al. 2003b; Andjic et al. 2007b, 2010, 2011; Hunter et al. 2011).

Many eucalypt pathogens in the Teratosphaeriaceae were previously treated in the genus *Mycosphaerella* and its associated asexual genera included *Colletogloeopsis*, *Phaeophleospora*, *Kirramyces* and *Readeriella* (Crous et al. 2007; Hunter et al. 2011; Quaedvlieg et al. 2014). However, the development of DNA-based technologies and application of the 'one fungus = one name' convention (Hawksworth et al. 2011; Wingfield et al. 2012) has resulted in significant changes in the taxonomy of fungi, including those in this group. Consequently, the majority of *Eucalyptus* pathogens previously in *Mycosphaerella* and its asexual genera now reside in *Teratosphaeria* (Crous et al. 2009b; Quaedvlieg et al. 2014).

The lesion morphology and the severity of infections caused by various *Teratosphaeria* spp. on *Eucalyptus* can provide useful clues regarding the identification of the causal pathogen (Andjic et al. 2007c). However, overlapping symptoms and the occurrence of multiple species within the same lesions can also lead to incorrect identifications (Crous et al. 2009a). Lesion severity is often also influenced by host and environmental conditions leading to confusion (Crous 1998; Andjic et al. 2007c; Hunter et al. 2011). Morphology of the asexual and sexual (when present) structures can also be useful but sizes and shapes of structures overlap for many species (Hunter et al. 2004, 2011; Taole et al. 2012).

Various *Teratosphaeria* species have been reported associated with stem and leaf diseases of *Eucalyptus* in South Africa. Of these, *Teratosphaeria zuluensis* is one of the most important stem pathogens responsible for a canker disease that has resulted in significant losses to the forestry industry (Wingfield et al. 1996b; van Zyl et al. 2002; Cortinas et al. 2010). Numerous species are known on leaves and shoots but very few of these species have been responsible for serious disease problems (Crous 1998; Hunter et al. 2004, 2011). Extensive surveys conducted by Hunter et al. (2004) found six species of *Teratosphaeria* associated with leaf disease symptoms on various plantation-grown *Eucalyptus* species. Of these, *T. nubilosa* was the most dominant species causing disease, mainly on *E. nitens*, in the temperate areas of the KwaZulu-Natal (KZN), Limpopo and Eastern Cape provinces of South Africa. Another species widespread throughout South Africa is *Teratosphaeria suttonii* (Crous et al. 1988). This fungus

infects virtually all *Eucalyptus* species, including *E. grandis* (Crous et al. 1988; Taole et al. 2012). The pathogen is not considered to be important, usually associated with mature leaves and leaves of stressed trees in many Southern Hemisphere countries including South Africa (Knipscheer et al. 1990), but it has been reported to cause serious damage in Indonesia and Australia (Old et al. 2003b; Carnegie 2007).

Eucalyptus species are amongst the most important plantation tree species used to establish intensively managed plantations globally (Turnbull 2000). In South Africa, *Eucalyptus* plantations comprise approximately 47% of the total hardwood plantation areas. The majority of these are situated in the KZN province, with approximately 67 000 ha situated in the subtropical Zululand region of KZN (Anonymous 2014). Plantations in Zululand are mostly established by planting clones derived from hybrid crosses between *E. grandis* and other *Eucalyptus* species, mainly *E. camaldulensis* and *E. urophylla* (Denison and Kietzka 1993). The introduction of non-native pathogens and the adaptation of native pests and pathogens to non-native hosts pose serious threats to these plantations, as well as those globally (Wingfield et al. 2015), where they can result in severe damage and significant economic loss (Wingfield et al. 2008).

In early 2015 lesions were noted on foliage of one-year-old *E. grandis* × *E. urophylla* hybrids in the Zululand region of South Africa. Black pycnidia exuding conidia in cirri suggested that the infections were by a *Teratosphaeria* species. However, the symptoms differed from those usually observed for *Teratosphaeria* species known in the area. The aim of this study was to identify the *Teratosphaeria* species associated with the leaf blight symptoms observed.

