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Characterisation of the Coniothyrium stem canker pathogen on *Eucalyptus camaldulensis* in Ethiopia

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Abstract. During a survey of *Eucalyptus* diseases in Ethiopia, a serious stem canker disease was discovered on *E. camaldulensis* trees at several localities in the south and south-western parts of the country. The disease was characterised by the presence of discrete necrotic lesions, stem cankers, cracking of stems, production of kino pockets in the wood, as well as malformation of stems. These symptoms are similar to those caused by *Coniothyrium zuluense* in South Africa. This study identified the causal agent of the disease in Ethiopia by sequencing the ITS regions of the rRNA operon for a representative set of isolates. Sequences for the Ethiopian isolates were compared with those from authenticated isolates collected in South Africa, Thailand and Mexico, as well as with *Coniothyrium*-like isolates collected from diseased *Eucalyptus* trees in Uganda. Pathogenicity trials were also conducted in the greenhouse to determine the virulence of Ethiopian isolates. Based on comparisons of sequence data, the pathogen causing the stem canker disease in Ethiopia was identified as *C. zuluense*. Isolates from Ethiopia, however, formed their own sub-clade, reflecting geographic isolation of the pathogen. Results, furthermore, also show that *C. zuluense* does not reside with other *Coniothyrium* spp., but rather within the genus *Mycosphaerella*. Small lesions were obtained from inoculated *Eucalyptus* trees, proving that the fungus is the cause of disease in Ethiopia. This study represents the first confirmed report of *C. zuluense* and the disease caused by it in Ethiopia and Uganda. It also shows that *C. zuluense* is closely related to species of *Mycosphaerella* and not other *Coniothyrium* spp. and that it will require a name change in future.

Additional keywords: Africa, coelomycete, disease, phylogeny, taxonomy.

Introduction

Eucalyptus species originate in Australia and nearby islands and have been introduced into many tropical and subtropical countries. Estimates indicate that plantations of exotic *Eucalyptus* spp. cover approximately 10 million ha of land world-wide (Eldridge *et al*. 1997). These plantations provide timber for building and furniture, distillates, tannins, essential oils, nectar, pollen and fibre for the production of paper, rayon and viscose. They are also a valuable source of fuel wood and construction timber (Poynton 1979; Turnbull 1991).

In Ethiopia, planting of exotic trees commenced with the introduction of *Eucalyptus* spp. in approximately the 1890s. *E. globulus*, *E. camaldulensis*, *E. saligna*, *E. grandis* and *E. citriodora* are the most commonly planted species and cover about 100000 ha of land. These *Eucalyptus* plantations provide wood for energy, construction material, transmission poles and fencing (Persson 1995; Pohjonen and Pukkala 1990).

Eucalyptus spp. have displayed great promise in most areas where they have been planted as exotics. However, fungal diseases pose a serious threat to these economically important plantation species. A number of important diseases have been recorded on different *Eucalyptus* spp. and clones. These diseases infect stems, roots and leaves. Cryphonectria canker caused by *Cryphonectria cubensis* (Hodges *et al*. 1986; Wingfield *et al*. 1989; Conradie *et al*. 1990), canker and die-back caused by *Botryosphaeria* spp. (Smith *et al*. 1994), vascular wilt of *Eucalyptus* caused by *Ceratocystis fimbriata* (Roux *et al*. 2000), pink disease caused by *Erythricium salmonicolor* (Sharma *et al*. 1984; Roux *et al*. 2001; Alemu *et al*. 2003*a*) and leaf blotch caused by

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Mycosphaerella spp. (Park and Keane 1982; Crous 1998) are examples of important diseases in commercial *Eucalyptus* plantations. Recently, a serious stem canker disease caused by *Coniothyrium zuluense* was also described causing losses to *Eucalyptus* trees in various countries (Wingfield *et al*. 1996; Roux *et al*. 2002; Van Zyl *et al*. 2002).

