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Morphological and molecular relatedness of geographically diverse isolates of *Coniothyrium zuluense* from South Africa and Thailand

Len M. VAN ZYL[1], Teresa A. COUTINHO[1]*, Michael J. WINGFIELD[1,2], Krisna PONGPANICH[3] and Brenda D. WINGFIELD[2]

[1] *Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.*
[2] *Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.*
[3] *Forest Research Office, Royal Forest Department, Chatuchak, Bangkok 10900, Thailand.*
E-mail: teresa.coutinho@fabi.up.ac.za

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Coniothyrium canker, caused by *Coniothyrium zuluense*, is a serious stem canker disease of *Eucalyptus* in subtropical parts of South Africa. A *Coniothyrium* associated with similar symptoms to those in South Africa was observed on *E. camaldulensis* in 1996 in Thailand. It was previously thought that *C. zuluense* was restricted to South Africa. This study compares South African isolates of *C. zuluense* with isolates of the *Coniothyrium* from Thailand. Results of morphological comparisons indicate that the South African and Thailand isolates are the same. This was further confirmed when all *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* spp. grouped together in a single major clade for both rDNA sequence data and AFLP analysis. This clade was distant from isolates of other *Coniothyrium* spp. included for comparative purposes. Although the *Coniothyrium* isolates from South Africa and Thailand resided in two separate clades, these were closely related and, we believe that the isolates from Thailand represent *C. zuluense*. This is the first record of the important *Eucalyptus* stem canker pathogen outside South Africa.

INTRODUCTION

Eucalyptus species are native to Australia, but approximately 8 M ha of *Eucalyptus* plantations have been established, mostly in tropical and subtropical countries of the world (Anon. 1995). The success of *Eucalyptus* propagation, however, is often hampered by fungal diseases. These diseases include both stem, root and leaf diseases, and have been shown to cause considerable economic losses on various *Eucalyptus* species, clones and hybrids (Park & Keane 1984, Florence, Sharma & Mohanan 1986, Hodges, Alfenas & Ferreira 1986, Ferreira 1989, Conradie, Swart & Wingfield 1990, Linde, Kemp & Wingfield 1994, Smith, Kemp & Wingfield 1994, Crous & Wingfield 1994, 1996).

Coniothyrium canker caused by *Coniothyrium zuluense* is a serious *Eucalyptus* stem canker pathogen from South Africa (Wingfield, Crous & Coutinho 1997). This fungus was first reported in 1988 in an isolated area in Zululand, KwaZulu-Natal, on a single clone of *E. grandis*. The earliest signs of infection by *C. zuluense* on trees are small, discrete, necrotic lesions on the young, green bark. These lesions coalesce to form large necrotic patches on the stems from which copius amounts of red kino exude. Epicormic shoots are commonly produced in the cankered areas, indicative of partial girdling of the stems. In severely infected clones, the tops of trees die, due to girdling (Wingfield *et al*. 1997).

Since its discovery in 1988, *C. zuluense* has become widespread throughout *Eucalyptus* growing areas of Zululand and occurs on a wide range of *E. grandis* clones and hybrids, as well as other species of *Eucalyptus* (Coutinho *et al*. 1997, Wingfield *et al*. 1997). An intensive disease survey undertaken during 1995 and 1996, showed that *C. zuluense* isolates differed considerably in surface colony colour and pathogenicity (van Zyl, Wingfield & Coutinho 1997). It was, therefore, thought that more than one species of *Coniothyrium* might be responsible for the disease in South Africa.

Several *Coniothyrium* species have been reported as pathogens of *Eucalyptus* leaves. In Australia, *C. eucalypticola*, *C. kallangurence*, *C. ovatum* and *C. parvum* are associated with leaf spots on various *Eucalyptus* species (Sutton 1975, 1980, Swart 1986). *C.*

* Corresponding author.

ahmadii has been isolated from eucalypt leaf spots in Pakistan (Sutton 1974). The only report of a pathogenic *Coniothyrium* from South Africa, prior to the discovery of *C. zuluense*, was of the leaf spot caused by *C. ovatum* (Crous, Knox-Davies & Wingfield 1988). In this case, *C. ovatum* was isolated from leaf spots occurring mainly on the lower branches of mature *E. cladocalyx* and *E. lehmannii* trees in the Western Cape province.

During 1996, a survey of diseases of eucalypt plantations in the Sinai area of Thailand was undertaken. This led to the discovery of a serious stem canker disease of *E. camaldulensis*. Disease symptoms were very similar to those caused by *C. zuluense* in South Africa, to which country it was thought to be endemic (Coutinho *et al*. 1997, Wingfield *et al*. 1997). Based on superficial morphological and cultural characteristics, the Thailand isolates were identified as representing a species of *Coniothyrium*. The aim of this study is to compare isolates of *C. zuluense* with those of the *Coniothyrium* from Thailand, based on morphological comparisons, rDNA sequence data, and Amplified Fragment Length Polymorphism (AFLP) analysis.