Materials and methods

Leaves bearing leaf spot symptoms were collected from one-year-old *E. grandis* × *E. urophylla* hybrids growing in a clonal screening trial planted in the Zululand region of KZN. Samples were collected in brown paper bags, packed in plastic bags and transported to the laboratory for further investigation. Isolations from diseased tissue were made by transferring single cirri or pycnidia to malt extract (20 g L^{-1}) agar (MEA) (Biolab) using a dissection microscope and sterile needle. They were allowed to hydrate for 1–5 min before conidia were streaked across the surface of the agar and single conidia transferred to fresh MEA plates. Cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI; http://www.fabinet.up.ac.za), University of Pretoria, South Africa.

Fungal structures were characterised by moistening leaf lesions with a drop of water to hydrate the tissue. Under a dissection microscope, the lower epidermis was peeled away to reveal the fungal fruiting structures. These structures were lifted from the tissue and mounted in 85% lactic acid for microscopic examination. Pieces of dried leaf displaying visible symptoms of disease and black spore masses were placed in 10% KOH to rehydrate. These were trimmed into smaller samples (approximately 3 mm × 3 mm), mounted on a disc with Jung tissue freezing medium™, and sectioned using a Leica CM1100 Cryostat microtome. Cut sections were mounted on glass slides in water. The morphology of the fungal structures were studied using a Nikon Eclipse N*i* compound microscope and a Nikon SMZ 18 stereo microscope. Images were captured with a Nikon DS-Ri2 camera.

DNA was extracted from cultures using Prepman Ultra™ (Applied Biosystems) following the manufacturer's instructions. DNA was then used as template for the amplification of the second internal transcribed spacer and part of the 5.8S rDNA (ITS2), partial β-tubulin gene (βT) and partial translation elongation factor 1α (TEF) gene regions using primers ITS-3 and ITS-4 (White et al. 1990), T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and EF1-728F and EF1-986R (Carbone and Kohn 1999), respectively. PCR reactions were performed in a final reaction volume of 25 µl containing 5× MyTaq™ Reaction Buffer (Bioline), 0.2 mM of each of the region-specific primers and 1 U MyTaq™ DNA polymerase. The PCR amplification protocol described by Andjic et al. (2007b) was used but annealing temperatures were modified as follows: an annealing temperature of 50 °C was used for the ITS-2 and TEF gene regions and an annealing temperature of 54 °C was used to amplify the βT gene region. Products were separated using gel electrophoresis and visualised using GelRed™ (Biotium).

PCR products for sequencing were purified using 4 U FastAP thermosensitive alkaline phosphatase (Fermentas) and 20 U Exonuclease 1 (Fermentas) following the manufacturer's instructions. Fragments were sequenced, using forward and reverse gene-specific primers as described above, using the ABI Prism® Big Dye™ Terminator 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and purified with Centri-sep Sephadex G-50 spin columns (Princeton Seperations) following the recommended protocols. Sequences were determined with an ABI PRISM™ 3100 genetic analyser (Applied Biosystems). DNA sequences of opposite strands were edited and consensus sequences obtained using CLC Main workbench 6.9 (CLC bio; http://www.clcbio.com). Sequences were used in BLAST searches on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and included in a data set with related sequences for subsequent phylogenetic analyses (Table 1).

DNA sequences for the ITS, βT and TEF regions were aligned using the online version of MAFFT (Katoh and Standley 2013) with default settings. In addition to the individual data sets, the aligned sequence matrices were concatenated to form a matrix referred to as the Combined data set. A nucleotide substitution model that fitted each data set was determined using the Akaike information criterion in jModelTest 2.1.6 (Darriba et al. 2012) and incorporated in the subsequent phylogenetic analyses. Phylogenetic trees were generated using maximum likelihood and Bayesian inference of phylogenies. Phylogenies based on maximum likelihood were determined using PHYML 20120412 (Guindon et al. 2010) and employed custom models obtained from jModelTest. Bootstrap analyses (with 1 000 replications) were performed to obtain statistical support for the clusters generated in the fundamental trees.

