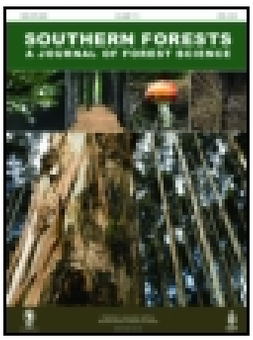

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Diseases of eucalypts in the central and northern provinces of Mozambique

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In the mid-1970s, Mozambique embarked on several forestry projects to promote and establish large plantation areas with eucalypt trees. The planted species included *Corymbia citriodora*, *Eucalyptus camaldulensis*, *E. saligna* and *E. tereticornis*. Similar to other regions of the world, pests and pathogens pose a significant threat to eucalypt plantations in Mozambique, but little is known regarding the diseases of trees in the country. The aim of this study was to provide the first detailed consideration of the possible disease problems affecting plantation-grown eucalypt species in the central and northern provinces of Mozambique. Isolates of fungal pathogens were obtained from material displaying disease symptoms collected during two field surveys conducted in 2010 and 2011. Representative fungal isolates were identified using DNA sequence data. Stem canker diseases caused by fungi in the Botryosphaeriaceae, Cryphonectriaceae and Teratosphaeriaceae, as well as leaf spots caused by species of *Calonectria*, *Pilidiella*, *Pilidium* and *Phakopsora myrtacearum* were commonly encountered. Their relative importance to the future of planation forestry in Mozambique is discussed.

Keywords: *Botryosphaeria*, *Calonectria*, *Chrysoporthe*, *Phakopsora*, *Teratosphaeria*

Introduction

Plantation forestry in Mozambique, particularly using eucalypt species, began towards the end of the nineteenth century (Ministério da Agricultura 2006). Since the independence of the country in 1975, the government has introduced several national forest projects in the Provinces of Gaza, Maputo, Nampula, Niassa, Sofala and Zambézia aimed at promoting the establishment of one million hectares of eucalypt plantations. Targeted species included *Corymbia citriodora*, *Eucalyptus camaldulensis*, *E. saligna* and *E. tereticornis* (Ministério da Agricultura 2006). These trees were planted to afforest degraded areas for soil conservation, to establish windbreaks and shelterbelts, to stabilise sand dunes and as amenity trees (Ministério da Agricultura 2006; FAO 2007). In 1999, it was estimated that the total area of non-native trees in Mozambique was c. 46 000 ha (Chitará 2003; Ministério da Agricultura 2006). Most of these areas were planted with *C. citriodora*, *E. camaldulensis*, *E. saligna*, *E. tereticornis*, *Pinus elliottii*, *P. patula* and *P. taeda*, in the central and northern provinces of the country (Ministério da Agricultura 2006). At the beginning of the twenty-first century, several private international forestry companies have been allowed access to land for plantation establishment in Mozambique. This has resulted in an additional 250 000 ha of eucalypts and *Pinus* species being established in the country (Ministério da Agricultura 2006; Impacto 2010; Overbreek 2010).

Similar to other regions of the world, pests and pathogens pose a significant threat to eucalypt plantations in Mozambique. Virtually no information is available regarding the diseases of trees in the country. Preliminary investigations of diseases of eucalypts in Mozambique in the period 2003–2004 (Roux et al. 2005; Nakabonge et al. 2006) identified several important tree pathogens and pests of eucalypts. The diseases included stem canker caused by *Chrysoporthe austroafricana*, occurring in the southern (Maputo) and central (Manica) provinces, *Chr. deuterocubensis* in the Manica province, *Teratosphaeria zuluensis* in the southern part of the country, and leaf diseases caused by species in the Capnodiales, including especially *Teratosphaeria suttonii*, in most of the areas inspected. Nakabonge et al. (2007) showed that isolates of *Chr. deuterocubensis* found in Mozambique had low genetic diversity, which suggests that the fungus was recently introduced to the country.

The aim of this study was to provide the first detailed consideration of disease problems affecting plantation-grown eucalypt species in the central and northern provinces of Mozambique. Putative fungal pathogens were identified to species level using multi-gene DNA sequence data.

Materials and methods

Field surveys

Surveys of eucalypt plantations in the central and northern provinces of Mozambique (Figure 1), including commercial stands, woodlots and nurseries, were conducted in July 2010 and August 2011 for the presence of diseases. The average rainfall for the sampled regions varies between 800 to 1 200 mm per annum and the mean annual temperatures between 18 and 28 °C. Specific compartments within larger afforested areas were selected based on age and species, to obtain a diversity of material for investigation. Areas for

sampling were also chosen based on reports of disease problems by foresters and farmers. Where available, species and clonal trial blocks were included in the study.

Samples collected included leaves, twigs, pieces of bark, segments of stems, and roots showing symptoms of disease or insect infestation. All the samples were placed in brown paper bags, which were sealed in larger plastic bags to retain moisture. Samples were processed on the same day where possible, but material that could last longer was transported to a laboratory for later processing. All samples that were not processed during the field trips were kept in cool dry conditions, or in a refrigerator, at approximately 4 °C. Soil samples were also collected from two different nurseries, Manica (IFLOMA/KLF) and Zambézia (ATFC Madeira e Agricultura Lda).

Isolations and preparation of cultures

Standard isolation media and techniques were used to grow putative pathogens associated with the observed disease symptoms. Pure cultures of all isolates considered in this study have been maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Leaf samples

To isolate fungi in the Mycosphaerellaceae and Teratosphaeriaceae (Capnodiales), small lesions with fruiting bodies, typical of symptoms caused by these fungi, were taken from leaves and placed in petri dishes with sterile water for 2 h. Using double-sided adhesive tape, the lesions were attached to the lids of 60 mm petri dishes above 2% malt extract agar (MEA) (20 g L^{-1} agar and 15 g L^{-1} malt extract, Biolab, Johannesburg, South Africa) containing 100 mg L^{-1} streptomycin sulphate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). These were incubated, upside down, at room temperature, for 24 h as described by Crous (1998) to allow ascospores to be shot upwards and onto the agar surface. Single germinating ascospores were individually transferred onto 2% MEA and pure cultures were obtained by transferring them to fresh plates containing 2% MEA and incubating plates at 25 °C under cool white light for 30 d.

Leaves showing blight symptoms, or where no fruiting bodies were visible, were examined under a dissection microscope and then placed in moist chambers and incubated at 25 °C to promote sporulation of fungi. Where present, single spore drops, or spore tendrils were transferred from conidiophores to 2% MEA and incubated for 8 d at 25 °C under continuous near-ultraviolet (UV) light.

Figure 1: Map of Mozambique indicating provinces where surveys for fungal pathogens on eucalypts were carried out (areas indicated by dark circles)

Stem and branch samples

To isolate possible pathogens from stem and branch samples, fungi were either isolated directly from fruiting bodies on the bark, or by transferring sections of plant material to agar. Where fungal fruiting bodies were present, single spore drops were transferred, using a sterile needle, onto 2% MEA and incubated at 25 °C until the onset of fungal growth. For some fungi, fruiting bodies were cut open using a sharp, sterile scalpel blade, and then exposed spore masses were transferred to sterile 2% MEA. Where no fresh spore drops were visible, tissue was incubated in moist chambers to induce spore production. Where no fungal fruiting bodies were present on lesions or cankers on symptomatic stem and branch samples, pieces of diseased tissue were plated onto 2% MEA. This was done by surface-disinfesting lesions with 70% ethanol and removing the epidermis to expose the fresh lesions. Small pieces of plant material (<5 mm) were taken from the leading edges of cankers and plated onto 2% MEA, after which the plates were incubated at 25 °C.

Soil samples

For isolations of fungi in the Nectriaceae from the soil collected in nurseries, alfalfa seeds were allowed to germinate in the soil, followed by incubation for 3 d at 25 °C under continuous near-UV light. Thereafter, isolations were made from alfalfa roots showing symptoms of infection by fungi, following the methods described by Crous (2002).

Fungal identification

For the identification of fungi obtained from disease symptoms, use was made both of morphological characteristics for each putative pathogen group, as well as DNA sequence data. Sequencing was considered necessary to identify pathogens accurately to the species level. Representative isolates of all possible pathogens and all morphotypes of unknown fungi, from different eucalypt species and geographic regions, were selected for identification based on DNA sequencing.