MATERIALS AND METHODS

Isolates

Six single conidial isolates of a *Coniothyrium* were collected from severely infected *Eucalyptus camaldulensis* trees from Rachaburi plantation in the Sinai region of Thailand (CMW 5231, CMW 5232, CMW 5233, CMW 5234, CMW 5235 and CMW 5236). Isolations were made from segments of symptomatic material that were placed in humidity chambers to induce the formation of fungal fruiting bodies. Single conidial isolates were obtained as described by van Zyl *et al*. (1997). Each isolate originated from stem cankers from a different tree.

Nine single conidial isolates of *C. zuluense* were collected from nine *Eucalyptus* plantation regions in Zululand, KwaZulu-Natal, South Africa (CMW 7399, CMW 7406, CMW 7442, CMW 7459, CMW 7465, CMW 7489, CMW 7491, CMW 7506 and CMW 7507). These isolates were chosen to be compared with those from Thailand, based on their differences in surface colony colour and pathogenicity to a susceptible *E. grandis* clone. Colony colour characteristics of these isolates varied between olive grey ($V23^{IIIIb}$), greenish glaucous (33^{IIIf}), isabella ($19^{II}I$) and grayish olive (21^{III}) (Rayner 1970). The selected isolates represented three non-pathogenic isolates, four isolates of intermediate pathogenicity and two isolates with high levels of pathogenicity.

Three *Coniothyrium* spp. other than *C. zuluense* were included for comparative purposes (Table 1): *C. ovatum*, a leaf-spotting pathogen isolated from *E. diversicolor* in Stellenbosch, Western Cape, South Africa; *C. palmarum* (CBS 758.73); and *C. fuckelii* (CBS 132.26) that cause stem cankers on various *Rosa* and *Rubus* species. *Massarina corni* (CBS 496.64) was included as an outgroup. All isolates were grown in Petri dishes containing 15 ml of an enriched 4% w/v Potato Dextrose Agar (PDA) (24 g Potato Dextrose extract (Difco); 1 g yeast extract (Difco); 1 g glucose (Difco); 40 g agar (Difco); 1 l distilled H_2O). Plates were incubated at 30 °C for 10 d. Isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, and representative isolates will be deposited in the Centraalbureau voor Schimmelcultures (CBS), Delft.

Morphological comparisons

Colony and conidial morphology

Coniothyrium isolates from Thailand were grown on PDA (39 g PDA (Difco); 1 l distilled H_2O) at 30 °. Measurements (length and width) using a Zeiss Axioskop light microscope were made of mature conidia (30 from each structure). Colony colour was rated using the charts of Rayner (1970). Colony colour and conidial measurements for the Thailand isolates were compared with those of *C. zuluense* isolates associated with *Eucalyptus* stem canker in South Africa as described by van Zyl *et al*. (1997).

Growth in culture

Growth rates and temperature requirements for the South African and Thailand isolates were determined on PDA. The PDA plates were inoculated with 5 mm

Table 1. Sequences of primers and adaptors used for AFLP analysis.

Name	Enzyme	Type	Sequence
CA	*Cfo*I	Adaptor	5′-GACGATGAGTCCTGAACG-3′ 3′-TACTCAGGACTT-5′
MA	*Mse*I	Adaptor	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
C-T	*Cfo*I	Primer + 1	5′-GATGAGTCCTGAACGCT-3′
C-TCC	*Cfo*I	Primer + 3	5′-GATGAGTCCTGAACGCTCC-3′
C-TGG	*Cfo*I	Primer + 3	5′-GATGAGTCCTGAACGCTGG-3′
M-A	*Mse*I	Primer + 1	5′-GATGAGTCCTGAGTAA-3′
M-AGT	*Mse*I	Primer + 3	5′-GATGAGTCCTGAGTAAGT-3′
M-AGC	*Mse*I	Primer + 3	5′-GATGAGTCCTGAGTAAGC-3′

mycelial plugs removed from the margins of actively growing colonies and placed, face down, at the centre of the plates. Plates were maintained at temperatures ranging from 10 to 35 °, at five degree intervals, in the dark for 30 d. Three plates were incubated for each isolate at each temperature. Two diameter measurements were obtained from each colony, perpendicular to each other. Six measurements were thus taken at each temperature for every isolate studied, and the means calculated.

Greenhouse pathogenicity trials

All six Thailand and nine South African isolates were used in greenhouse inoculation trials. Isolates were grown on enriched 4% PDA for 2 weeks prior to inoculation. Pathogenicity tests were conducted on six-month-old *E. grandis* trees of the clone ZG 14, which is known to be highly susceptible to Coniothyrium canker under natural conditions (Wingfield *et al.* 1997). Twenty trees were inoculated for each isolate tested. A small wound (10 mm diam) was made on the stem of each tree by removing the bark and exposing the cambium. Mycelial plugs, of similar size, overgrown with the test fungi, were placed into each wound with the mycelium facing the cambium. Inoculation wounds were covered with masking tape to prevent desiccation of the inoculum. For control inoculations, sterile PDA plugs were used and inserted into wounds on the stems of five trees. Mean lesion length was assessed after six weeks and statistical differences for each isolate determined using Tukey's studentised range test ($P = 0.05$). The experiment was repeated once.