Table 1: Host and locations for *Teratosphaeria* sequences considered in this study, including GenBank accession numbers for the three gene regions ITS, β-tubulin (βT) and translation elongation factor 1α (TEF)

Species	Culture no.[a]	Origin	Host	Genbank accession number		
				ITS	βT	TEF
T. destructans	CMW 44957	Zululand, South Africa	*E. grandis* × *E. urophylla*	KT343569	KT343563	KT343575
	CMW 44958	Zululand, South Africa	*E. grandis* × *E. urophylla*	KT343570	KT343564	KT343576
	CMW 44959	Zululand, South Africa	*E. grandis* × *E. urophylla*	KT343571	KT343565	KT343577
	CMW 44962	Zululand, South Africa	*E. grandis* × *E. urophylla*	KT343574	KT343568	KT343580
	CBS 111370	Indonesia	*E. grandis*	KF901574	KF903000	KF903301
	CMW 17919	Guangzhou, China	*E. urophylla*	DQ632701	DQ632622	DQ632729
T. pseudoeucalypti	MUCC 607	Queensland, Australia	*E. grandis* × *E. camaldulensis*	FJ793220	EU101542	EU101598
	MUCC 615	Queensland, Australia	*Eucalyptus* sp.	FJ793231	EU101556	EU101613
T. eucalypti	CMW 19453	Settlement Rd, New Zealand	*E. nitens*	FJ793234	EU101529	EU101585
	CMW 19455	Coxs, New Zealand	*E. nitens*	FJ793260	EU101571	EU101628
T. viscidus	CBS 121156, MUCC 452	Mareeba, Australia	*E. grandis*	EF031471	EF031483	EF031495
	CBS 121157, MUCC 453	Mareeba, Australia	*E. grandis*	EF031472	EF031484	EF031496
T. suttoni	CMW5348	Indonesia	*Eucalyptus* sp.	AF309621	DQ240117	DQ240170
	CBS 119973	Vietnam	*E. pellita*	KF901784	KF903055	KF903359
T. zuluensis	CBS 120301	South Africa	*E. grandis*	KF901735	KF903064	KF903368
	CBS 120302	South Africa	*E. grandis*	KF901736	KF903065	KF903369
T. molleriana	CBS 117927	Tasmania, Australia	*E. globulus*	KF901767	KF903030	KF903333
	CBS 117926	Australia	*E. globulus*	KF901766	KF903029	KF903332

[a] CBS = CBS Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC = Collection Pedro Crous housed at the CBS; MUCC = Murdoch University Culture Collection, Perth, Western Australia

Phylogenies based on Bayesian inference were obtained using MrBayes 3.2.3 (Huelsenbeck and Ronquist 2001). For the Combined data set, nucleotide substitution models specific to each of the partitions were incorporated in the analyses. Four Markov Chain Monte Carlo (MCMC) runs of 10 million generations and sampling size of every 100th tree were used to search tree space, after which 25% of the trees were discarded. The remaining trees from the individual runs were combined to form a consensus tree with posterior probability values at the nodes. Effective sampling size (ESS) values were assessed in Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) as a measure of convergence. Consensus trees and posterior probability values were viewed in FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

The leaf spot disease was first observed in a clonal trial planting (6 × 6 tree row plots, four replicates) near KwaMbonambi in KZN. Early symptoms of the leaf spot disease on 8- to 12-month-old *E. grandis* × *E. urophylla* hybrids were visible as chlorotic/bleached lesions (Figure 1a and d). Spots were irregular, circular or semi-circular and formed along midribs, veins or margins of the leaves. Older spots developed red-brown margins and black pycnidia exuding spores in brown to black cirri were abundant on the undersides of both juvenile and mature leaves (Figure 1b and e). Symptoms were visible in multiple plots and varied between clones. Most trees presented only leaf spots, but in a minority of cases entire leaves were affected, but no defoliation was encountered. Cultures obtained from fresh leaf symptoms were white to pink, producing black spore masses on the upper surfaces of the colonies (Figure 1c). On the reverse side, colonies were olive-green to black at their centres (Figure 1c).