Stem canker caused by *C. zuluense* was reported for the first time in 1989 from an *E. grandis* clone in South Africa (Wingfield *et al*. 1996). Trees affected by Coniothyrium stem canker develop small, discrete, necrotic lesions on the young, green bark (Wingfield *et al*. 1996; Roux *et al*. 2002; Van Zyl *et al*. 2002). The canker disease has been found on several *E. grandis* clones, on hybrids of *E. grandis* with *E. urophylla* and on *E. camaldulensis*, which is generally believed to be a relatively disease-tolerant species (Wingfield *et al*. 1996). Initially, the pathogen was believed to be native to South Africa. It has, however, recently been described from *Eucalyptus* spp. in Thailand (Van Zyl *et al*. 2002), Mexico (Roux *et al*. 2002) and Hawaii (Cortinas *et al*. 2004).

During a disease survey of plantation forestry species in western and south-western Ethiopia, several pathogens were identified (Alemu *et al*. 2003*b*). Symptoms of stem canker similar to those of Coniothyrium canker were observed on *E. camaldulensis* trees at a number of localities (Alemu *et al*. 2003*b*). *Coniothyrium* spp. are difficult to identify and morphological characteristics are generally considered insufficient to identify *C. zuluense* with certainty. This study was, therefore, conducted to confirm the identity of the causal agent of the canker disease of *E. camaldulensis* and to determine the phylogenetic relationship between the fungus occurring in Ethiopia and isolates from other parts of the world.

Methods

Symptoms, sample collection and isolation

Samples were collected from infected *E. camaldulensis* trees planted in southern and south-western Ethiopia. Samples were collected from symptomatic plant parts including twigs, branches and stems of infected trees. Collections were made from plantations, community woodlots, and from *E. camaldulensis* trees planted around farmlands and homesteads. Segments of plant parts with disease symptoms were incubated in moist chambers at room temperature to induce sporulation. Masses of spores emerging from pycnidia were transferred to Petri dishes containing MEA (20 g/L Biolab malt extract, 15 g/L Biolab agar), spread on the agar surface with sterilised water and incubated at 25°C for 2 weeks. To purify the cultures, the resultant fungal colonies were transferred to Petri dishes containing MEA. Cultures collected from Ethiopia are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Deoxyribonucleic acid extraction

Total genomic DNA was extracted from isolates (Table 1) grown in liquid MY medium (2 g/L Biolab malt extract, 0.3 g/L Biolab yeast extract agar) for 2 weeks at 25°C. Mycelium was harvested by centrifugation (8000 rpm, 30 min), freeze-dried and ground to a fine powder in liquid nitrogen using a pestle and mortar. The DNA extraction method described by Raeder and Broda (1985) was used to isolate DNA from the mycelium. Deoxyribonucleic acid was precipitated using standard sodium acetate (0.1 v/v) (3 M NaAc, pH 5.5) and ethanol precipitation techniques. Nucleic acids were washed with 70% ethanol and pellets were vacuum dried to remove excess ethanol and resuspended in 50 µL sterilised water. RNase A (1 mg/mL) (Roche Diagnostics, South Africa) was added to the DNA solution to remove the contaminating

Table 1. List of fungal isolates used in this study

Isolate[A]	Origin	Species	Host	Collector	GenBank accession number
		Botryosphaeria dothidea			AF464951
		Coniothyrium fuckelii			AJ293813
CMW13324	South Africa	*C. zuluense*	*E. grandis*	M.J. Wingfield	AY73214
CMW11221	South Africa	‘	‘	‘	AF376816
CMW11225	Ethiopia	‘	*E. camaldulensis*	Alemu Gezahgne & J. Roux	AY244415
CMW11226	Ethiopia	‘	‘	‘	AY244413
CMW11227	Ethiopia	‘	‘	‘	AY244414
CMW11228	Uganda	‘	*E. grandis*	J. Roux	AY244416
CMW11230	Mexico	‘	‘	M.J. Wingfield & J. Roux	AY385610
CMW11231	Mexico	‘	‘	‘	AF385611
CMW5232	Thailand	‘	*E. camaldulensis*	M.J. Wingfield & L.M. van Zyl	AF376828
CMW5234	Thailand	‘	‘	‘	AF376825
		Fusarium ambrosium			AF178397
	Australia	*Mycosphaerella ambiphylla*	*Eucalyptus* sp.		AY150675
CMW8575	Chile	*M. molleriana*	*E. globulus*	R. Ahumada	AY738215
CMW3032	South Africa	*M. nubilosa*	*E. bicostata*	P.W. Crous	AY738216
	Australia	*M. vespa*	*Eucalyptus* sp.		AY045499
	Australia	*M. vespa*	*Eucalyptus* sp.		AY045500