Molecular comparisons

DNA extractions

Nucleic acid was extracted from all *Eucalyptus* stem canker isolates, as well as isolates included for comparative purposes. Total genomic, high molecular weight DNA was extracted from all isolates by culturing them in 250 ml enriched Potato dextrose broth (24 g Potato Dextrose extract (Merck); 1 g yeast extract (Merck); 1 g glucose (Merck); 1 l distilled H_2O) in 500 ml Erlenmeyer flasks. Cultures were incubated at 30 ° on rotary shakers for 7 d. Mycelium was then harvested by filtration through Whatman No. 1 filter paper and lyophilised.

DNA was isolated using the technique of Raeder & Broda (1985) with some amendments. One gram of dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. Ten ml of extraction buffer (100 mM Tris–HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25% SDS; 10 mM β-mercaptoethanol; 4 mM spermidine; 1 mM spermine; 1 mM Phenylmethylsulfonyl fluoride (PMSF)), maintained at 65 °, was added to each isolate and incubated in a water bath at 65 ° for 60 min with frequent mixing. The aqueous phase was collected after centrifugation and phenol/chloroform (1:1 phenol to chloroform) extractions were performed until the interface was completely clean. Nucleic acids were precipitated using 3 M NaAc (0.1 v/v) and isopropanol (0.6 v/v) followed by overnight incubation at −20 °. After centrifugation, to harvest the nucleic acids, and washing with 70% EtOH, the pellet was air-dried and re-suspended in 200 µl sterile water. One µl of RNaseA (10 mg ml^{-1}) was added to the re-suspended sample and left at 37 ° overnight to degrade all RNA in the sample. All DNA extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng μl^{-1}.

DNA sequence comparisons

Polymerase Chain Reaction

The ITS1 and ITS2, as well as the 5.8S gene of the ribosomal RNA operon, were amplified using the polymerase chain reaction (PCR) (Saiki *et al.* 1988). Amplifications were performed using ITS primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCTGCGTTCTTCATCGATGC-3′) (White, Bruns & Taylor 1990). The PCR reaction mixture (100 µl) included two units of *Taq* DNA Polymerase (Boehringer Mannheim, Germany), reaction buffer (Boehringer), 4.5 mM $MgCl_2$ (Boehringer), 250 mM dNTPs and 0.5 µl of each primer (100 pM). Amplification reactions were carried out in a Hybaid Omnigene Temperature Cycler (Hybaid, Middx). Denaturation was performed at 95 ° for 30 s, followed by primer annealing at 55 ° for 45 s, and elongation at 72 ° for 2 min. These steps were repeated for 35 cycles. Final chain elongation took place at 72 ° for 5 min. PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and visualised using UV light. Amplification reactions were done in duplicate.

DNA sequencing and data analysis

All PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with *Amplitaq*® DNA Polymerase, FS (Perkin–Elmer, Warrington) on a ABI PRISM™ 377 DNA Autosequencer (Perkin–Elmer). Primers ITS1 and ITS4 were used in the sequence reaction. Phylogenetic analysis was done using midpoint rooting. Phylogenetic relationships among species were determined using the Heuristic search option in PAUP* with gaps treated as missing data (Swofford 1998). Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony) (1000 replicates) (Felsenstein 1993). Sequences from all isolates were deposited in GenBank with the following accession numbers: CMW 7506 (AF376815), CMW 7459 (AF376816), CMW 7465 (AF376817), CMW 7507 (AF376818), CMW 7442 (AF37819), CMW 7489 (AF376820), CMW 7491

Table 2. Comparison between morphological characteristics, growth rate and lesion lengths of *Coniothyrium* isolates from South Africa and Thailand.