Scattered sub-epidermal pycnidia were present in the spongy mesophyll (abaxial surfaces of the leaves) (Figure 1h). They were unilocular to occasionally multilocular, 102–129 × 95.5–179 μm. Conidiophores were reduced to conidiogenous cells, which were discrete, pale brown, verruculous, doliiform to subcylindrical, 6.5–8 × 3.5–5 μm. Conidia exuded in cirri formed black hydrophobic masses on the leaf surfaces (Figure 1g). They were subhyaline when young, becoming pale brown when mature, brown in mass, cylindrical, tapering toward the apex, with blunt bases and curved in various ways (Figure 1i). Conidia were verrucose (distinctly visible under ×1 000), 0–3-septate, septation inconspicuous and the middle septum mostly submedian, (55–) 71–74 (–88) × (1.5–)2–2.5(–3) μm (mean 72.2 × 2.5 μm).

BLAST searches of the different gene regions showed the South African sequences to be most similar to sequences belonging to *Teratosphaeria destructans* with 100% identity. Phylogenetic trees generated from the Combined data set grouped the newly collected isolates from South Africa with sequences belonging to *Teratosphaeria destructans* (ML bootstrap support = 98%, posterior probability (PP) = 1) (Figure 2). The South African isolates with *T. destructans* formed a sister group with *T. viscidus* (ML bootstrap support = 99%, PP = 1). Together, the isolates from South Africa, *T. destructans*, *T. viscidus, T. pseudo-eucalypti* and *T. eucalypti* formed a well-supported cluster (ML bootstrap support = 100%, PP = 1). This cluster was placed distant from *T. suttonii* and *T. zuluensis*, but without statistical support.