DNA extraction

For DNA extraction, mycelium was separately harvested from actively growing cultures of the selected isolates and placed in 1.5 ml sterile Eppendorf tubes and freeze dried overnight. Mycelium was then ground to a fine powder using sterile metal beads on a Mixer Mill (Type MM 301, Retsch® Tissue Lyser, Retsch, Germany) for 2 min at 30 cycles per second. DNA was extracted and purified using the cetyl trimethyl ammonium bromide (CTAB) method, described by Möller et al. (1992). The nucleic acids were pelleted using centrifugation and washed with 70% ethanol, followed by suspension in sterilised distilled water. Two microliters of RNaseA (10 μg μl^{-1}) were added to each tube and incubated at room temperature for 24 h to digest any residual RNA. The concentrations of the extracted DNA were determined using a Nanodrop ND-1000 Spectrophotometer V3.6 (Thermo Fisher Scientific, Wilmington, DE, USA).

PCR amplification and purification

The polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS1 and ITS2) regions, including the 5.8S gene of the ribosomal RNA (rRNA) operon, with the primer pair ITS1 and ITS4 (White et al. 1990) for all isolates. Depending on identities based on the ITS and 5.8S sequence results, sequence data were also obtained for the β-tubulin 1 and β-tubulin 2 regions (Bt) with primers Bt1a/1b, Bt2a/2b (Glass and Donaldson 1995) and T1/Bt2b, T10/Bt2b (O'Donnell and Cigelnik 1997), the translation elongation factor 1-α (TEF-1α) gene region with primers EF1-728F/EF1-986R (Carbone and Kohn 1999), the Histone 3 (His 3) gene region with primers H31a/1b (Glass and Donaldson 1995), the Calmodulin gene region with primers Cal228F/I737R (Carbone and Kohn 1999) and the actin (ACT) gene region with primers ACT-512F/783R (Carbone and Kohn 1999). The PCR reaction mixtures used to amplify the different loci consisted of 2.5 U FastStart *Taq* polymerase (Roche Applied Science, Indianapolis, IN, USA), 1× PCR buffer, 1–1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μm of each primer and approximately 50–100 ng fungal genomic DNA, made up to a total reaction volume of 25 μl with sterile deionised water. The amplification conditions included an initial denaturation of the double-stranded DNA at 96 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, annealing for 1 min at 54 °C to 58 °C (depending on primers and pathogens) and extension for 90 s at 72 °C, and a final elongation step of 10 min at 72 °C. The PCR amplification products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide in TAE buffer and visualised under UV light. Amplified fragments were purified using Centri-Sep mini spin columns (Princeton Separations, Adelphia, NJ, USA) containing 6% Sephadex G-50 (Sigma, Steinhein, Germany) following the manufacturer's instructions.

DNA sequencing and phylogenetic analyses

The purified PCR products were used as template DNA for cycle sequencing reactions using an iCycler Thermal Cycler, which generated sequences in both (forward and reverse) directions using 10 μl mixes. The composition of the mixtures was 2 μl Sabax water, 2 μl ready reaction buffer (BigDye), 1 μl 5× reaction buffer, 1 μl primer (10 mM) and 4 μl PCR product. The BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) was used for sequencing reactions. For all pathogens studied, additional sequences for comparison were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and TreeBASE (http://www.treebase.org). Sequence alignments were compiled using the online interface (http://align.bmr.kyushu-u.ac.jp/mafft/software/) of MAFFT 5.667 (Katoh et al. 2002), incorporating the G-INS-i alignment algorithm. All sequence alignments were checked and adjusted manually where necessary.

The software PAUP* 4.0 (Swofford 2002) was used to reconstruct phylogenies from the aligned sequences. Partition homogeneity tests (PHT) (Farris et al. 1994) were done for the multi-gene sequence data sets of each pathogen group to determine whether these data sets could be combined for analyses. For phylogenetic analyses, all gaps were coded as missing data and characters were assigned equal weight. The heuristic search option with random stepwise addition and tree bisection reconnection (TBR) was used as the swapping algorithm. The Mulpar option was in effect and branches collapsed if they equalled zero. Confidence levels of the branching points were determined using 1 000 bootstrap replications (50% majority rule). For phylogenetic analyses, out-group taxa were defined and treated as paraphyletic sister groups to the in-groups. In parsimony analyses, the tree length (TL), retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy indices (HI) were determined.

Results

Field surveys

Several disease symptoms were observed on plantation-grown eucalypt trees in Mozambique. Diseases encountered included stem cankers, leaf spots/blights and rot of cuttings. Stem canker symptoms (Figure 2) on *Eucalyptus* species included those typical of the Cryphonectriaceae (Figure 2a–c) on *E. grandis* and *Eucalyptus* species in Manica (Ifloma and Messica) and Zambézia (Gurué), Teratosphaeria stem canker (Figure 2d) caused by *T. zuluensis* in Zambézia and Botryosphaeria stem canker (Figure 2e and f). Stem canker symptoms typical of those caused by species of *Valsa* were observed in Sofala. The symptoms mentioned above were observed in forestry plantations on trees between the ages of 2- to 25-years old. Signs and symptoms of ophiostomatoid fungal infection, long-necked black ascomata and sap stain were observed on stem wounds on *Eucalyptus* species in Zambézia (Gurué).

Leaf symptoms were commonly observed during the study. These included symptoms of a rust disease caused by *Phakopsora myrtacearum* (Figure 3a), symptoms of Calonectria leaf blight (Figure 3b) on *Eucalyptus grandis* × *E. camaldulensis* (GC) clones in Manica, leaf spot symptoms typical of species in the Capnodiales (Figure 3c) on *E. grandis* × *E. urophylla* (GU) and several *Eucalyptus*

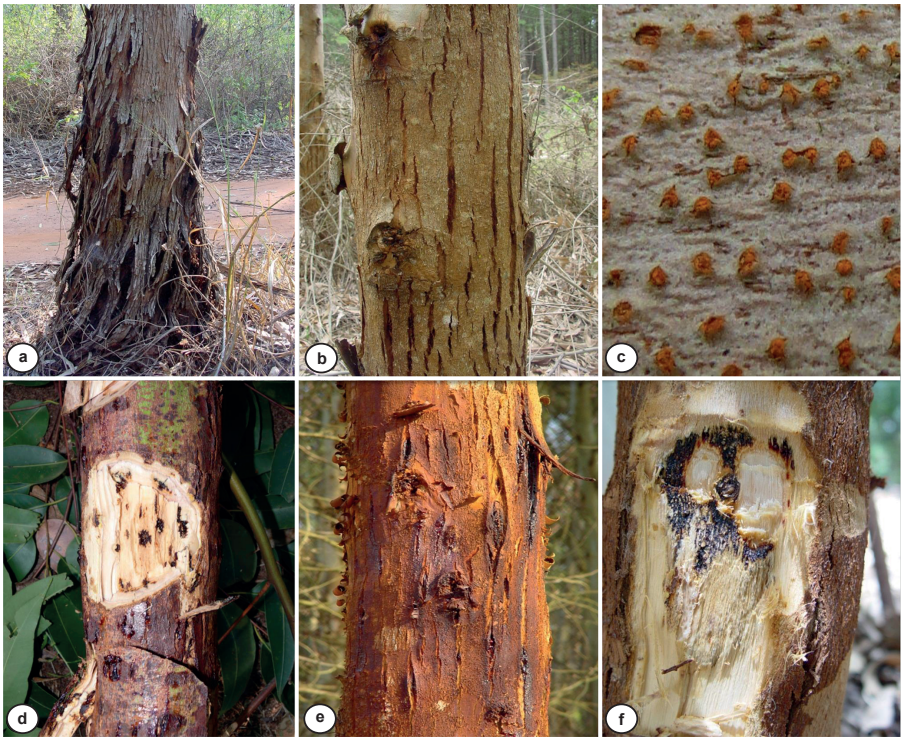

Figure 2: Symptoms of fungal stem canker diseases of eucalypts in northern and central Mozambique. (a) Bark cracking and swelling at the base of a tree caused by *Chrysoporthe austroafricana*, (b) superficial bark cracks caused by *Holocryphia eucalypti*, (c) orange fruiting bodies of *Holocryphia eucalypti*, (d) spots, kino exudation and internal kino pockets caused by *Teratosphaeria zuluensis*, (e) bark cracking, kino exudation and (f) internal kino ring caused by *Neofusicoccum kwambonambiense* and *N. parvum*