		Morphological characteristics			Growth studies[c] (average colony diameter (mm)						Pathogenicity[fg]
Fungus	Isolate	Colony colour[a]	Conidium length[b]	Conidium width	10 °	15 °	20 °	25 °	30 °	35 °	Lesion length (mm)
Coniothyrium zuluense	CMW 7489	V23^{IIIIb}	4–4.5 (4.3)	2.5–3 (2.5)	0 a[de]	11.8M b	24.1 ab	34.5 ab	53.8 b	26.5 b	37.6 d[h]
	CMW 7491	19^{II}I	4–4.5 (4.2)	2.5–3 (2.5)	0 a	15.2 bc	30.9 c	50.5 d	75.4 e	40.1 d	10.0 a
	CMW 7459	21^{IIII}	4.5–5.0 (4.5)	2.5–3 (3)	0 a	7.5 b	16.5 a	25.4 a	39.4 a	16.2 a	23.6 b
	CMW 7506	V23^{IIIIb}	4.5 (4.5)	2.5–3 (3)	0 a	11.7 b	23.8 ab	36.5 b	59.6 bc	27.9 bc	10.0 a
	CMW 7465	33^{IIIf}	4.5–5.0 (4.5)	2.5–3.5 (3)	0 a	14.2 b	26.4 bc	42.3 bc	66.8 cd	35.6 c	56.4 e
	CMW 7507	33^{IIIf}	4–5 (5)	2.5–3.5 (3)	0 a	10.4 b	19.8 a	28.4 a	42.1 a	19.8 ab	23.1 b
	CMW 7442	21^{IIII}	4.5–5 (5)	2–3 (3)	0 a	9.8 b	20.1 a	32.1 ab	50.6 b	24.5 b	27.8 c
	CMW 7406	33^{IIIf}	3.5–4 (4.0)	2.5–3 (3)	0 a	8.9 b	17.5 a	27.8 a	43.6 a	23.5 b	10.0 a
	CMW 7399	V23^{IIIIb}	3.5–4.5 (4)	2.5–3.5 (3)	0 a	15.4 bc	30.2 c	53.6 d	80.1 e	40.1 d	17.8 b
Coniothyrium sp.	CMW 5231	21^{IIII}	4–4.5 (4)	2.5–3 (2.5)	0 a	0 a	27.4 bc	38.7 bc	62.4 c	15.8 a	10.0 a
	CMW 5232	21^{IIII}	4–4.5 (4.5)	2.5–3 (2.5)	0 a	0 a	23.5 ab	35.8 b	58.4 bc	14.4 a	10.0 a
	CMW 5233	21^{IIII}	4.5 (4.5)	2.5–3(3)	0 a	0 a	28.4 bc	41.6 c	66.5 cd	16.9 a	10.0 a
	CMW 5234	21^{IIII}	4.5–5 (4.5)	2.5–3.5 (3)	0 a	0 a	23.3 ab	40.2 c	58.9 bc	14.8 a	10.0 a
	CMW 5235	21^{IIII}	4.5–5 (5)	2.5–3 (3)	0 a	0 a	24.0 ab	41.5 c	62.4 c	15.7 a	10.0 a
	CMW 5236	21^{IIII}	4.5–5 (4.5)	2.5–3 (3)	0 a	0 a	24.2 ab	38.2 bc	59.9 bc	14.8 a	10.0 a
Control											10.0 a

[a] Colour classes follow Rayner (1970); represent the following: olive grey (V23^{IIIIb}), greenish glaucous (33^{IIIf}), Isabella (19^{II}I) and greyish olive (21^{IIII}).

[b] All measurements are in μm; values in brackets are means of 30 measurements.

[c] Growth was measured after incubating cultures for 30 d in the dark.

[d] Each value represents an average of six measurements.

[e] Each value with a different letter differs significantly at $P = 0.05$ from the others for that specific temperature range (CV = 13.45 %).

[f] Six-month-old trees of a susceptible *Eucalyptus grandis* clone (ZG 14) were inoculated under glasshouse conditions.

[g] Each value is an average of 20 measurements: CV = 11.3 %.

[h] Values followed by different letters differ significantly at $P = 0.05$.

(AF376821), CMW 7406 (AF376822), CMW 7399 (AF376823), CMW 5231 (AF376824), CMW 5234 (AF376825), CMW 5235 (AF376826), CMW 5236 (AF376827), CWM 5233 (AF376828), and CMW 5232 (AF376829).

Amplified Fragment Length Polymorphism (AFLP) analysis

Restriction, ligation, and amplification reactions were performed as described by Vos *et al.* (1995). Genomic DNA (500 ng) from each sample was incubated for 16 h at 25 ° in a solution containing 10 Uμl^{-1} *Cfo*I and 5 Uμl^{-1} *Mse*I (Boehringer Mannheim), 1 × restriction-ligation buffer (10 mM Tris-HAc, pH 7.5; 10 mM MgAc; 50 mM KAc; 5 mM DTT), 1 Uμl^{-1} T4 DNA Ligase, 50 pmol μl^{-1} *Cfo*1-adaptors, 50 pmol μl^{-1} *Mse*I-adaptors (Table 2), 10 mg ml^{-1} RNaseA and 100 mM spermidine. The final sample volume was increased to 100 μl with sterile water. After ligation the reaction mixture was diluted 10-fold with TE buffer (10 mM TRIS–HCl, pH 8.0; 10 mM EDTA) and stored at –20 °. These ligated fragments served as templates in the amplification reaction.