[A]CMW numbers refer to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

RNA and incubated at 37°C in a water bath over night. The presence of DNA in the samples was detected by comparison with a standard in a 1% agarose gel stained with ethidium bromide and visualised under UV light.

PCR amplification

The internal transcribed spacer (ITS) regions of the rRNA operon and the 5.8S gene were amplified using the polymerase chain reaction (PCR). PCR was conducted using primers ITS 1 (5′ TCC GTA GGT GAA CCT GCG G ′3) and ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC ′3) (White *et al*. 1990).

The PCR reaction mixtures contained DNA polymerase (*Taq*, 2.5 U/μL, Roche Diagnostics, South Africa), 2.5 mM DNTPs, 10× PCR Buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 2.5 mM $MgCl_2$, 0.15 mM of each primer, approximately 1 μL of DNA and 37 μL of sterilised water to make up a final volume of 50 μL. PCR reaction conditions consisted of an initial denaturation step at 96°C for 1 min, followed by 35–40 cycles of primer annealing at 55°C for 30 s, chain elongation at 72°C for 1 min and denaturation at 92°C for 1 min. Final chain elongation was carried out at 72°C for 5 min. All PCR products were detected on 1% agarose gels stained with ethidium bromide and visualised under UV illumination.

Deoxyribonucleic acid sequencing and sequence analysis

The PCR products were purified using the High Pure PCR Product Purification Kit (QIAGEN, GmbH, Hilden, Germany). The PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with Amplitaq DNA Polymerase FS (Perkin-Elmer, Warrington, UK), according to the manufacturer's protocols on an ABI PRISM 3100 DNA Autosequencer (Perkin-Elmer). The same primers used for the PCR amplification reactions were used for sequencing of the respective gene areas.

The sequences for the Ethiopian isolates were compared with ITS rDNA sequences obtained from GenBank [National Centre for Biotechnology Information (NCBI), USA National Institute of Health Bethesda (http:/www.ncbi.nlm.nih.gov/BLAST)]. Once the possible identity of the fungus was determined using a BLAST search, additional published sequence data of *Coniothyrium* spp. and *Mycosphaerella* spp. were included in the study and the ITS data analysed. *Botryosphaeria dothidea* and *Fusarium ambrosium* were included as outgroup taxa.

The ITS gene sequences were aligned manually and analysed using PAUP 4.0 (Swofford 1998). Gaps were inserted manually and treated as missing data. The sequences were analysed using parsimony with trees generated by heuristic searches, simple addition and Tree Bisection Reconstruction (TBR) branch swapping. Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony; 1000 replicates) (Felsenstein 1993).

Pathogenicity tests

An inoculation study was conducted on an 18-month-old *E. grandis* clone (ZG 14) (~1 cm diameter) in the greenhouse at a temperature of ~25°C. Prior to inoculation, plants were allowed to acclimatise to greenhouse conditions for 1 week. Seven of the *Coniothyrium* isolates (CMW11223, CMW11225, CMW11226, CMW11233, CMW11234, CMW11235, CMW11238) from Ethiopia were randomly selected for the inoculation study. Cultures were grown on MEA for 2 weeks before inoculation. A 9-mm-diameter cork borer was used to remove the bark and expose the cambium from the stems of trees to be inoculated. Mycelial plugs of equal size, overgrown with the test cultures, were placed into each wound with the mycelium facing the xylem. All the wounds were covered with Parafilm (Pechiney Plastic Packaging, Chicago) to prevent desiccation of the wood and the inoculum. Each isolate was inoculated on ten trees and an additional ten trees were inoculated with sterile MEA to serve as controls. After 6 weeks, development of symptoms was examined by measuring the lesion lengths on inoculated trees. A one-way ANOVA was conducted using Statistica for Windows (Statsoft Inc. 1995) to compare lesion development associated with the isolates and the control.