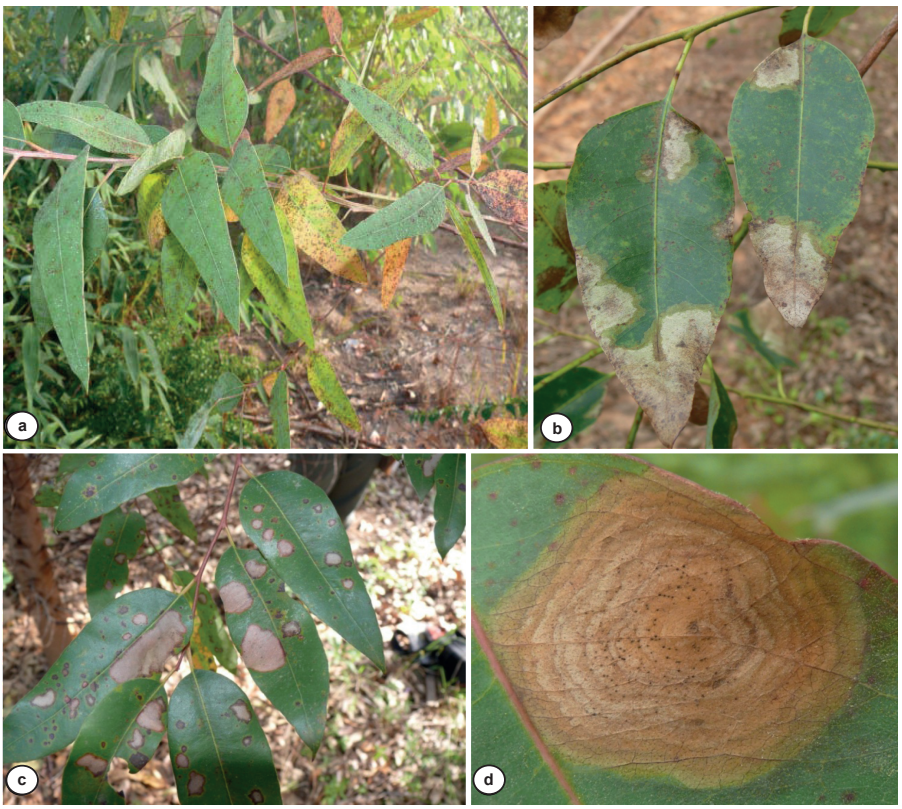

Figure 3: Symptoms of fungal leaf diseases of *Eucalyptus* spp. in Mozambique. (a) Leaf spot and discolouration caused by the rust fungus *Phakopsora myrtacearum*, (b) leaf blight caused by *Calonectria mossambicensis*, (c) leaf spot caused by *Mycosphaerella acaciigena* and (d) Pilidiella leaf spot showing typical concentric rings on leaf surface

species in Manica, Nampula, Niassa and Zambézia provinces, *Pilidiella* species (Figure 3d) in Nampula and Sofala, and *Pilidium* species in Sofala. Diseases in eucalypt nurseries in Manica, Nampula and Zambézia included leaf blight and cutting rot in Manica and Zambézia provinces. Leaf spot caused by *T. suttonii* was also common in all nurseries; however, damage was restricted to older leaves and was not of economic importance.

Fungal identification

DNA sequencing and phylogenetic analyses

The fungal pathogens obtained from diseased *Eucalyptus* species in Mozambique were identified based on multiple-genomic region analyses. In phylogenetic analyses both individual genome regions, as well as combined genome regions were considered (Tables 1 and 2). Partition homogeneity tests were conducted prior to combination of data from different regions. This was done for the Cryphonectriaceae (β-tubulin and ITS) and Nectriaceae (CAL, His, ITS and TEF-1α) groups of fungi. The PHT resulted in a *P*-value of 0.01 for both the Nectriaceae and the Cryphonectriaceae. This *P*-value is lower than the conventionally accepted value of 0.05 required for combining data, but *P*-values greater than 0.001 have been accepted in several studies (Cunningham 1997; Dettman et al. 2003).

Stem canker pathogens in the Cryphonectriaceae were identified as belonging to the genera *Chrysoporthe* and *Holocryphia* (Figure 4). *Chrysoporthe austroafricana* was detected on *E. grandis* in the Manica and Zambézia provinces and *Eucalyptus* species in the Zambézia province. *Chrysoporthe deuterocubensis* was identified from *E. grandis* in Zambézia province and *Holocryphia eucalypti*

Table 1: Identity and GenBank accession numbers of fungal pathogens obtained from *Eucalyptus* species in Mozambique

Fungal species	Isolate number	Origin	GenBank accession number[a]					
			ITS	TEF	CAL	His3	BT1	BT2
Calonectria mossambicensis	CMW36327	Manica	JX570730	JX570718	JX570722	JX570726		
C. mossambicensis	CMW36329	Zambézia	JX570729	JX570717	JX570721	JX570725		
C. mossambicensis	CMW36331	Manica	JX570728	JX570716	JX570720	JX570723		
C. mossambicensis	CMW38040	Manica	JX570727	JX570715	JX570719	JX570723		
Chrysoporthe austroafricana	CMW36293	Zambézia	KC915394				KF143807	KF156754
Chr. austroafricana	CMW36311	Zambézia	KC915392				KF143805	KF156756
Chr. austroafricana	CMW36315	Zambézia	KC915393				KF143806	KF156755
Chr. austroafricana	CMW37259	Manica	KC915391				KF143804	KF156752
Chr. deuterocubensis	CMW36312	Zambézia	KC915395				KF143808	KF156753
Holocryphia eucalypti	CMW37257	Manica	KF156758				KF156757	KF309270
Huntiella savannae	CMW37494	Zambézia	KJ778643	KJ925135			KJ939695	
H. savannae	CMW37471	Zambézia	KJ778642	KJ925134			KJ939696	
Mycosphaerella acaciigena	CMW36322	Zambézia	KF420413					
M. acaciigena	CMW38312	Niassa	KF420412					
Mycosphaerella sp.	CMW37288	Niassa	KF420415					
Mycosphaerella sp.	CMW37296	Niassa	KF420414					
M. intermedia	CMW37290	Niassa	KF420416					
Neofusicoccum kwambonambiense	CMW37256	Nampula	KF432944	KF454685				KF454699
N. kwambonambiense	CMW37291	Niassa	KF432948	KF454689				KF454703
N. kwambonambiense	CMW37298	Niassa	KF432945	KF454686				KF454700
N. kwambonambiense	CMW38311	Manica	KF432949	KF454690				KF454704
N. parvum	CMW37251	Zambézia	KF432950	KF454691				KF454705
N. parvum	CMW37263	Nampula	KF432951	KF454692				KF454706
Neofusicoccum sp.	CMW37237	Manica	KF432946	KF454687				KF454701
Neofusicoccum sp.	CMW37260	Manica	KF432947	KF454688				KF454702
Ophiostoma tsotsi	CMW36317	Zambézia	KF143801					
O. tsotsi	CMW36318	Zambézia	KF143803					
O. tsotsi	CMW36319	Zambézia	KF143802					
Pilidiella eucalyptorum	CMW36324	Nampula	KF060275					
P. eucalyptorum	CMW36334	Sofala	KF060274					
Pilidium concavum	CMW36330	Sofala	KF060276					
P. concavum	CMW37238	Sofala	KF060278					
P. concavum	CMW37239	Sofala	KF060279					
P. concavum	CMW37240	Sofala	KF060280					
P. concavum	CMW37243	Sofala	KF060281					
Teratosphaeria zuluensis	CMW36320	Zambézia	KF454697	KJ925133				
T. zuluensis	CMW37252	Zambézia	KF454693	KJ925132				
Valsa variostromatica	CMW37242	Manica	KF309269					

[a] ITS = internal transcribed spacer (ITS1 and ITS2) and the 5.8S gene of the rRNA operon, TEF = translation elongation factor 1-α, CAL = Calmodulin, His3 = Histone 3, BT1 = β-tubulin 1, BT2 = β-tubulin 2

Table 2: Statistics from phylogenetic analyses of fungal pathogens identified from *Eucalyptus* species in Mozambique