A pre-selective PCR (+1 reaction) amplification reaction was performed in 20 μl PCR reaction mix containing 5 μl of the diluted DNA, 0.5 μl of each +1 primer (10 pmol μl^{-1}) (Table 2), 100 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 25 mM $MgCl_2$, 5 Uμl^{-1} *Taq* DNA Polymerase (Boehringer Mannheim) and 250 μM of dNTP. Initial denaturation was performed at 94 ° for 1 min, followed by 30 cycles of 30 s at 94 °, 60 s

at 56 ° (primer annealing) and 60 s at 72 ° (final chain elongation). The amplification PCR products were diluted 10-fold in 1 × T.1E buffer and used as templates in the second amplification.

The second amplification (+3 reaction) was performed using primers derived from the first set of primers, with additional nucleotides at the 3′ end (Table 1). The *Cfo*I primer was HEX fluorochrome-labelled and the *Mse*I primer was FAM fluorochrome-labelled (AmpFlSTR Profiler PCR Kit, Perkin–Elmer, Norwalk, CN). Fluorescent AFLPs were amplified under the following conditions: 0.2 μl of the HEX fluorochrome-labeled *Cfo*I +3 primer (50 pmol μl^{-1}) was added to a 20-μl PCR reaction solution containing 5 μl of the diluted +1 pre-amplification mix, 0.4 μl of the FAM fluorochrome-labeled *Mse*I+3 primer, 5 U/μl *Taq* DNA Polymerase (Boehringer Mannheim), 10 × Buffer (100 mM Tris–HCl, pH 8.0; 15 mM $MgCl_2$; 500 mM KCl, pH 8.3), 25 mM $MgCl_2$ and 250 μM of dNTP. The following temperature profile was used: 12 cycles of 30 s at 94 °, 30 s at 65 °, 60 s at 72 °, where the annealing temperature was subsequently reduced by 0.7 ° after each cycle. The amplification was continued for 22 cycles of 30 s at 94 °, 30 s at 56 °, and 60 s at 72 °. All amplification reactions were performed using a Hybaid Omnigene thermocycler (Hybaid, Middx).

Electrophoresis and visualisation of AFLP PCR products

PCR products (1.5 μl) were combined with 3 fmol TAMRA fluorescent-labelled GeneScan 500 internal size standard (ABI), 1.6 μl formamide, and 0.3 μl 25 mM EDTA (pH 8.0) containing 50 mg ml^{-1} blue dextran. This mixture was heat denatured for 3 min at 95 °, and immediately cooled on ice. Samples were loaded on a 5% denaturing polyacrylamide gel in 1 × TBE (Tris-borate EDTA, pH 8.0) and electrophoresed for 2.5 h at 1680 w using the GS 36A-2400 run module. Data were processed by GeneScan Analysis software (version 2.02) to produce a gel image. PCR fragments for individual samples were automatically sized by the GeneScan software using a comparison of the mobility of the internal lane size standard to that of the sample fragment.

AFLP data analysis

Each polymorphic AFLP fragment was treated as a unit character and scored as present (1) or absent (0) across all isolates. The experiments were repeated, and only reproducible bands were scored. The index of genetic similarities was calculated following the Nei & Li (1979) definition of similarity. Unweighted pair-group mean arithmetic (UPGMA) was used for cluster analysis of the pairwise similarity matrix that generated a dendrogram representing the genetic similarity among fungal isolates. UPGMA analysis was carried out using NCSS97 (Visual Components).

RESULTS

Morphological comparisons

Colony and conidial morphology

Conidia of the Thailand *Coniothyrium* isolates were 4–5 × 2.5–3.5 μm (Table 2), and those of *C. zuluense* from South Africa (van Zyl *et al.* 1997) were 3.5–5.5 × 2–3.5 μm. All conidia used in this study were thick-walled, smooth, and broadly ellipsoid. The apices were obtuse, and the bases subtruncate to bluntly rounded.

The colour of the *Coniothyrium* isolates from Thailand were all greyish olive (21^{IIII}) (Table 2). South African isolates of *C. zuluense* vary from an olive grey ($V23^{IIII}b$), isabella ($19^{II}i$), greenish glaucous ($33^{III}f$) to a greyish olive (21^{IIII}) colour (Table 3) (van Zyl *et al.* 1997). All South African and Thailand isolates viewed from below were black or rust coloured with white margins.

Growth studies

In growth studies, isolates of the *Coniothyrium* from Thailand and *C. zuluense* had growth optima at 30 ° (Table 2). Thailand isolates failed to grow at 10 ° and 15 °. *Coniothyrium zuluense* isolates were, however, able to grow at 15 °, although very slowly.

Pathogenicity tests

None of the Thailand isolates screened for pathogenicity, was able to cause disease (Table 2). Results were consistent between repetitions. Significant differences in lesion development were, however, evident among *C. zuluense* isolates from South Africa. Isolates previously defined as having high levels of pathogenicity (van Zyl 1999) produced significantly larger lesions ($P = 0.05$) than those isolates having intermediate and low levels of pathogenicity (Table 2). These isolates also differed significantly among each other in their capacity to cause disease. There was no significant difference ($P = 0.05$) between lesion lengths for the isolates of intermediate pathogenicity. Significant differences in lesion development were, however, observed for isolates previously described as having intermediate and low levels of pathogenicity (van Zyl 1999). Isolates previously defined as being non-pathogenic produced no lesions in this study (van Zyl 1999). No symptoms developed on trees inoculated as controls (Table 2). The inoculated pathogen was consistently re-isolated from the lesions on inoculated trees and never from control trees.