Results

Symptoms, sample collection and isolation

Symptoms of Coniothyrium stem canker were observed in several localities growing *E. camaldulensis* in south and south-western Ethiopia. These areas were between Woliso and Jima and between Wolkite and Sodo. *E. camaldulensis* trees in Jiren plantation near Jima, and *E. camaldulensis* trees planted in woodlots as well as around farms and homesteads were seriously affected by the stem canker. About 50% of *E. camaldulensis* trees growing at these localities had symptoms of the disease. Stem malformation and extensive discoloration of the stems was evident on most infected trees. Initially, small discrete lesions developed on young green bark. When these lesions coalesced, large necrotic lesions developed on the stems, branches and twigs. Kino pockets were observed in the wood associated with the bark lesions on infected trees.

After 1 day in moisture chambers, pycnidia producing slimy spore masses were found in the sunken necrotic lesions collected from infected trees. A *Coniothyrium* sp. was consistently isolated from these lesions and this fungus was morphologically similar to *C. zuluense* described from South Africa. In culture, isolates grew slowly and colonies were olive green in colour. The colonies of most isolates were similar in growth and colour.

PCR amplification

Amplification of the ITS regions and 5.8S gene for the *Coniothyrium* isolates used in this study yielded a fragment of about 500 base pairs (bp) in size.

Deoxyribonucleic acid sequencing and sequence analysis

The ITS regions and 5.8S gene were sequenced and after alignment yielded a total of 533 characters, of which 125 were parsimony informative. Comparison of the ITS and 5.8S data with sequences available in the GenBank database revealed that the Ethiopian isolates were most similar to *C. zuluense* (98%), followed by *Mycosphaerella ambiphylla*, *M. vespa* and *M. molleriana* (96% homology) and *M. nubilosa* (94% homology). Analysis of the ITS sequence data, using sequences obtained from GenBank and the dataset from Van Zyl *et al*. (2002), produced four most parsimonious trees with a length of 411 steps. The majority-rule consensus tree (Fig. 1) had a CI (consistency index) = 0.893 and RI (retention index) = 0.807, and showed that the *Coniothyrium* isolates from Ethiopia and Uganda grouped together in the larger *C. zuluense* clade (95% bootstrap). Deoxyribonucleic