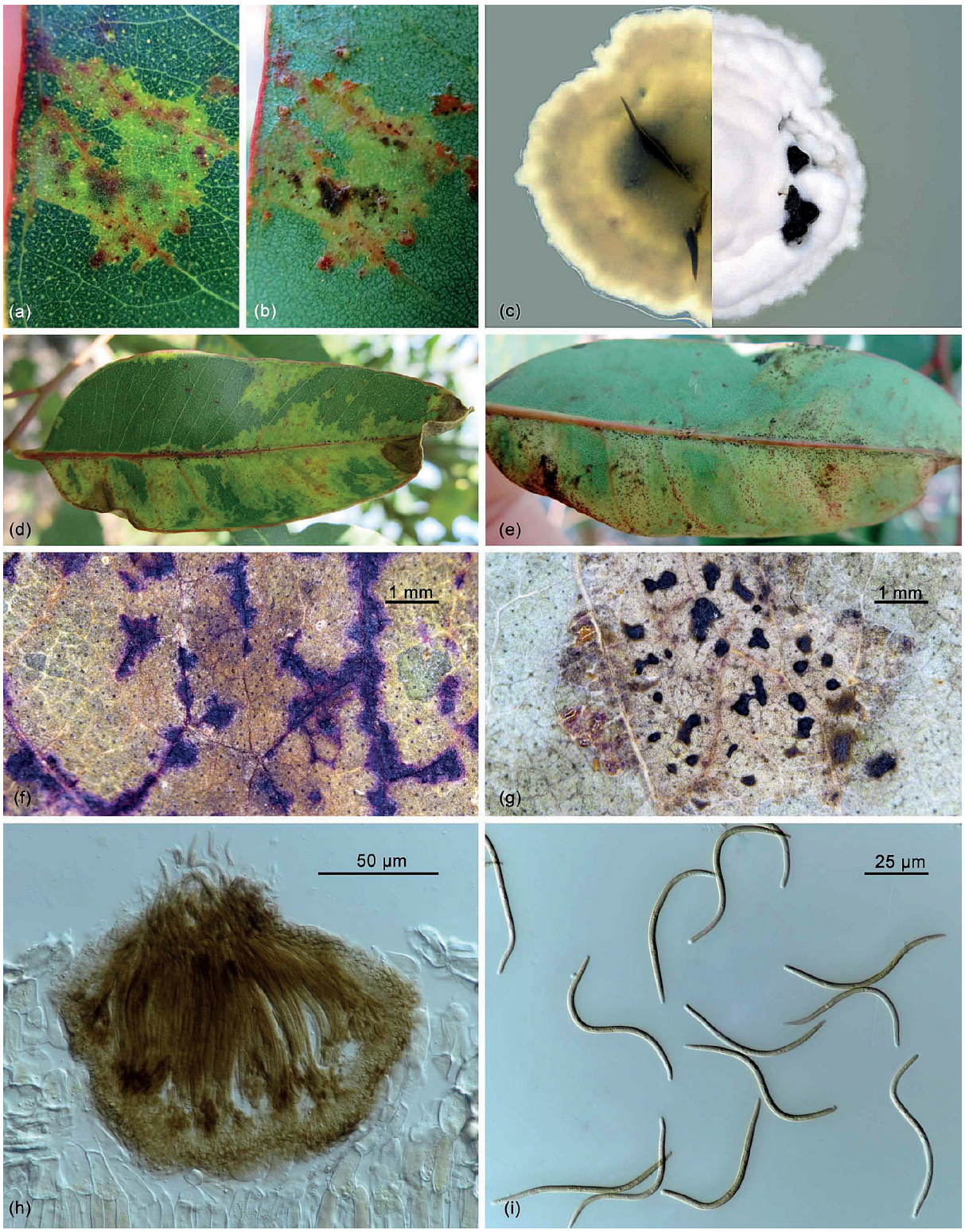

Figure 1: Leaf lesions of infected *E. grandis* × *E. urophylla* hybrid (a, b, d and e), culture characteristics on MEA (c), close-up of leaf lesion (f and g) and microscopic features of causal agent, *Teratosphaeria destructans* (h and i). (a and b) Early infection; (d and e) advanced infection. (f) Infected veins showing purplish discolourations on adaxial surface of leaf. (g) Black conidial masses formed on abaxial surface of leaf. (h) Cross-section of fungal fruiting structure embedded in spongy mesophyll. (i) Conidia. (a and d) Adaxial surface of leaf; (b and e) abaxial surface of leaf