Molecular comparisons

DNA sequence comparisons

Alignment of the DNA sequence data within the ITS1 and ITS2 regions proved variable between all the

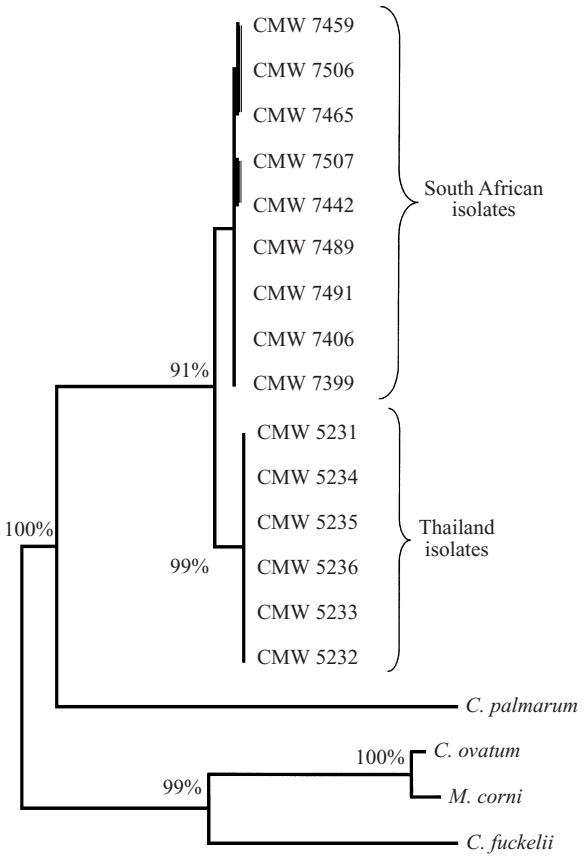

Fig. 1. Phylogram generated with PAUP analysis based on the DNA sequence data from ITS1, ITS2 and the 5.8S RNA gene for isolates of *Coniothyrium zuluense* from South Africa and Thailand. *C. palmarum* (CBS 758.73), *C. fuckelii* (CBS 132.26), *Massarina corni* (CBS 496.64) and *C. ovatum* are included for comparative purposes. Bootstrap values are indicated at each branch point.

different species studied. An heuristic search from the aligned DNA sequence data (573 characters) of the ITS1, 5.8S, and ITS2 regions of the rRNA operon, produced one most parsimonious tree (Fig. 1) of 458 steps (CI = 0.930, HI = 0.070, RI = 0.934). Three major clades emerged. All the *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* grouped together in a single major clade. This clade was clearly distant from isolates of other *Coniothyrium* species included. Bootstrap analysis showed that the branch point separating *Eucalyptus* stem canker isolates from the other *Coniothyrium* species, had a confidence interval of 100 %.

Isolates representing *C. zuluense* and the *Coniothyrium* from Thailand could be divided into two distinct subgroups: (i) *C. zuluense* isolates from South Africa (91 % bootstrap); and (2) *Coniothyrium* isolates from Thailand (99 % bootstrap). *C. palmarum* formed a single, strongly supported (100 % bootstrap) clade. The three remaining *Coniothyrium* species grouped in a third clade (99 % bootstrap). This clade was subdivided into two subgroups. *Massarina corni* and *C. ovatum* grouped together (100 % bootstrap) with *C. fuckelii*, but in a separate though strongly supported (99 % bootstrap) subgroup.

AFLP analysis

177 scorable AFLP markers were included in this study. Of these, 9 (5.1 %) were monomorphic for all the species while 168 (94.9 %) displayed informative polymorphisms. Fragment size ranged from 40 to 499 bp. A matrix based on the similarity coefficient of Nei & Li (1979) was produced using all 177 (polymorphic and monomorphic) fragments (Table 3). Pairwise genetic similarity revealed an average similarity of 82.3 % (17.7 % dissimilar) among isolates of the *Coniothyrium* from Thailand and 77.6 % similarity (22.4 % dissimilar) among the *C. zuluense* isolates. The average percentage similarity shared between isolates of *C. zuluense* from South Africa and those of the *Coniothyrium* from Thailand was 76.1 % (23.9 % dissimilar). Genetic similarity ranged from 61.7 %–86.2 %. UPGMA analysis of the similarity matrix yielded a dendrogram (Fig.

Table 3. Pairwise distance matrix based on the F-statistic of Nei & Li (1979) converted to a percentage, i.e. % S*ij* = 1-*Sij*.