Pathogen group	Data set	No. of taxa	No. of bp[a]	Maximum parsimony[b]					
				PIC	No. trees	Tree length	CI	RI	HI
Cryphonectriaceae	β-tubulin/ITS	30	1 352	355	48	322	0.854	0.908	0.146
Huntiella	β-tubulin/TEF/ITS	22	1 373	1 040	1	2 959	0.750	0.849	0.250
Mycosphaerella	ITS	24	475	128	1	200	0.825	0.929	0.175
Neofusicoccum	β-tubulin/ITS/TEF	24	1 232	160	17	195	0.887	0.926	0.113
Ophiostoma	β-tubulin	44	226	68	10	195	0.641	0.892	0.359
Pilidiella	ITS	19	517	75	1	99	0.838	0.936	0.162
Pilidium	ITS	14	422	79	1	98	0.939	0.968	0.061
Teratosphaeria	β-tubulin/TEF/ITS	22	777	153	6	184	0.891	0.950	0.109
Valsa	ITS	30	577	121	255	370	0.522	0.751	0.478

[a] bp = Base pairs

[b] PIC = Number of parsimony informative characters, CI = consistency index, RI = retention index, HI = homoplasy index

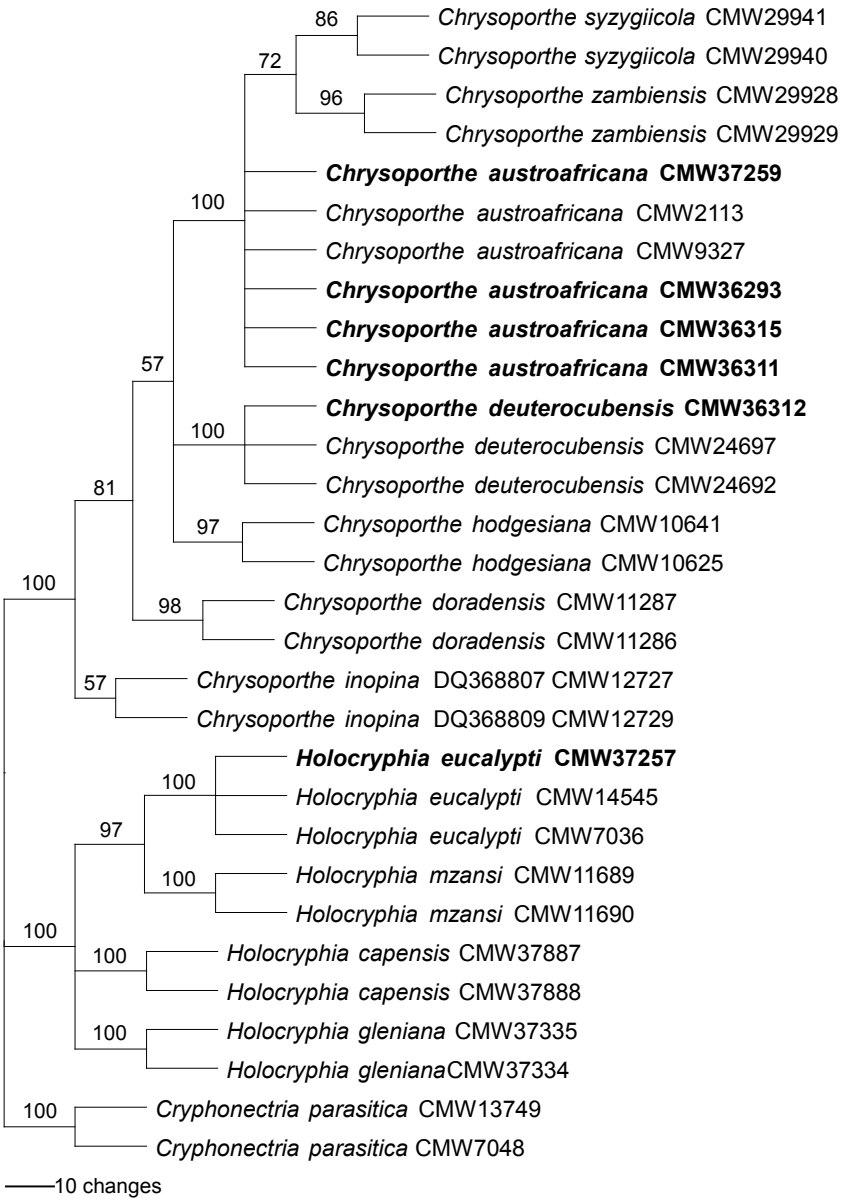

Figure 4: Phylogram of the Cryphonectriaceae indicating the phylogenetic positions of isolates from Mozambique (in bold). The most parsimonious tree obtained from a heuristic search of the combined β-tubulin (tub2 and tub1) and ITS regions of the rDNA sequence data (TL = 469, CI = 0.938, RI = 0.988, RC = 0.927, HI = 0.062). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Cryphonectria parasitica*

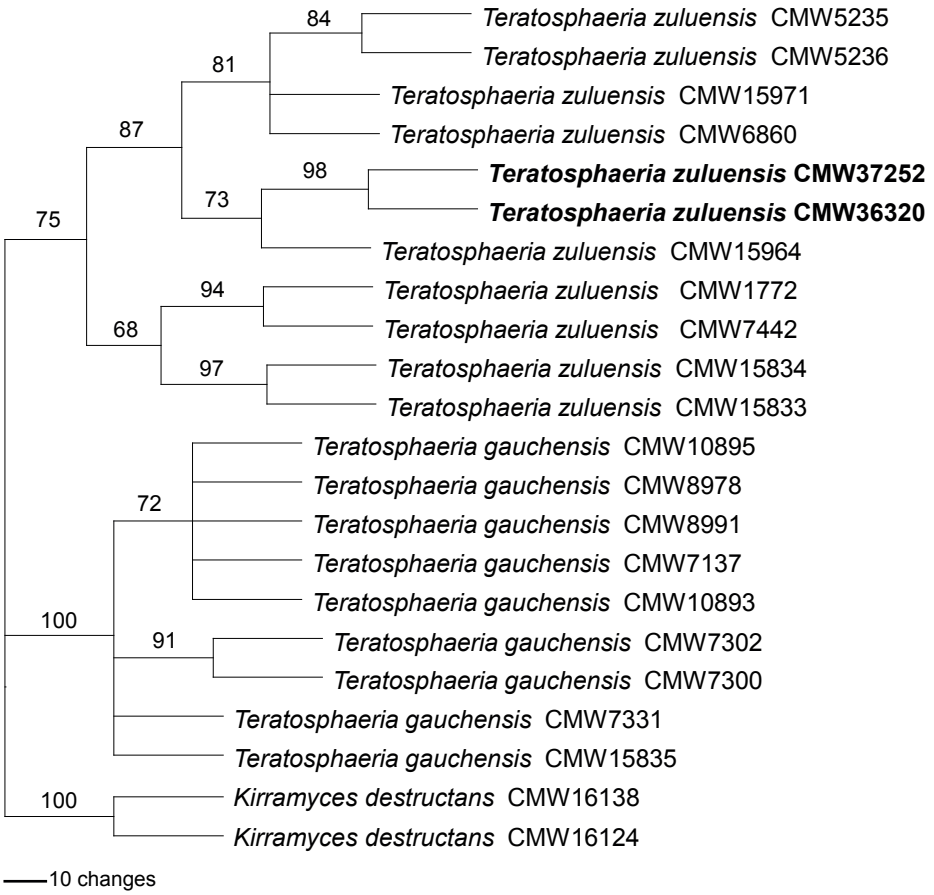

Figure 5: Phylogram of Teratosphaeriaceae species indicating the phylogenetic positions of the fungus *Teratosphaeria zuluensis* and two undescribed species occurring on *Eucalyptus* in Mozambique. The most parsimonious tree obtained from a heuristic search of the combined β-tubulin, ITS and TEF-1α gene regions of the rDNA sequence data (TL = 184, CI = 0.891, RI = 0.950, RC = 0.847, HI = 0.109). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. Isolates sequenced in this study are in bold

on a GC clone in Manica. These pathogens were identified based on β-tubulin and ITS gene sequence data.