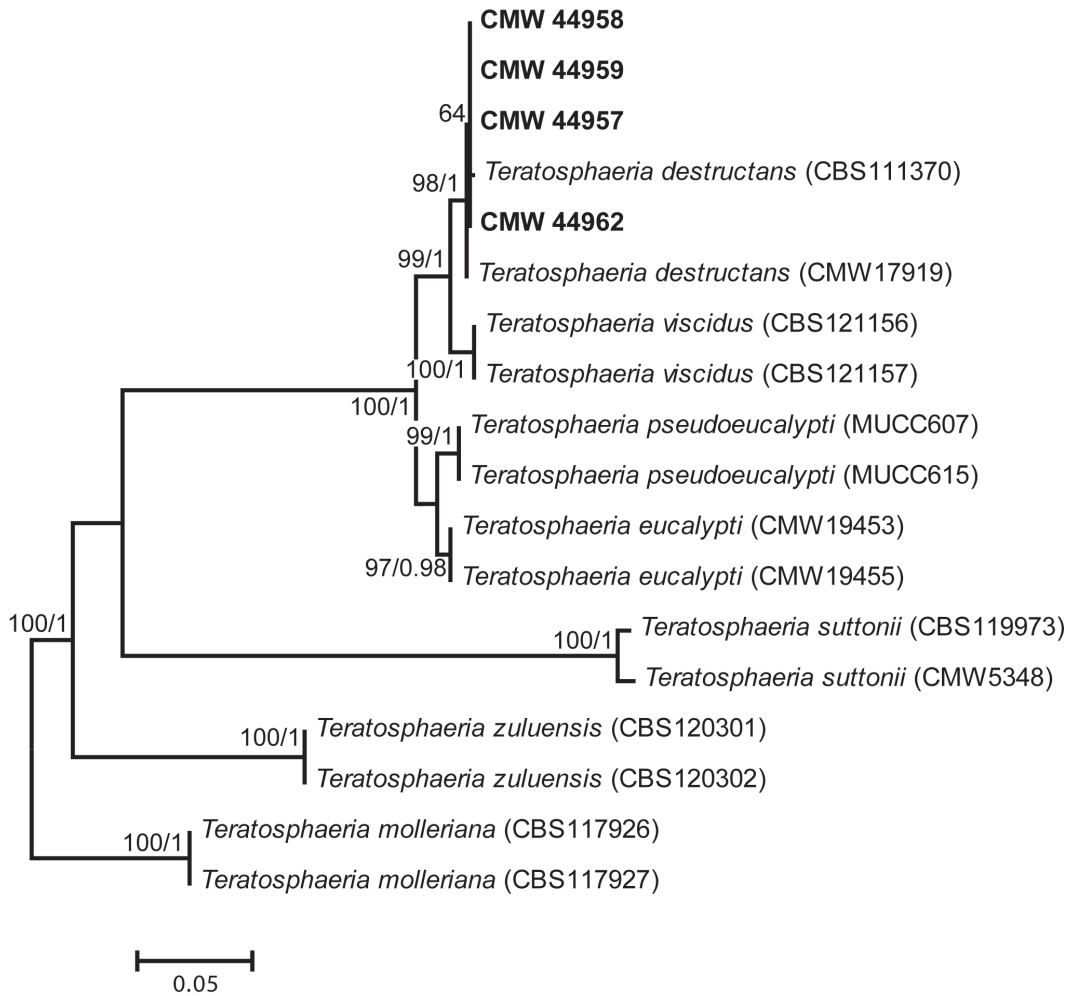

Figure 2: Maximum likelihood tree generated from combined ITS, βT and TEF sequences for isolates from South Africa and sequences for selected species of *Teratosphaeria*. Bootstrap values (>60%) and Bayesian posterior probabilities values (≥0.95) are shown at the nodes. The tree was rooted with *T. molleriana* as the outgroup species. Scale bar indicates the number of nucleotide substitutions per site

Discussion

The results of this study have shown conclusively that the destructive leaf and shoot pathogen *T. destructans* has appeared in South Africa and for the first time on *Eucalyptus* outside its previously known range in South-east Asia and Australia. This was verified based on conidial morphology, culture characteristics and phylogenetic comparisons for three gene regions. The discovery of *T. destructans* is of concern because of the damage that it has caused to *Eucalyptus* plantations elsewhere in the world and its recognition as one of the most destructive leaf pathogens of *Eucalyptus* species (Wingfield et al. 1996a; Andjic et al. 2011).