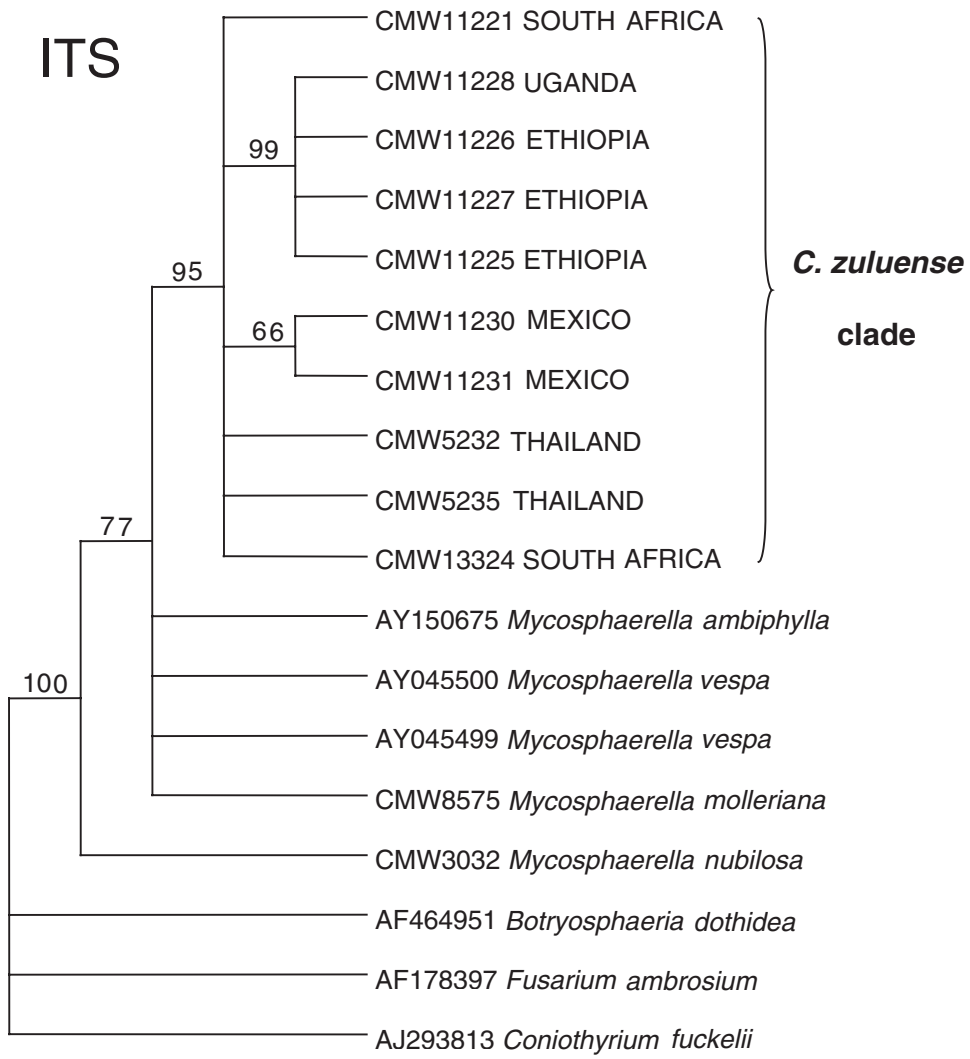

Fig. 1. Bootstrap consensus tree of the ITS sequence data of *Coniothyrium* spp. and *Mycosphaerella* spp. (CI = 0.893 and RI = 0.807). Bootstrap values are shown above the branches. *Coniothyrium fuckelii*, *Botryosphaeria dothidea* and *Fusarium ambrosium* were used as outgroup taxa.

acid sequences from Uganda and Ethiopia were identical with the exception of isolate CMW11227 where two substitutions were found in the ITS region. *C. zuluense* isolates grouped more closely with *M. ambiphylla*, *M. vespa*, *M. molleriana* and *M. nubilosa* than with available species of *Coniothyrium* (*C. fuckelii*) or the two outgroup taxa (Fig. 1).

Pathogenicity tests

Small lesions developed on *E. grandis* trees inoculated with Ethiopian *Coniothyrium* isolates after 6 weeks. Lesion lengths differed statistically from those of the control ($P < 0.0001$) ($R^2 = 0.48$). No distinct differences were observed in lesion development between the *C. zuluense* isolates used in the inoculation study (Table 2).

Table 2. Results of inoculation of a *Eucalyptus grandis* clone with Ethiopian *Coniothyrium* isolates

Isolates	Mean lesion length (mm)[A]	95% Confidence limits
CMW11223	17.2 a	15.65–18.75
CMW11234	17.9 a	16.35–19.45
CMW11233	16.6 a	15.05–18.15
CMW11226	16.7 a	15.15–18.25
CMW11238	17.9 a	16.35–19.45
CMW11225	16.8 a	15.25–18.35
CMW11235	18.8 a	17.25–20.35
Control	11.0 b	9.45–12.55

[A]Each mean lesion length is the average of 10 measurements; $R^2 = 0.48$; mean values followed by the same letters did not differ significantly at $P = 0.05$.