Coniothyrium (Teratosphaeria) stem canker in central and northern Mozambique was found to be caused by *Teratosphaeria zuluensis* (Figure 5). This pathogen was identified from *E. grandis* (Niassa province) and from a *Eucalyptus* sp. (Zambézia province). A stem canker disease caused by *Valsa variostromatica* (Figure 6) was identified from *Eucalyptus* in the Manica province. In the Botryosphaeriaceae group (β-tubulin, ITS and TEF-1α), samples collected in Mozambique grouped with *Neofusicoccum kwambonambiense* from *E. camaldulensis* (Ribaué/ Nampula), *E. grandis* (Lichinga/Niassa) and *Eucalyptus* species (Manica and Niassa). Isolates from three other samples were related to *N. parvum* on *E. grandis* and *Eucalyptus* sp. in Manica province, and on *Eucalyptus* sp. in Nampula and Zambézia (Figure 7).

Several leaf pathogens were identified from leaf spots on *Eucalyptus* species in Mozambique. Based on sequence data of the ITS genome regions, the causal agents of Mycosphaerella leaf blotch (MLB) disease on *Eucalyptus* species in the surveyed areas was identified as being most closely related to *Mycosphaerella acaciigena* (Figure 8) on *E. grandis* (Niassa) and GU clones (Niassa and Zambézia). *Pillidiela eucalyptorum* (Figure 9) was identified from *Eucalyptus* species in Nampula and Sofala provinces and *Pilidium concavum* (Figure 10) from *Eucalyptus* species in Sofala province. The recently described rust fungus, *P. myrtacearum*, was common on *Eucalyptus* species in Manica, Niassa, Sofala and Zambézia provinces.

The fungus isolated from eucalypt stem cuttings in nurseries at Manica and Zambézia provinces resembled a species of *Calonectria*. Isolates collected in the Nectriaceae did not group with any known clade, suggesting that they represented a previously undescribed species. This species was recently described as *C. mossambicencis* (Maússe-Sitoe et al. 2013) based on multiple gene sequence data.

Material collected from wounds and cut stumps of trees grouped into two broad groups, the Ophiostomatales and the Microascales. Isolates of fungi in the Ophiostomatales, from *Eucalyptus* sp. in Gurue (Zambezia), were identified as *Ophiostoma tsotsi* based on β-tubulin sequence data (Figure 11). Those in the Microascales, from *Eucalyptus* sp. in Zambézia province, belonged to the genus *Huntiella* and were identified as *H. savannae* based on β-tubulin, ITS and TEF-1α sequence data (Figure 12).

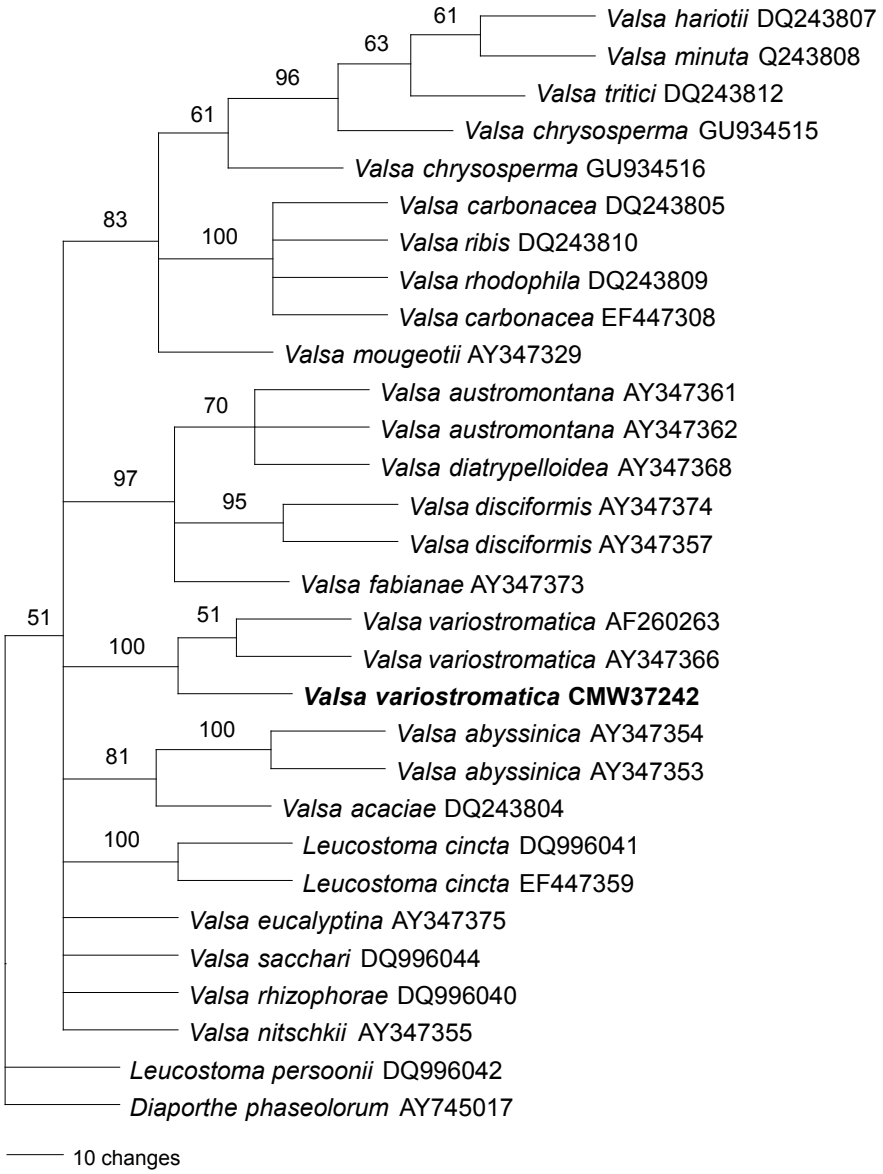

Figure 6: Phylogram of *Valsa* species indicating the phylogenetic position of the isolates occurring on *Eucalyptus* species in Mozambique. The most parsimonious tree obtained from a heuristic search of the ITS1 and ITS2 regions of the rDNA sequence data (TL = 370, CI = 0.522, RI = 0.751, RC = 0.392, HI = 0.478). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Diaporthe phaseolorum*. Isolates sequenced in this study are in bold

Discussion

This study represents the first species-level identification of fungal pathogens or putative pathogens of plantation-grown *Eucalyptus* species in the central and northern provinces of Mozambique. It is also the most extensive investigation of eucalypt tree diseases for the country, following the preliminary investigations in 2003 and 2004 (Roux et al. 2005; Nakabonge et al. 2006). Most of the pathogens identified in this study represent first reports for Mozambique. Consequently, these results will contribute to a better understanding of the impact, distribution and origin of these pathogens in Africa and worldwide.

Three species of stem canker pathogens in the Cryphonectriaceae, *Chr. austroafricana*, *Chr. deuterocubensis* and *H. eucalypti*, were obtained from *Eucalyptus* trees in central and northern Mozambique. This is the first report of *H. eucalypti* from Mozambique. The disease associated with this pathogen was not common and was observed only in Manica province, causing minor and superficial damage to tree bark. Species in *Holocryphia* were recently revised by Chen et al. (2013) and *H. eucalypti* is now known from Mozambique, South Africa, Swaziland and Uganda. *Chr. austroafricana* and *Chr. deuterocubensis* have both previously been reported from Mozambique (Roux et al. 2005) and results from this study show that *Chr. austroafricana* is widespread in the country. In contrast, *Chr. deuterocubensis* was found only in Zambézia province. All available evidence suggests that *Chr. austroafricana* is native to Africa where it occurs on native *Syzygium* species