CMW 5232	100																	
CMW 5233	91.27	100																
CMW 5236	74.96	68.75	100															
CMW 5235	82.17	74.96	83.82	100														
CMW 5234	83.82	78.7	88.43	86.94	100													
CMW 5231	91.96	86.18	71.95	77.79	81.33	100												
CMW 5877	82.17	82.17	72.97	82.17	78.7	79.59	100											
CMW 5879	80.47	76.86	70.91	78.7	76.86	75.92	85.41	100										
CMW 5878	71.95	71.95	62.93	73.98	69.84	70.91	77.79	71.95	100									
CMW 5691	79.59	69.84	60.4	69.84	71.95	76.86	73.98	69.84	61.68	100								
CMW 5690	82.17	80.47	68.75	72.97	72.97	77.79	88.43	80.47	71.95	83	100							
CMW 5881	79.59	75.92	60.4	81.33	65.34	76.86	89.16	81.33	78.7	66.5	83	100						
CMW 5880	85.41	76.86	78.7	82.17	85.41	75.92	86.94	85.41	71.95	67.64	80.47	77.79	100					
CMW 5972	76.86	72.97	64.15	70.91	72.97	79.59	78.7	76.86	67.64	79.59	82.17	69.84	74.96	100				
CMW 5973	77.79	69.84	71.95	79.59	79.59	78.7	81.33	83	68.75	68.75	73.98	78.7	79.59	75.92	100			
Massarina corni	38.08	29.49	19.26	48.82	29.49	36.06	38.08	41.91	47.17	27.11	24.62	53.47	33.96	41.91	43.72	100		
Coniothryium ovatum	48.82	38.08	24.62	51.96	38.08	47.17	45.47	41.91	47.17	40.03	33.96	56.35	38.08	45.47	43.72	95.23	100	
Coniothyrium fuckelii	24.62	−21.27	−1.15	19.26	6.57	10.04	13.29	6.57	10.04	10.04	29.49	16.36	13.29	−1.15	16.36	−506	−506	100
Coniothyrium palmarum	2.85	−15.44	−5.48	2.85	10.04	6.57	−5.48	2.85	−56.2	−21.3	−27.9	−21.3	2.85	−27.9	−10.2	−506	−431	−506	100

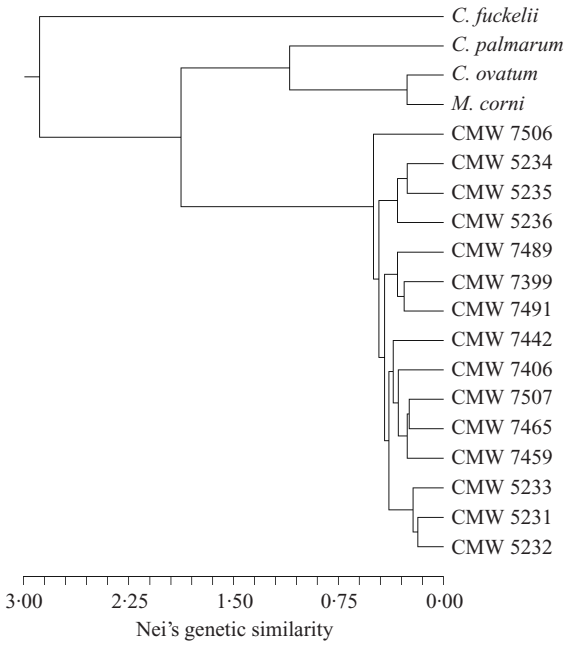

Fig. 2. Dendrogram generated of *Coniothyrium zuluense* isolates from South Africa (CMW 7399, CMW 7406, CMW 7442, CMW 7459, CMW 7465, CMW 7489, CMW 7491, CMW 7506 and CMW 7507) and Thailand (CMW 5231–5236), together with four related species (see Fig. 1) based on AFLP data using UPGMA cluster analysis of pairwise distance data. The scale represents genetic similarity obtained using the equation of Nei & Li (1979).

2) which grouped the South African and Thailand isolates together in a single clade.

UPGMA analysis of the similarity matrix grouped all *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* separate from the four species included for comparative purposes. This is in agreement with results obtained for sequence data. Average percentage similarity between the *Eucalyptus* stem canker pathogens and the other *Coniothyrium* spp. was 11.2% for *C. fuckelii*, 18.2% for *C. palmarum*, 37.3% for *M. corni* and 43.4% for *C. ovatum*.

DISCUSSION

The morphology, pathogenicity tests, and molecular comparisons, strongly support the view that the *Coniothyrium* from Thailand is the same as *C. zuluense*, which causes *Eucalyptus* stem canker in South Africa. This is the first report of *C. zuluense* outside South Africa.