Teratosphaeria destructans was first described from Indonesia in the late 1990s (Wingfield et al. 1996a). It subsequently spread through South-east Asia to Thailand (Old et al. 2003a), Vietnam (Old et al. 2003a), Lao (Barber et al. 2012) and China (Burgess et al. 2006). In these countries, it has caused severe damage to susceptible *E. grandis*, *E. camaldulensis*, *E. urophylla* and various hybrids of these species (Wingfield et al. 1996a; Barber 2004). It was also reported from northern Australia in 2007 (Burgess et al. 2007), but this report has since been shown to be incorrect. *Teratosphaeria destructans*, therefore, has not yet been confirmed from Australia (TA Burgess pers. comm.). The most likely origin of the pathogen is Australia or Indonesia where eucalypts are native. This view is supported by the greater number of haplotypes found in isolates from Indonesia compared with Thailand, Vietnam and China (Burgess et al. 2006). The pattern of spread of *T. destructans* throughout these countries has been attributed to the movement of infected plant material, most likely nursery stock, as the primary pathway for its introduction into these areas (Andjic et al. 2011).

Some variation in morphology and DNA sequence data was observed for *T. destructans* isolates from South Africa compared with those of other regions. Conidia from diseased leaf material were, on average, slightly longer than those reported in previous studies (Wingfield et al. 1996a; Andjic et al. 2007a). There were no differences between sequences from South Africa and other

T. destructans sequences in the TEF gene region. Two base-pair differences were noted between sequences from South Africa and one other *T. destructans* sequences in the βT gene region, although sequence misreads could account for these differences. No differences were noted between South African ITS sequences and those from Indonesia, Thailand, Vietnam and China. This suggests that *T. destructans* in South Africa shares a similar origin to those from South-east Asia, but further studies are needed to confirm this supposition.

It is unknown as to how *T. destructans* might have entered South Africa. Neither is it known how long the pathogen might have been present in the country. It seems unlikely that its pathway of entry would have been on plant material, the movement of which is strictly regulated in the region. A more likely source of entry would have been with seed, which recently has been shown to carry *Teratosphaeria* spp. (Jimu et al. 2015). Seed importations of *Eucalyptus* spp. are common globally and important in order to broaden the genetic diversity of planting stock in new regions. Such new genetic material provides opportunities to avoid disease and pest problems but ironically also represents a threat in terms of new pest introductions.

An important cause for concern is the fact that once a pathogen has become established in a new region, the likelihood of its movement to new areas is substantially increased. This is well-known for *Eucalyptus* and is often referred to as a 'beachhead effect' (Wingfield et al. 2011; Garnas et al. 2012). Having now become established in South Africa, the possibility of *T. destructans* moving to other African countries as well as South and Central America, where *Eucalyptus* species are intensively propagated, is significantly increased. Surveillance, monitoring and other strategies to restrict such movement should be seriously considered.

It is not possible at the present time to predict how important *T. destructans* might become in South Africa. This will depend on many factors, including the environment and the susceptibility of planting stock. However, it is clear that one of the most important pathogens known on *Eucalyptus* has entered the country and that this will need to be considered seriously. The best possible management options will most likely lie in breeding and selection of resistant or tolerant planting stock (Wingfield et al. 2013). Observations in the area where this pathogen has appeared suggest that there are differences in susceptibility between clones. This is encouraging but it implies that costly screening procedures will need to be established and most likely that some valuable genetic material will inevitably be lost.

Conclusions

This study represents the first record of the serious leaf and shoot pathogen *Teratosphaeria destructans* from South Africa. It is unknown how long the pathogen has been present in the country, but regular surveys suggest that this is a relatively new introduction. Intensified surveys and monitoring efforts will be needed to determine the extent of infections in plantations and the impact that the pathogen might have. Differences in clone susceptibility have been observed in the field but screening studies will be necessary to identify disease-tolerant planting stock and understanding the genetic diversity of the pathogen in South Africa will inform issues such as those relating to durability of resistance. The arrival of *T. destructans* in South Africa enhances the likelihood that it will move further to areas, such as South and Central America, where *Eucalyptus* species are widely planted in tropical and subtropical regions. This is an important threat to commercial forestry and it deserves serious consideration as there is regular movement of seed between eucalypt-producing countries.

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