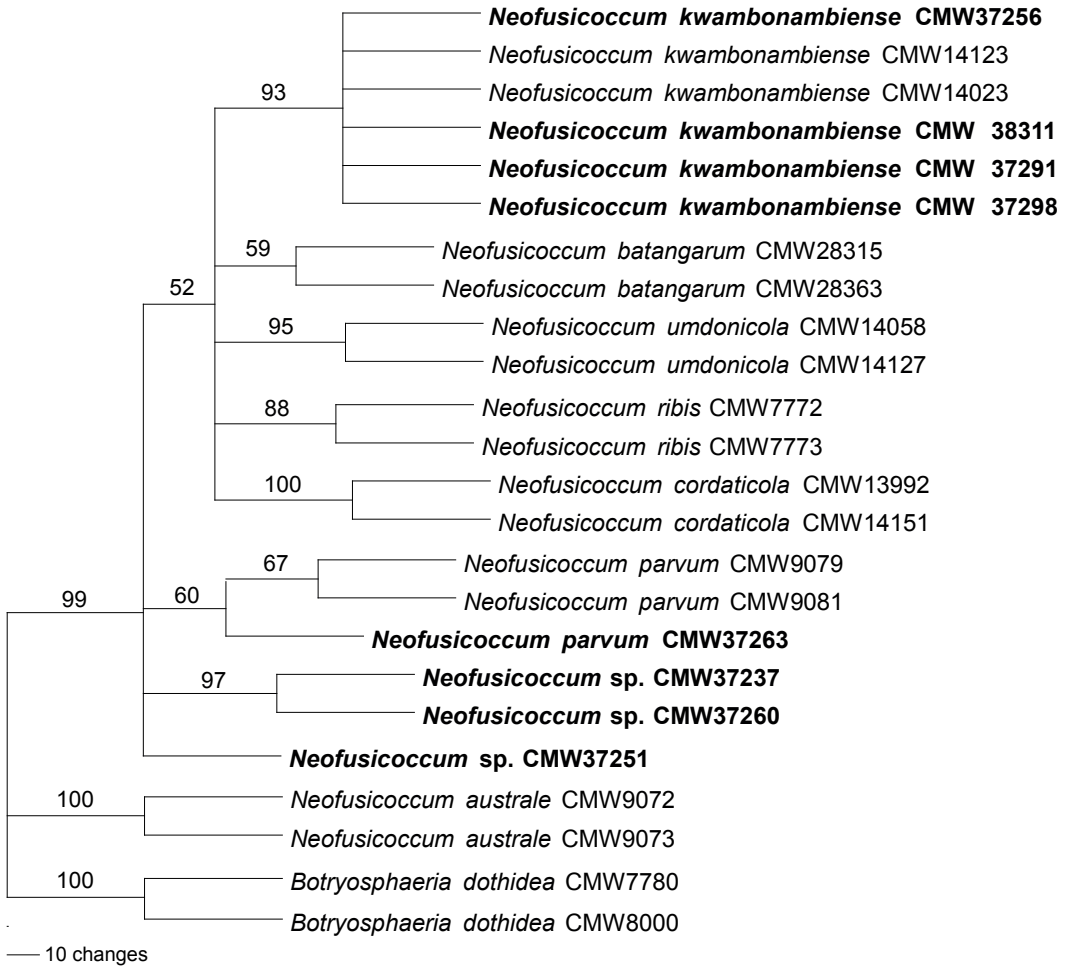

Figure 7: Phylogram of Botryosphaeriaceae species indicating the phylogenetic positions of *Neofusicoccum kwambonabiensis*, one undescribed species related to *N. parvum* and another two undescribed species occurring on *Eucalyptus* in Mozambique. The most parsimonious tree obtained from a heuristic search of the combined BT2, ITS and TEF-1α gene regions of the rDNA sequence data (TL = 195, CI = 0.887, RI = 0.926, RC = 0.821, HI = 0.113). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Bothryosphaeria dothidea*. Isolates sequenced in this study are in bold

(Heath et al. 2006; Nakabonge et al. 2006). In contrast, *Chr. deuterocubensis* was introduced to Africa and most recently to Mozambique (Nakabonge et al. 2007). To date, only nine records of trees being infected with *Chr. deuterocubensis* have emerged from Mozambique, one from the current study. This supports the hypothesis that the pathogen has been introduced into the country.

The disease known as Coniothyrium canker on *Eucalyptus* can be caused by two different species of *Teratosphaeria*, *T. gauchensis* and *T. zuluensis* (Cortinas et al. 2006a). Only *T. zuluensis* was found in this study. *Teratosphaeria zuluensis* was first reported and described from South Africa, where it has caused serious damage to the stems of clonally propagated *E. grandis* trees (Wingfield et al. 1997). The pathogen and the associated serious stem canker disease is now known from Argentina (Gezahgne et al. 2003), China (Cortinas et al. 2006b), Mexico (Roux et al. 2002), Mozambique (Roux et al. 2005), Thailand (Van Zyl et al. 2002), Uganda (Jimu et al. 2014) and Vietnam (Gezahgne et al. 2003). During the course of the survey in Mozambique, *T. zuluensis* was commonly found causing stem canker disease in the southern Mozambique (Maputo), Manica, Niassa and Zambézia provinces.

Symptoms of Botryosphaeria stem canker were observed in all of the areas sampled in this study. The associated pathogens were identified as *N. kwambonambiense*, *N. parvum* and two groups close to *N. parvum*. *Neofusicoccum kwambonambiense* was only recently described, from native *Syzygium cordatum* (Myrtales) in South Africa (Pavlic et al. 2009), but has since also been found on three other continents (Sakalidis et al. 2013). *Neofusicoccum parvum* was described from New Zealand (Pennycook and Samuels 1985) and has been found on a wide range of hosts in many different parts of the world. It has previously been reported to cause cankers on *Eucalyptus* in several African countries (Gezahgne et al. 2004; Slippers et al. 2004; Pavlic et al. 2009; Chungu et al. 2010; Jimu et al. 2015). The Botryosphaeriaceae are opportunistic pathogens that exist as endophytes in healthy plant tissue and cause disease when trees are exposed to stresses such as

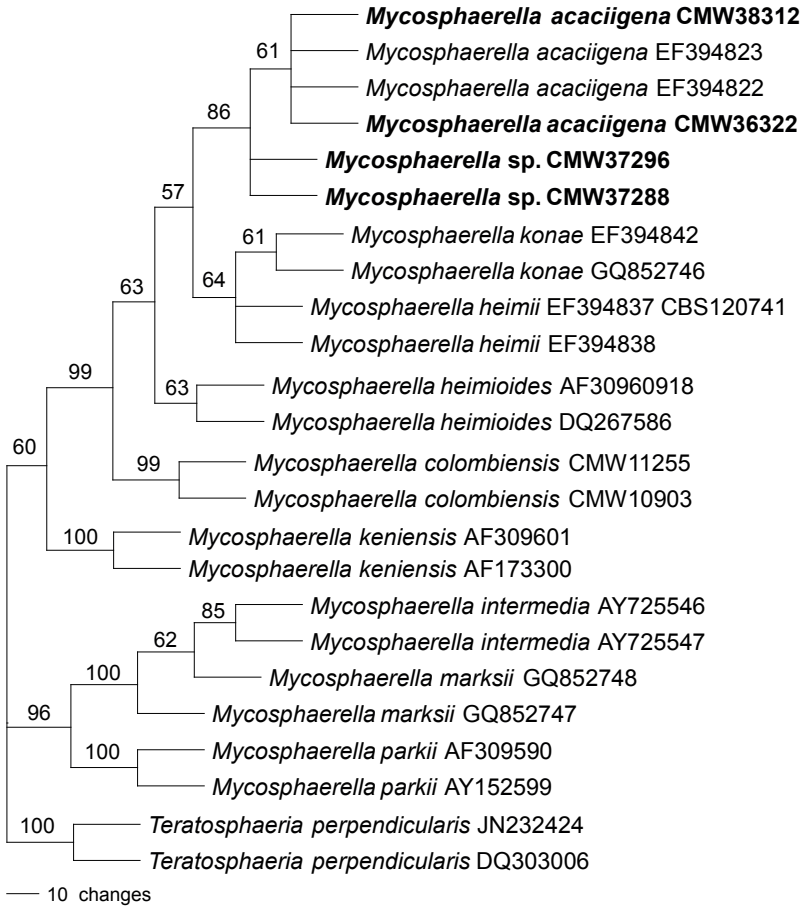

Figure 8: Phylogram of Mycosphaerellaceae species indicating the phylogenetic positions of the fungal *Mycosphaerella acaciigena*, *Mycosphaerella* sp. occurring on *Eucalyptus grandis* and *Eucalyptus grandis* × *Eucalyptus urophylla* clones in Mozambique. The most parsimonious tree obtained from a heuristic search of the ITS gene region of the rDNA sequence data (TL = 200, CI = 0.825, RI = 0.929, RC = 0.767, HI = 0.175). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to the out group *Teratosphaeria perpendicularis*. Isolates sequenced in this study are in bold

those emerging from frost, hail, drought and physical damage (Slippers and Wingfield 2007). They have significant potential to cause increasingly common problems to *Eucalyptus* plantings under conditions of changing climate, particularly drought situations, and where appropriate silviculture is not applied timeously.