Discussion

Coniothyrium stem canker, caused by *C. zuluense*, is considered to be one of the most important new threats to plantation grown *Eucalyptus* species. This disease was known only from South Africa (Wingfield *et al*. 1996), Thailand (Van Zyl *et al*. 2002), Mexico (Roux *et al*. 2002) and Hawaii (Cortinas *et al*. 2004). Although observations based on symptoms and morphology of the fungus have led to suggestions that the disease is present in Ethiopia (Alemu *et al*. 2003*b*), this study provides the first clear evidence of its occurrence in the country and it expands the geographic distribution of this important disease. This is particularly important, as it is virtually impossible to identify *C. zuluense* with certainty without DNA sequence comparisons.

Symptoms of Coniothyrium stem canker were first observed on *E. camaldulensis* in Ethiopia during a survey of plantation forestry diseases in 2000 and 2001 (Alemu *et al*. 2003*b*). The disease is restricted to specific areas in southern and south-western Ethiopia, and is causing large-scale damage to trees in plantations, woodlots and around homesteads. It has not been found on other species of *Eucalyptus* in Ethiopia. This is probably due to the fact that they are planted in cooler areas, which would not be conducive to the development of *C. zuluense*. It is also consistent with the fact that in South Africa, Coniothyrium stem canker is a problem only in warmer sub-tropical areas (Wingfield *et al*. 1996), while the only other reports of this disease is from tropical and sub-tropical areas such as Thailand (Van Zyl *et al*. 2002), Mexico (Roux *et al*. 2002) and Hawaii (Cortinas *et al*. 2004).

Comparison of ITS and the 5.8S gene sequences showed that Ethiopian isolates were most similar to those of *C. zuluense.* The next closest relatives were *Mycosphaerella* spp., including *M. ambiphylla*, *M. vespa*, *M. molleriana* and *M. nubilosa.* This is particularly interesting as the *Coniothyrium* sp. for which sequence data was available, *C. fuckelii,* was more distantly related to *C. zuluense* than the group of *Mycosphaerella* spp. noted above. Van Zyl *et al*. (2002) provided the first DNA sequence data for *C. zuluense* and used *C. ovatum* and *C. fuckelii* as outgroup taxa. Our study, however, strongly suggests that *C. zuluense* is more closely related to *Mycosphaerella* spp. than to other *Coniothyrium* spp. for which sequence data are available. It was for this reason that we included *Mycosphaerella* spp. in this study and used *B. dothidea* and *F. ambrosium* as outgroup taxa. Our data provide preliminary evidence to suggest that *C. zuluense* is an anamorph of *Mycosphaerella.* This is particularly interesting, as many *Mycosphaerella* species are pathogens of *Eucalyptus* leaves and stems.

Pathogenicity tests showed that Ethiopian *Coniothyrium* isolates are pathogenic to *E. grandis*. Only very small lesions were produced, but they differed significantly from the controls. Wingfield *et al*. (1996) reported similar results for South African isolates in artificial inoculations. In an extensive survey of *Eucalyptus* diseases in western and southern Ethiopia (Alemu *et al*. 2003*b*), no Coniothyrium stem canker was observed on *E. grandis*, or any species other than *E. camaldulensis*. The pathogenicity of *C. zuluense* under field conditions and on *E. camaldulensis*, however, needs to be investigated further.

E. camaldulensis is one of the most widely planted *Eucalyptus* spp. in Ethiopia. This species appears to be highly susceptible to Coniothyrium stem canker. Currently the disease is resulting in stunted growth and reduction of timber quality and strength. This greatly affects the use of these trees for construction purposes. The disease is widespread in areas growing *E. camaldulensis* between Wolkite and Sodo, and between Woliso and Jima. Near Jima, the disease was found on most *E. camaldulensis* trees in the Jiren plantation, east of Jima, whereas *E. camaldulensis* planted on the other side of the town showed no signs of infection. This might suggest that different seed sources of *E. camaldulensis* differ in their susceptibility and it raises the possibility of being able to select disease tolerant planting stock in the future. We recommend more intensive surveys for this disease and disease screening trials in the future.

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