Based on morphological comparisons, the *Coniothyrium* from Thailand is virtually indistinguishable from *C. zuluense* from South Africa. Conidial measurements of the Thailand isolates were within size ranges published for *C. zuluense* (van Zyl *et al.* 1997, Wingfield *et al.* 1997). Thailand isolates, however, differed from *C. zuluense* in that they were greyish olive colour and failed to grow at 15 °. This was in contrast to characteristics published for *C. zuluense* from South Africa (van Zyl *et al.* 1997), which varied between an olive grey, greenish glaucous, isabella or greyish olive colour and, was able to grow at temperatures ranging from 15–35 ° (van Zyl *et al.* 1997). Optimal growth temperature for both *Coniothyrium* species was 30 °. Although some differences in colony colour and temperature requirement for growth were observed, we do not believe that these are sufficient to separate the isolates from the two regions into different taxa.

Pathogenicity tests on young trees in the greenhouse showed that the Thailand *Coniothyrium* was not pathogenic to *E. grandis*. Its role in tree disease in Thailand is, therefore, uncertain. However, only a small number of isolates from Thailand were available for study. Pathogenicity studies conducted in South Africa (van *Zyl et al.* 1997), showed that only 22% of 344 *C. zuluense* isolates were able to cause lesions on a susceptible *E. grandis* clone (van Zyl *et al.* 1997). Most of these isolates (78%) collected from severely infected trees in the field were, thus, not able to cause disease. In the future, we hope to collect more isolates in Thailand and conduct pathogenicity tests on established trees in that country. Such tests will expand our understanding of the role that *C. zuluense* has in *Eucalyptus* disease in Thailand.

Phylogenetic analysis of sequence data from the ribosomal RNA operon, confirmed that the Thailand isolates and *Coniothyrium zuluense* are the same. The ribosomal RNA operon is well known to be an useful source of genetic data for taxonomic comparisons at species level (Blanz & Unseld 1986, White *et al.* 1990, Kurtzman 1992, Wingfield & Wingfield 1993, Mitchell, Roberts & Moss 1995, Wingfield *et al.* 1996a, b, Witthuhn *et al.* 1998). Data analysis of the present study showed that the South African and Thailand isolates produced a single clade, separate from the other *Coniothyrium* species used. However, *Coniothyrium* isolates from the two regions formed subgroups within this major clade. This might suggest that *C. zuluense* in Thailand is in the process of diverging from *C. zuluense* in South Africa.

Genetic similarity between the Thailand isolates and *C. zuluense*, as determined by AFLP analysis, confirmed that they are the same. AFLP analysis is a novel PCR fingerprinting technique which selectively amplifies DNA fragments, corresponding to unique positions on the genome (Zabeau & Vos 1993, Vos *et al.* 1995). This technique has previously been shown to be extremely useful in determining genetic similarities between different fungal populations (Majer *et al.* 1996, Majer, Lewis & Mithen 1998, Pongam, Osborn & Williams 1999). Data suggest that genetic differences between the Thailand isolates and *C. zuluense* are evident, but that they share a common origin.

This report represents the first record of *Coniothyrium zuluense* outside South Africa. It is, however, intriguing to consider the possible origin of *C. zuluense* on *Eucalyptus*. Wingfield *et al.* (1997) suggested that the fungus was native to South Africa. This hypothesis was based on the fact that the disease was not known

elsewhere in the world, especially in Australia where *Eucalyptus* is native. They suggested that the fungus might have originated from native *Myrtaceae*, and had developed the capacity to infect *Eucalyptus*. This is similar to the situation with *Eucalyptus* rust, caused by *Puccinia psidii*, which is not known in Australia, but is common and damaging in South and Central America where it apparently originated from native *Myrtaceae* (Coutinho *et al*. 1998). This study has shown that *C. zuluense* occurs elsewhere in the world, surveys to find possible alternative hosts, both native and introduced, need to be conducted in subtropical and tropical *Eucalyptus* growing areas.

South African *Coniothyrium zuluense* isolates display considerable variation in colony colour and pathogenicity (van Zyl *et al*. 1997). Large variations in pathogenicity are widely associated with diverse pathogen populations that are influenced by a number of factors, including the capacity for sexual reproduction. Organisms capable of sexual reproduction generally have higher genetic diversity than those reproducing only asexually (McDonald & McDermott 1993, Wolf & McDermott 1994, Milgroom 1996). *C. zuluense* is known to reproduce only asexually (Wingfield *et al*. 1997), thus, the large variation in virulence was not expected. One hypothesis has been that more than one species of *Coniothyrium* might be responsible for the disease in South Africa. Our sequencing results, however, show that only a single, yet variable species is responsible for cankers on *Eucalyptus* species in South Africa. In the future, pathogenicity tests with a wider range of isolates should be undertaken in Thailand. Such tests will allow the susceptibility of different *Eucalyptus* species, clones, and hybrids to the different strains to be assessed. The ultimate aim of avoiding the disease, however, could be achieved through the selection of disease-resistant planting stock.

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Corresponding Editor: D. W. Hollomon