Cytospora canker, associated with *V. variostromatica*, was commonly observed on *Eucalyptus* stems. Although not considered economically important pathogens of commercially propagated *Eucalyptus* species, *Valsa* species can cause disease and death of plants that are subjected to stressful conditions (Adams et al. 2006). Several species of *Valsa* have been reported from *Eucalyptus* species in the Republic of Congo (Roux et al. 2000), South Africa (Adams et al. 2006) and Uganda (Roux et al. 2001). In the present study, *V. variostromatica* was found only associated with superficial bark cankers on *Eucalyptus* species.

Pillidiela eucalyptorum found in this study belongs to the *Coniella/Pilidiella*-complex that has previously been reported to infect *Eucalyptus* species in Brazil, Indonesia and South Africa where it can occasionally defoliate trees (van Niekerk et al. 2004). Although commonly found in this study, no associated leaf drop was found in Mozambique.

Pilidium species are known to cause disease on crop plants and trees such as strawberry, *Eucalyptus*, *Bergenia crassifolia* and *Paeonia suffruticosa* (Debode et al. 2011; Geng et al. 2012). *Pilidium concavum*, found in this study, has previously been reported on *Eucalyptus* species in Australia (Simpson et al. 1997), in nurseries in South Africa (Lundquist and Foreman 1986) and also from *E. globulus* in Uruguay (Bettuccii and Saravay 1993), but it does not appear to be an important pathogen in Mozambique.

The *Mycosphaerella* species found on leaves in this study was identified as *M. acaciigena*. The genus belongs to a group of fungi previously collectively known under the generic name *Mycosphaerella* (Hunter et al. 2011), but which has now been shown to represent multiple genera in several distinct families in the order Capnodiales (Crous et al. 2009). Some species in the order, such as *Kirramyces destructans* and *Teratosphaeria nubilosa*, can cause defoliation, leaf blotch and shoot die-back on *Eucalyptus* species and are considered of economic importance in some countries (Crous 1998). *Mycosphaerella acaciigena* was

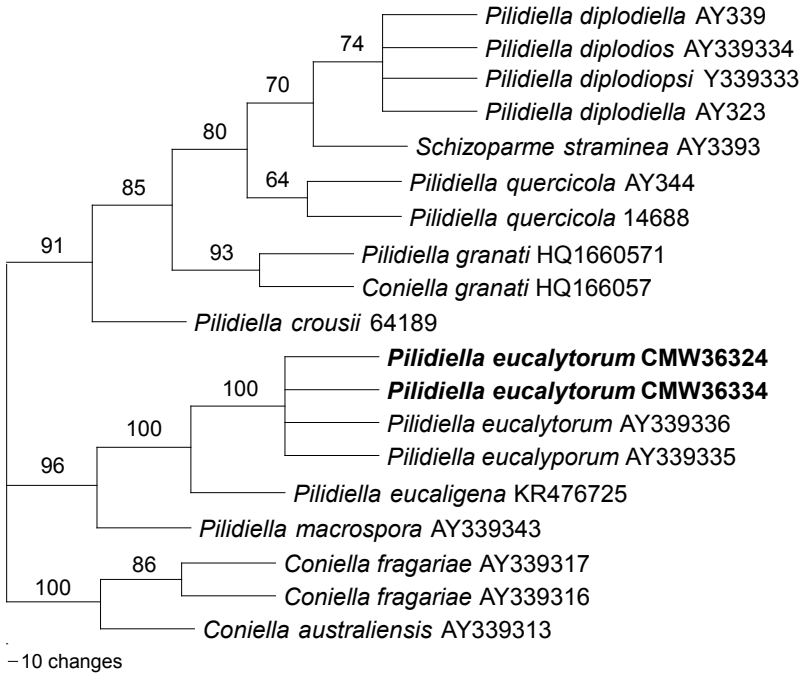

Figure 9: Phylogram of *Pilidiella* species indicating the phylogenetic positions of the isolates occurring on *Eucalyptus* species in Mozambique. The most parsimonious tree obtained from a heuristic search of the ITS1 and ITS2 regions of the rDNA sequence data (TL = 99, CI = 0.838, RI = 0.936, RC = 0.785, HI = 0.162). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Coniella* species (Melanconidaceae). Isolates sequenced in this study are in bold

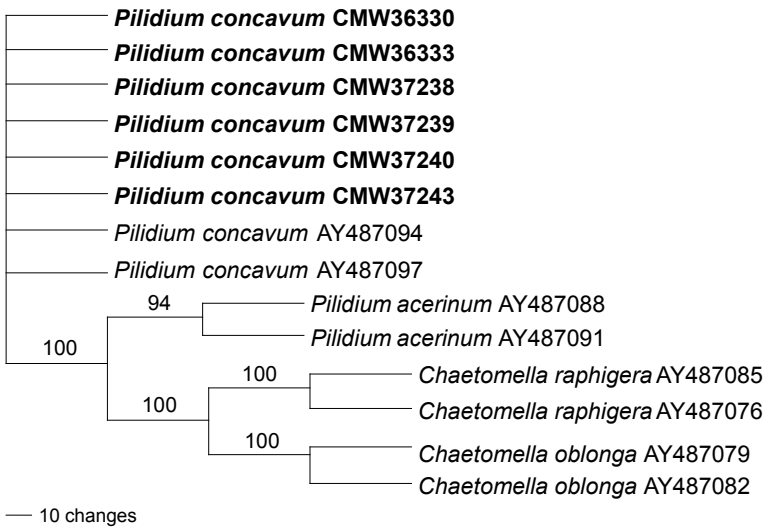

Figure 10: Phylogram of *Pilidium* species indicating the phylogenetic positions of the isolates occurring on *Eucalyptus* species in Mozambique. The most parsimonious tree obtained from a heuristic search of the ITS1 and ITS2 regions of the rDNA sequence data (TL = 98, CI = 0.939, RI = 0.968, RC = 0.909, HI = 0.061). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Chaetomella* species (Chaetomiaceae). Isolates sequenced in this study are in bold

first described causing disease on *Eucalyptus* in Australia by Crous et al. (2007), but in Mozambique this pathogen was found associated with only mild leaf spot symptoms.

Ophiostomatoid fungi (Wingfield et al. 1993) in the orders Microascales and Ophiostomatales are common wound-inhabiting fungi on *Eucalyptus* species globally (Kile et al. 1996; Roux and Wingfield 2009). Although *C. eucalypticola* and *C. fimbriata sensu lato* have been reported as important pathogens of *Eucalyptus* trees in Brazil (Roux et al. 2000), the Republic of Congo (Roux et al. 2000) and Uganda (Roux et al. 2001), no disease was found associated with ophiostomatoid fungi in the Mozambique surveys. All ophiostomatoid fungi collected in the present study originated from tree wounds and were associated with

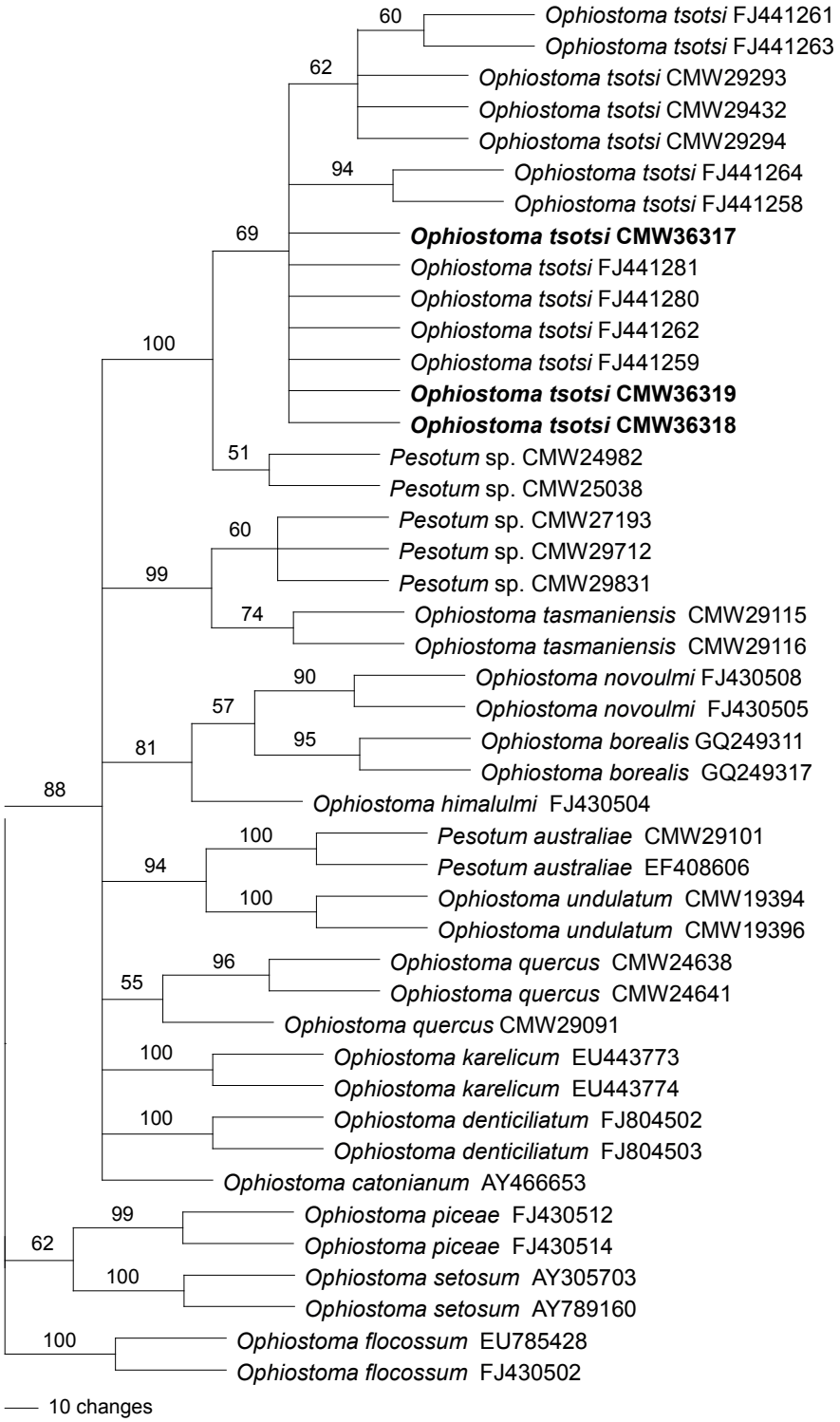

Figure 11: Phylogram of *Ophiostoma* species indicating the phylogenetic positions of *Ophiostoma tsotsi* occurring on *Eucalyptus* in Mozambique. The most parsimonious tree obtained from a heuristic search of the ß-tubulin gene region of the rDNA sequence data (TL = 195, CI = 0.641, RI = 0.892, RC = 0.572, HI = 0.359). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Ophiostoma flocossum*. Isolates sequenced in this study are in bold

superficial staining of wound surfaces. *Ophiostoma tsotsi* is a recently described species in the *O. picea* complex that was treated for many years collectively with *O. quercus* (Grobbelaar et al. 2010). It has previously been reported from *Eucalyptus* in Malawi, South Africa (Grobbelaar et al. 2009) and China (Grobbelaar et al. 2011). There was, however, no indication that it is causing any disease problems on eucalypts in Mozambique.

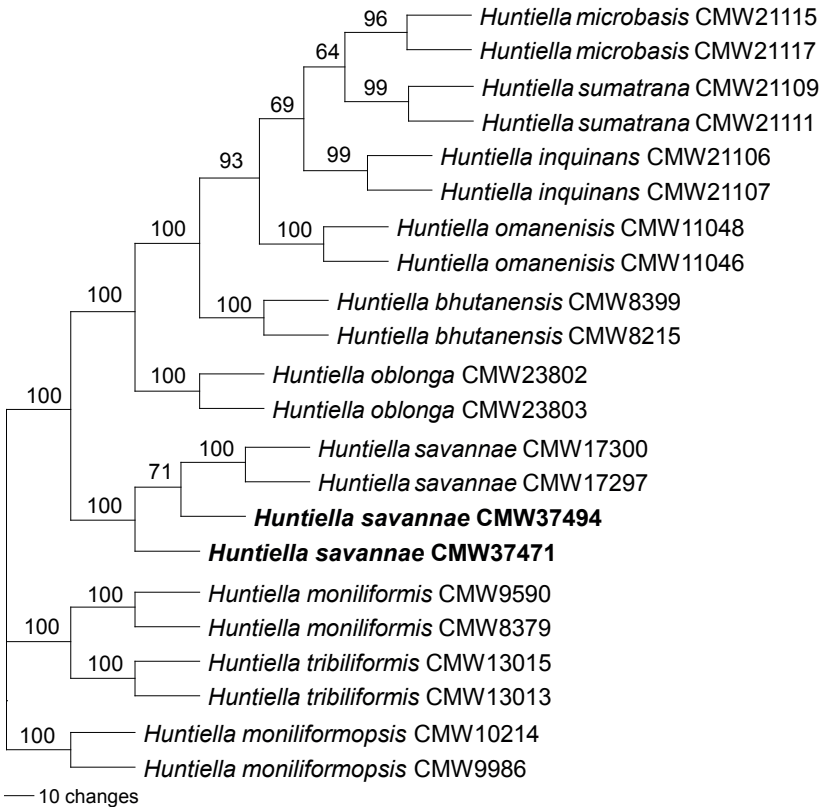

Figure 12: Phylogram of *Huntiella* species indicating the phylogenetic positions of undescribed *Huntiella* occurring on *Eucalyptus* in Mozambique. The most parsimonious tree obtained from a heuristic search of the combined β-tubulin, TEF and ITS gene regions of the rDNA sequence data (TL = 2959, CI = 0.750, RI = 0.849, RC = 0.637, HI = 0.250). Bootstrap confidence values (1 000 replicates) are indicated above the internodes. The tree is rooted to *Huntiella moniliformis*. Isolates sequenced in this study are in bold

Huntiella, previously treated in the *Ceratocystis moniliformis sensu lato* complex (de Beer et al. 2014), represents a large genus that commonly infect wounds or the cut surfaces of recently felled trees (Kile 1993; Roux and Wingfield 2009). In the present study, an apparently undescribed species, most closely related to *Huntiella savannae* (Kamgan Nkuekam et al. 2008), was found.

The most commonly observed nursery disease found in this study was leaf blight and cutting rot caused by *Cal. mossambicensis.* This fungus was recently described from Mozambique (Maússe-Sitoe et al. 2013) and is not known from any other country. As is common for all *Calonectria* species, it was isolated from both nursery soil and from cuttings. Species of *Calonectria* have a wide distribution as forestry pathogens in tropical and subtropical areas of the world (Crous 2002). In nurseries, *Calonectria* species can have devastating effects and great care should be taken to ensure optimal hygiene conditions to avoid infections by this group of fungi (Crous 2002; Lombard et al. 2010).

A rust disease, caused by the recently described species *P. myrtacearum* (Maier et al. 2016), was commonly found in the Manica, Niassa, Sofala and Zambézia provinces. It was mostly found affecting older leaves of trees and no associated defoliation was observed. Nothing is known regarding the potential impact or origin of this fungus. Although it is not closely related to the important eucalypt pathogen *Puccinia psidii* (Maier et al. 2016), *P. myrtacearum* and its impact should be monitored closely.

Conclusions

This study represents the most comprehensive study to consider diseases of eucalypts in Mozambique. Results presented here should be important to plantation owners and managers, as well as to quarantine agencies in the country. Management of the diseases reported in this study will need to rely on sound breeding programmes and silvicultural practices as part of an integrated management strategy. The report of a number of opportunistic, stress-associated pathogens highlights the importance of careful site and genotype matching, which will be possible only through sound selection and monitoring programmes. Forest companies, private growers and the government will need to invest substantially in technologies enabling management of the diseases and pests affecting forestry in Mozambique.

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do Niassa, Tree Farm, and Lurio Green Resources (Nampula and Niassa).

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